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System for efficacy and cytotoxicity screening of inhibitors targeting intracellular Mycobacterium tuberculosis

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Abstract:	Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is a leading cause of morbidity and mortality worldwide. With increased spread of multi drug-resistant TB (MDR-TB), there is a real urgency to develop new therapeutic strategies against M. tuberculosis infections. Traditionally, compounds are evaluated based on their antibacterial activity under in vitro growth conditions in broth; however results are often misleading for intracellular pathogens like M. tuberculosis since in vitro phenotypic screening conditions are significantly different from the actual disease growth conditions within the human body. Screening for inhibitors that work inside macrophages has been traditionally difficult due to its complexity, variability in infection and slow replication rate of M. tuberculosis. In this study, we report a new approach to rapidly assess the effectiveness of compounds on viability of M. tuberculosis in a macrophage infection model. In combination with a cytotoxicity assay and an in vitro M. tuberculosis viability assay we are able to create a screening system that generates comprehensive analysis of compounds of interest. This system is capable of producing quantitative data at a low cost that is within reach of most labs and yet highly scalable to fit large industrial setting.
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Dear Editor of JoVE

Enclosed is our manuscript titled "System for efficacy and cytotoxicity screening of inhibitors targeting intracellular *Mycobacterium tuberculosis*". Please accept our submission as a candidate for publication in Journal of Visualized Experiments.

Our protocol addresses a long standing issue in Tuberculosis drug development where a simple high throughput intracellular screening method was not available. This deficiency has been a major hurdle in tuberculosis drug discovery since tuberculosis is characterized mainly as intracellular infection of human macrophages. Our manuscript describes a simple, low cost, and highly scalable *ex vivo* high throughput compound screening method against intracellular *M. tuberculosis.* We feel this manuscript is suitable for publication in JoVE as it is a method that would benefit the field of Tuberculosis drug development.

Sincerely

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KEYWORDS:
Mycobacterium tuberculosis, high-throughput screening, luciferase assay, THP1, macrophage, intracellular, MTT, infection

SHORT ABSTRACT:
We have developed a modular high-throughput screening system for discovering novel compounds against *Mycobacterium tuberculosis*, targeting intracellular and in-broth growing bacteria as well as cytotoxicity against the mammalian host cell.

LONG ABSTRACT:
Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is a leading cause of morbidity and mortality worldwide. With the increased spread of multi drug-resistant TB (MDR-TB), there is a real urgency to develop new therapeutic strategies

against *M. tuberculosis* infections. Traditionally, compounds are evaluated based on their antibacterial activity under *in vitro* growth conditions in broth; however, results are often misleading for intracellular pathogens like *M. tuberculosis* since in-broth phenotypic screening conditions are significantly different from the actual disease conditions within the human body. Screening for inhibitors that work inside macrophages has been traditionally difficult due to the complexity, variability in infection, and slow replication rate of *M. tuberculosis*. In this study, we report a new approach to rapidly assess the effectiveness of compounds on the viability of *M. tuberculosis* in a macrophage infection model. Using a combination of a cytotoxicity assay and an in-broth *M. tuberculosis* viability assay, we were able to create a screening system that generates a comprehensive analysis of compounds of interest. This system is capable of producing quantitative data at a low cost that is within reach of most labs and yet is highly scalable to fit large industrial settings.

INTRODUCTION:

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is a leading cause of morbidity and mortality worldwide. Drug-sensitive TB is a treatable disease that requires multiple antibiotics for a period of 6 months. Despite being a treatable disease, TB mortality was estimated to be 1.5 million in 2015[1]. In the past 10 years, there have been increasing concerns over the prevalence of drug-resistant *M.tuberculosis*. Multidrug-resistant TB (MDR-TB) is defined as TB that is resistant to at least Isoniazid (INH) and Rifampicin (RMP), and most MDR-TB strains are also resistant to select second-line TB drugs, thus limiting treatment options. The effects of drug resistance create a greater challenge for treating patients co-infected with Human Immunodeficiency Virus (HIV); 400,000 patients worldwide died of HIV-associated TB in 2015[1]. Disappointingly, the United States Food and Drug Administration has approved only one new TB drug against MDR-TB, bedaquiline, in the past 40 years[2]. Advances in finding better and shorter TB therapies are urgently needed in order to stay ahead in the fight against TB and MDR-TB.

Traditionally, TB drug screens are performed under *in vitro* growth conditions in growth medium, whereby compounds are added to a growing culture and their effectiveness in eradicating the microorganisms are determined by counting colony forming units (CFU) on solid medium. As CFU counts are labor intensive, time consuming, and costly, various indirect methods have been developed to alleviate this problem. Such methods include the Alamar Blue viability assay[3], the determination of fluorescence[4] from green fluorescent protein (GFP) or luminescence[5] from luciferase-expressing bacteria, and the estimation of total adenosine triphosphate (ATP)[6,7].

Typical TB is characterized by an *M. tuberculosis* infection of the lung, where the bacteria reside and replicate inside the phagosomes of alveolar macrophages[8]. The simple in-broth phenotypic screen may suit extracellular pathogens; however, in the historical perspective, hit compounds against *M. tuberculosis* identified using this method often fail to live up to expectations during downstream validation steps in infection models. We propose that TB drug is best performed in an intracellular host cell infection model. Nevertheless, intracellular models possess many technological and

biological barriers to high-throughput screening (HTS) development. A big hurdle is the complexity of the infection process, exemplified by numerous steps and the elaborate removal of extracellular bacteria by in-between washing. A second major hurdle is the lengthy time requirements, as growth detection, normally done by CFU counting on culture plates, is a process that takes over 3 weeks to complete. One solution to replace CFU counts has been provided by automated fluorescent microscopy in combination with fluorescent bacteria. However, this solution requires an initial equipment investment that is out of reach for many research labs. A simple, low-cost, and disease-relevant HTS method would greatly enhance the drug discovery process.

In this study, we report a new, modular HTS system that is aimed at providing a rapid, and highly scalable, yet economical, assay suitable for determining the activity of compounds against intracellular *M. tuberculosis*. This system is composed of three modules: (i) intracellular, (ii) cytotoxicity, and (iii) in-broth assays. The combined final result provides a comprehensive description of the compound properties, with additional information as to the potential mode of action. This screening system has been used in several projects with various compound libraries that target mode of action, including the analysis of drug synergy[9], the stimulation of autophagy[10], and the inhibition of *M. tuberculosis*-secreted virulence factor (unpublished). Compounds of unknown mode of action have also been studied[11]. A modified version of this method was also adopted by our industrial partner as the primary screening method to identify new compounds against intracellular *M. tuberculosis*[11].

PROCEDURE:

1. Bacterial strain and growth medium

1.1 Make albumin dextrose and salt stock solution (ADS) by solubilizing 25 g of bovine serum albumin, 10.0 g of dextrose, and 4.05 g of sodium chloride in 460 mL of deionized water. Filter-sterilize the ADS and store at 4 °C.

1.2 Make 7H9 broth by adding 4.7 g of 7H9 powder and 2 mL of glycerol to 900 mL of purified water. Autoclave the 7H9 broth at 121 °C for 10 min and allow it to cool to room temperature before proceeding. Make 7H9ADST by adding 100 mL of ADS and 0.5 mL of Tween80 to 900 mL of 7H9 broth. Store at 4 °C.

1.3 Weigh 50 mg of kanamycin sulfate and dissolve in 1 mL of deionized water; the final concentration is 50 mg/mL. Filter-sterilize and store at -20 °C. Add 0.5 mL of kanamycin stock solution per 1 L of 7H9ADST.
Note: This medium should be made fresh, so scale the volumes appropriately according to the culture size.

1.4 Grow *M. tuberculosis* in 7H9ADST supplemented with kanamycin in standing culture. Shake the culture daily and dilute it before the OD600 reaches 1.0 to avoid clumping.
Note: The *M. tuberculosis* strain used for the development of this method was H37Rv transformed with pJAK2.A plasmid[12]. pJAK2.A is an integrative plasmid based on the

pMV361 vector, which allows high-level expression of the firefly luciferase gene from the *hsp60* promoter and can be selected using kanamycin.

2. THP-1 medium and maintenance

2.1 Add 50 mL of heat-inactivated fetal bovine serum (FBS) and 5 mL of 200 mM L-glutamine to 500 mL of RPMI 1640 to make RPMI incomplete medium (approximately 10% FBS and 2 mM glutamine).

2.2 Maintain an THP-1 cell culture according to standard protocol[13]. Briefly, grow THP-1 cells in RPMI incomplete medium while maintaining a cell density of 0.2 to 1 million per mL of medium between passages.

3. High-throughput intracellular screening using luciferase-expressing *M. tuberculosis* H37Rv

3.1 Measure the optical density of an actively growing bacterial suspension in a spectrophotometer at a wavelength of 600 nm. Calculate the bacterial density using the conversion factor of 0.1 OD_{600} = 3 x 10^7 bacteria per mL.

3.2 Pipette out sufficient bacteria for a multiplicity of infection (MOI) of 10:1 into a new centrifuge tube. Pellet at 3,000 x g for 10 min and aspirate the liquid. Add 50 µL of human serum to 450 µL of RPMI1640. Scale the volume to appropriate values for the experiment.

3.3 To opsonize the bacteria, resuspend the pellet at a density of 1 x 10^8 bacteria per 500 µL of RPMI1640 containing 10% human serum. Allow the mixture to incubate at 37 °C for 30 min. Determine the THP-1 cell culture density by counting with a hemocytometer and an inverted microscope.

3.4 Pellet the cells in sterile centrifuge tubes at 100 x g and 37 °C for 10 min. Aspirate the supernatant and resuspend the cells in RPMI incomplete at a density of 1 million cells per mL. Add phorbol-12-myristate-13-acetate (PMA) to a 40 ng/mL final concentration. NOTE: This will be referred to as the differentiation mix.

3.5 Combine opsonized *M. tuberculosis* with THP-1 differentiation mix at a MOI of 10:1 and aliquot the final mix at 100 µL per well in a 96-well flat-bottom white plate. Regularly stir the mixture to ensure uniformity. Allow the differentiation and infection to proceed overnight at 37 °C in a humidified incubator containing 5% CO_2 .

3.6 Wash the wells twice with 100 µL of RPMI each. Add compounds diluted to the desired concentrations in RPMI incomplete and incubate for 3 days.

3.7 Aspirate the medium from the wells. Add 50 µL of luciferase assay reagent to each well. Seal the plates with transparent adhesive plate sealers. Allow 5 min of incubation at 22 °C and then obtain a readout in a luminometer at 1 s per well.

4. Cytotoxicity analysis using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[14]

4.1 Differentiate THP-1 cells in RPMI incomplete supplemented with 40 ng/mL of PMA in clear 96-well plates. Maintain a cell density of 1 million per mL and aliquot 100 µL per well. Allow differentiation to proceed overnight at 37 °C in a humidified incubator containing 5% CO_2 .

4.2 Aspirate the medium from the wells and wash them twice with RPMI 1640. Add compounds diluted in RPMI incomplete to the wells. Incubate for 3 days.

4.3 Dissolve 0.5 g of MTT in 100 mL of phosphate-buffered saline (PBS) to make a stock solution of 5 mg/mL. Sterile filter and store at -20 °C, away from light ; it is best to make this solution fresh.

4.4 2.5 h before the end of the 3-day incubation period, add 25 µL of MTT solution to each well and complete the incubation period.

4.5 Prepare 50% N,N-dimethyl formamide (DMF) by mixing 250 mL of DMF with 250 mL of deionized water.

4.6 Prepare MTT extraction buffer as follows: Weigh 100 g of SDS in a 500-mL bottle and add 300 mL of 50% DMF. Apply low heat to allow the SDS to dissolve. Add 10 mL of pure acetic acid and 12.5 mL of 1 M HCl. Fill up to the 500-mL mark with 50% DMF; the final composition of the extraction buffer is 50% DMF, 20% SDS, 2.5% acetic acid, and 2.5% 1 M hydrochloric acid.

4.7 At the end of the treatment period, add 100 µL of extraction buffer (warmed to 45 °C to dissolve any crystals) to each well. Allow the mixture to incubate overnight at 37 °C in a humidified incubator containing 5% CO_2. Read the absorbance at 570 nm.
Note: The cytotoxicity assay is best performed in parallel with an intracellular screen using same-batch and age of THP-1 cells.

5. In-broth activity analysis using a resazurin assay[3]

5.1 Grow *M. tuberculosis* in 7H9ADST to the mid-log phase (~0.5-0.8 OD_{600}). Dilute the culture with the 7H9ADST to 0.01 OD_{600}. Dilute the compounds in 7H9ADST to 2x the testing concentrations and aliquot 100 µL of each diluted compound into each well.

5.2 Transfer 100 µL of the diluted bacterial suspension into each well. Allow the plates to incubate at 37 °C in a humidified incubator for 5 days. Dissolve 10 mg of resazurin in 100 mL of deionized water and sterile filter.

5.3 Add 30 µL of resazurin solution and monitor the color change after 48 h; bacterial growth is indicated by a color conversion from blue to pink.

Note: A quantitative analysis can also be performed by measuring either the fluorescence at 590 nm with excitation at 530-560 nm or the absorbance at 570 nm and 600 nm[15].

REPRESENTATIVE RESULTS:

High-throughput intracellular screening using *M. tuberculosis* expressing the luciferase gene.

Figure 2A and Table 1 contain the raw data collected by the luminometer, expressed in relative luminescent units (RLU), showing the effect of an increasing concentration of the TB drug rifampicin on *M. tuberculosis* inside THP-1 cells. Figure 2A is a scatter plot of the raw luminescence measured in RLU for various concentrations of rifampicin. The error bars indicate the standard error of the mean (SEM). Figure 2B shows the percent reduction in luminescence in treated wells compared to the untreated wells. The data shows that rifampicin is capable of reducing >99.9% of RLU from intracellular *M. tuberculosis* at a concentration of 0.1 µg/mL. This is in accordance with the previously published MIC between 0.1 and 0.4 µg/mL[16]. Luminescence produced in each well is an indication of the total luciferase expressed by *M. tuberculosis* and thus is an indicator of the metabolic status of *M. tuberculosis* inside the well. It is normal for raw luminescent levels to vary between experiments. As such, a comparison of raw data would generate unreliable conclusions. Thus, the data should be normalized against a defined negative control, which would be the samples treated with DMSO only. The resulting values can be expressed as the percent reduction in *M. tuberculosis* in the wells (Figure 2B).

Cytotoxicity analysis using the MTT assay

The MTT assay is a well-established assay for eukaryotic cytotoxicity. This colorimetric assay indicates live cells through the conversion of MTT (yellow) to purple-colored formazan. This assay is performed without an *M. tuberculosis* infection because the bacteria are capable of metabolizing MTT, which reduces the observed toxicity of the compounds. A common suggestion for the MTT assay is to use media without the pH indicator phenol red due to the absorption at 570 nm[17]. However, the acidified extraction buffer described in this method is able to minimize interference caused by phenol red[17].
Figure 3 illustrates the cytotoxicity caused by an increasing concentration of a test compound coded G1-1H. Normally, a 50% inhibitory concentration (IC_{50}) is employed to indicate the level of cytotoxicity. In the case of G1-1H, concentrations of 10 µM and 3 µM are clearly below the IC_{50} concentration.

In-broth activity analysis using the resazurin assay

The resazurin assay is commonly used for the analysis of cytotoxicity in eukaryotic cells, but it can also be used to monitor live bacteria in broth[3]. The resazurin assay is a redox-based assay similar to the MTT assay. It measures NADPH levels and NADPH dehydrogenase activity through the conversion of resazurin into resorufin, a red fluorophore. The easiest and quickest way to determine drug efficacy is to look for the lowest treatment concentration where the wells remain blue in color. Figure 4 shows part of a 96-well plate showing the effects various concentrations of the antibiotic apramycin have on resazurin conversion by *M. tuberculosis*. The in-broth MIC was determined to be between 2.5 and 5 µg/mL. This is slightly higher than the previously

published value of 1.5 µg/mL[18], but it is still well within the acceptable range. In many cases, the color change is rather gradual, so it is difficult to make a confident determination. Under these conditions, it is better to quantify the actual amount of resazurin conversion using either absorbance or fluorescence. Due to the similar absorbance characteristics of resazurin and resorufin, MIC determination using absorbance requires measurements using two different wavelengths and complex calculations[15]. Therefore, the best method is to measure fluorescence using 530- to 560-nm excitation wavelengths and a 590-nm emission wavelength, as described in the manufacturer's literature[15].

Figure 4 illustrates a potential source of inconsistency associated with multiday incubations that could drastically alter screening results. Due to improper humidifying of the 37 °C incubator used for this experiment, wells on the edges of the plates suffered significantly more evaporation. All 15 DMSO-treated wells are supposed to be identical, but the wells along the left and top edges had reduced volumes. These wells also appear to have different colors than the other DMSO-treated wells in the middle of the plate. The same inconsistency can also be observed in 2.5 µg/mL apramycin-treated wells. In this case, the reduced volume would also lead to a quantitative reading by a spectrophotometer and fluorometer. Evaporation becomes significant for all experiments with prolonged incubation times, so care must be taken to keep incubators well-humidified to avoid this source of inconsistency.

FIGURE LEGENDS:

Figure 1: Method schematics. Diagram depicting assay schematics for 3 separate modules of the high-throughput screening system.

Figure 2: Representative data from an intracellular experiment to examine the effectiveness of rifampicin in eliminating *M. tuberculosis* inside THP-1 cells. (A) Graph of the mean luminescence reading (in relative luminescence units) for each treatment (rifampicin) concentration. The error bars denote the standard error of the mean for each triplicate. (B) Graph of the calculated percent reduction in luminescence caused by a treatment of rifampicin, where higher values indicate greater effectiveness. The 90% inhibitory concentration (IC_{90}) in this case is somewhere between 0.01 and 0.1 µg/mL.

Figure 3: Representative data from a cytotoxicity (MTT) assay to examine the toxic effects of compound G1-1H on differentiated THP-1 cells. Graph of the calculated "percent of control" values for each concentration of the treatment compound G1-1H, where higher values indicate healthier THP-1 cells. The IC_{50} in this case is somewhere between 10 and 30 µM.

Figure 4: Representative data from a resazurin assay to examine the effectiveness of apramycin at inhibiting *M. tuberculosis* in-broth. Only qualitative data is collected in this case due to equipment limitations in the biosafety level 3 (BSL3) lab. The photo shows part of a 96-well plate containing *M. tuberculosis* treated with either DMSO or varying amounts of apramycin. The DMSO-treated wells exhibited a

conversion of resazurin to resorufin, as indicated by the color conversion from blue to pink. The apramycin-treated samples clearly underwent the color conversion below 5 µg/mL.

Table legends:
Table 1: Raw data from the luciferase assay for the determination of the intracellular IC_{90} of rifampicin.

Table 2: Raw data from the MTT assay for the determination of the cytotoxicity of test compound G1-1H at different concentrations.

DISCUSSION:
The goal of this study was to create a simple and cost-effective HTS method using a human intracellular infection model for *M. tuberculosis*. Tuberculosis is a human disease characterized by the infection of alveolar macrophages by *M. tuberculosis*. Due to biosafety issues, research involving biological models of both the bacterium and the host cells has been used in the past. However, it has been shown that the usage of surrogate bacteria and non-human models are poor predictors of hit-to-lead success in drug development, indicating that drug screening is best done with human cells infected with *M. tuberculosis*[19-22].

In this method, we have advanced and adapted current state-of-the-art screening protocols for the human macrophage-like THP-1 cell-line. In order to achieve high-throughput, we introduced several technical improvements to the infection protocol. First and foremost, we replaced all steps involved in CFU determination and substituted them with a luciferase-based reporter system. Firefly luciferase was chosen due to the simple end-point assay, the rapid degradation by host cell lysosome enzymes, and the minimal equipment requirements. This substitution effectively eliminated a 30-day incubation period, as well as the labor and consumables costs associated with plating and colony-counting steps.

A second major improvement we introduced was batch processing and infection, which further improves throughput and consistency between the wells with a simpler infection protocol. By combining the differentiation and infection steps into a single step, we were able to shorten the protocol by one day. At the same time, we were able to reduce the three rounds of washing that would normally occur between differentiation and infection, which is a source of possible THP-1 cell loss due to detachment.

This protocol was developed for screening inside the THP-1 cell line, which confers several advantages. THP-1, as an immortalized cell line, can be reliably cultured *in vitro* for over 20 passages[23]. This is especially important for large screening campaigns, where it can be challenging to maintain enough cells to supply a high-throughput setup. Additionally, testing in THP-1 provides a homogenous genetic background that minimizes variability in results. This is highly beneficial for testing compounds that influence host cell responses[10]. As an added bonus, gene expression in the THP-1 cell line can be down-regulated by small interfering RNAs (siRNAs)[24]. This provides a

valuable tool for downstream investigations into the mode of action of hit compounds. Although this method was designed using THP-1 cell line, it can be easily adapted for human primary cells, such as peripheral blood mononuclear cells (PBMC), as was previously shown[10].

To best mimic the actual interaction between alveolar macrophages and *M. tuberculosis*, the intracellular assay protocol includes a step to opsonize the bacteria. Opsonization with human serum coats *M. tuberculosis* and facilitates cell entry via complement receptor 3 (CR3)[25]. Naked bacteria are more likely to enter macrophages via the lectin receptor[8]. Given that the bronchoalveolar fluid is known to contain components of human serum[25], opsonization, or the lack thereof, may have a fundamental impact on the screening outcome. However, some may choose to skip this step in order to further simplify the infection process[16], or it may not be feasible to obtain enough human serum due to the size of the library being screened[11].

The intracellular assay includes a step to remove all liquid containing unattached material from the wells prior to the addition of the luciferase reagent. This step is designed to increase the signal-to-noise ratio while reducing the amount reagent used per well. However, the inclusion of this step may generate false-positive data for compounds that kill THP-1 but not *M. tuberculosis*, since detached and lysed THP-1 and free-floating *M. tuberculosis* would be removed. Therefore, the manufacturer's suggested protocol of adding equal amounts of luciferase reagent (100 µL) to each well should be followed in order to assay for *M. tuberculosis* survival in wells containing cytotoxic compounds.

In contrast to the firefly luciferase system, the bacterial lux system does not require external reagents for signal generation[26]. However, the firefly system is preferred over the lux system for the following reasons: First, the bacterial system may be toxic in mycobacteria[26]. Second, a more complex reporter system (5 genes for lux versus 1 gene for firefly) is more susceptible to signal inhibition by test compounds. Lastly, commercially available reagents for the firefly luciferase assay provide the necessary conditions for optimal signal generation. On the other hand, the bacterial luciferase system relies on ATP production and the cofactors present inside the bacteria for signal generation. These can vary between different treatments and are not as easy to control. Therefore, the addition of the luciferase reagent to the firefly system standardizes the reaction conditions and provides more reliable measurements of luciferase activity across all treatments.

CFU determination has long be the gold standard for quantifying bacterial density. In contract, bioluminescence, like most reporters, is not a direct measure of bacteria. Instead, RLU is a function of both CFU and the metabolic state of the bacteria. Others have demonstrated the mostly linear relationship between RLU and CFU for bioluminescent mycobacteria under specific conditions[5]. In any case, a significant reduction in the luciferase assay signal, no matter the underlying cause—other than the actual inhibition of luciferase enzyme activity—would indicate a reduction of the *M. tuberculosis* fitness inside the host cell. Therefore, these compounds would be of

interest from a drug screening standpoint and should not be excluded in method development.

An alternative to the luciferase-based intracellular screening protocol is the automated fluorescent microscopy-based approach[11,27-29]. The luciferase assay output is measured by a luminometer, and the data obtained is quantitative, whereas fluorescent microscopy generates images that are qualitative. However, through clever computer programming, images can be analyzed to generate quantitative data. Furthermore, fluorescent microscopy allows multiple fluorophores to be used concurrently, which is very helpful for gathering valuable parameters such as cell viability, cell counts, and actual rate of infection. As one might predict, these benefits come with some setbacks. The initial investment on automated fluorescent microscopy equipment is many times greater than the cost of a luminometer or fluorometer and is therefore out of reach for many research labs. For those who have access to the equipment, the sample processing must be considered prior to image acquisition and data analysis. Those two steps affect the overall time investment with increases in library size. The inclusion of additional fluorescent labels in host cells requires fixing, staining, and washing steps, thus necessitating additional user intervention and time investments. Furthermore, fluorescent microscopy data collection and analysis, although automated, still requires significantly more time and resources than the simple luciferase assay readout. Therefore, the luciferase reporter-based intracellular screening method is simpler and capable of higher throughput.

The luciferase-based intracellular screening method has one significant limitation compared to fluorescent microscope-based screening methods. This is due to the fact that the luciferase assay provides no data regarding the health status of host macrophages. Cytotoxic compounds would cause the death of macrophages, and thus live bacteria may be released into the medium and would no longer contribute to the final luciferase assay signal. As a result, cytotoxic compounds would appear to cause the death of intracellular *M. tuberculosis* and thus generate a great number of false positives. To address this issue, we have complemented our method with an MTT assay to assess the cytotoxicity of compounds on host macrophages. This module of the screening method gives us additional information about the drugability of compounds of interest and allows the early elimination of less-than-ideal drug candidates.

Alternatively, one may also use the luciferase-based intracellular assay prior to performing automated fluorescent microscopy. In screens of large compound libraries, this allows for the quick and efficient assessment of compound effectiveness in the macrophage infection model. As a result, automated fluorescent microscopy can be reserved for detailed studies on better candidates, as illustrated by a previously published study[11].

The low cost and simple nature of the luciferase-based intracellular assay also greatly benefits researchers who wish to test smaller chemical libraries. Overall, the luciferase-

based intracellular assay has proven to be an extremely flexible tool for research labs of all calibers and for screening projects of various sizes.

DISCLOSURES:
The authors declare no competing financial interests for this work.

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Figure 1
Figure 1

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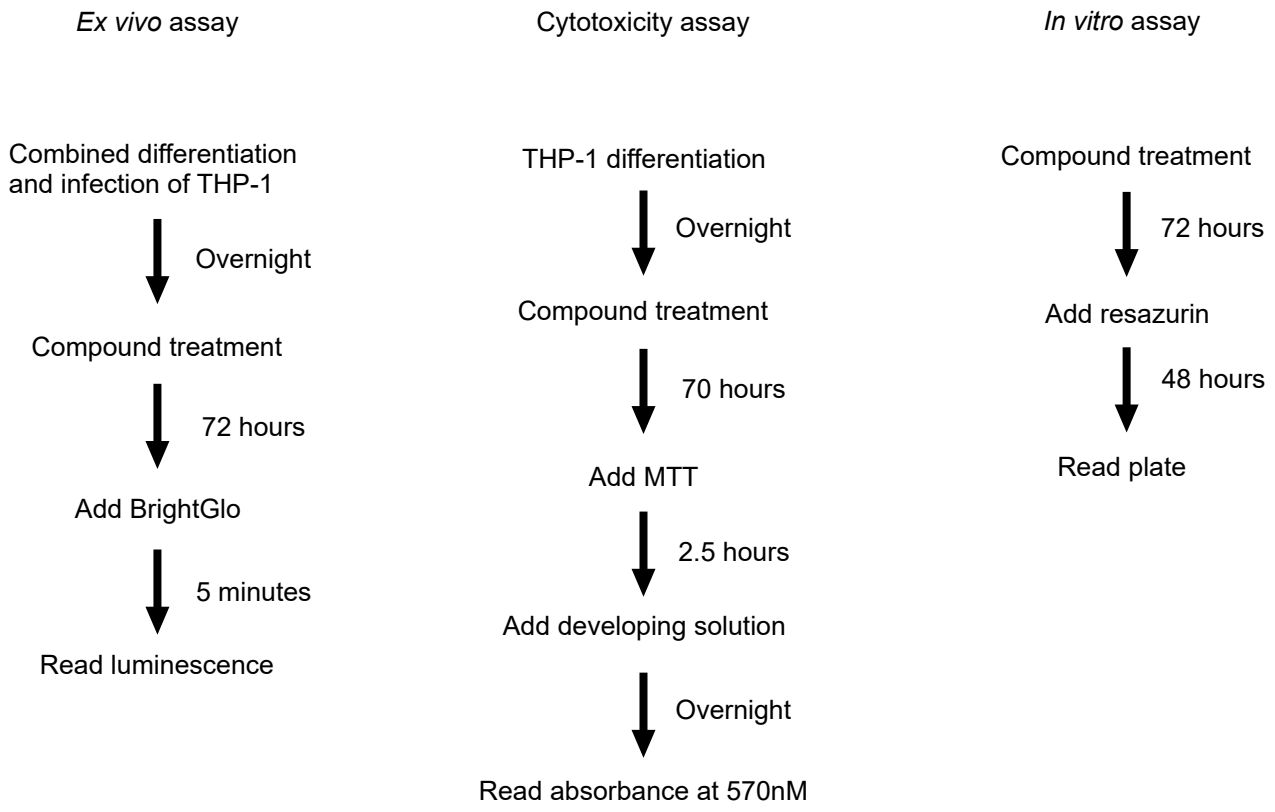

Figure 2

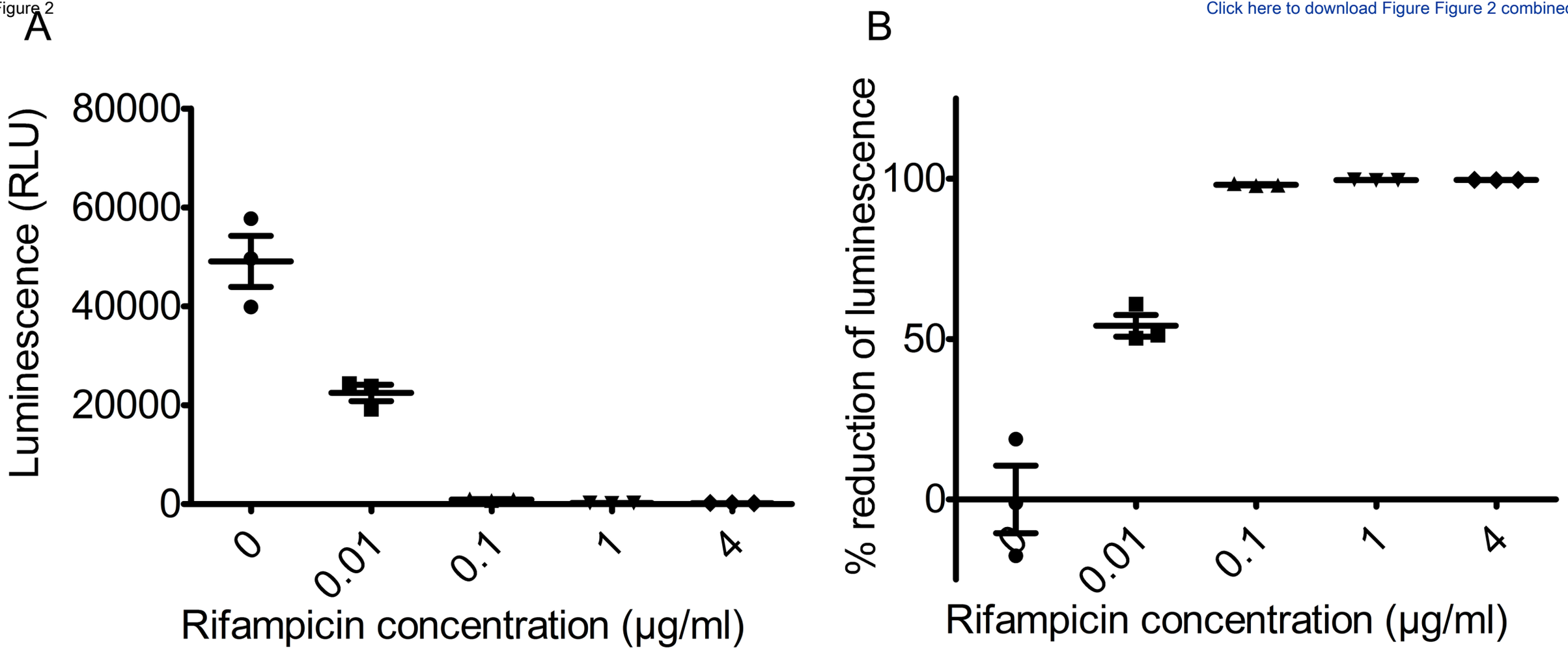

Figure 3

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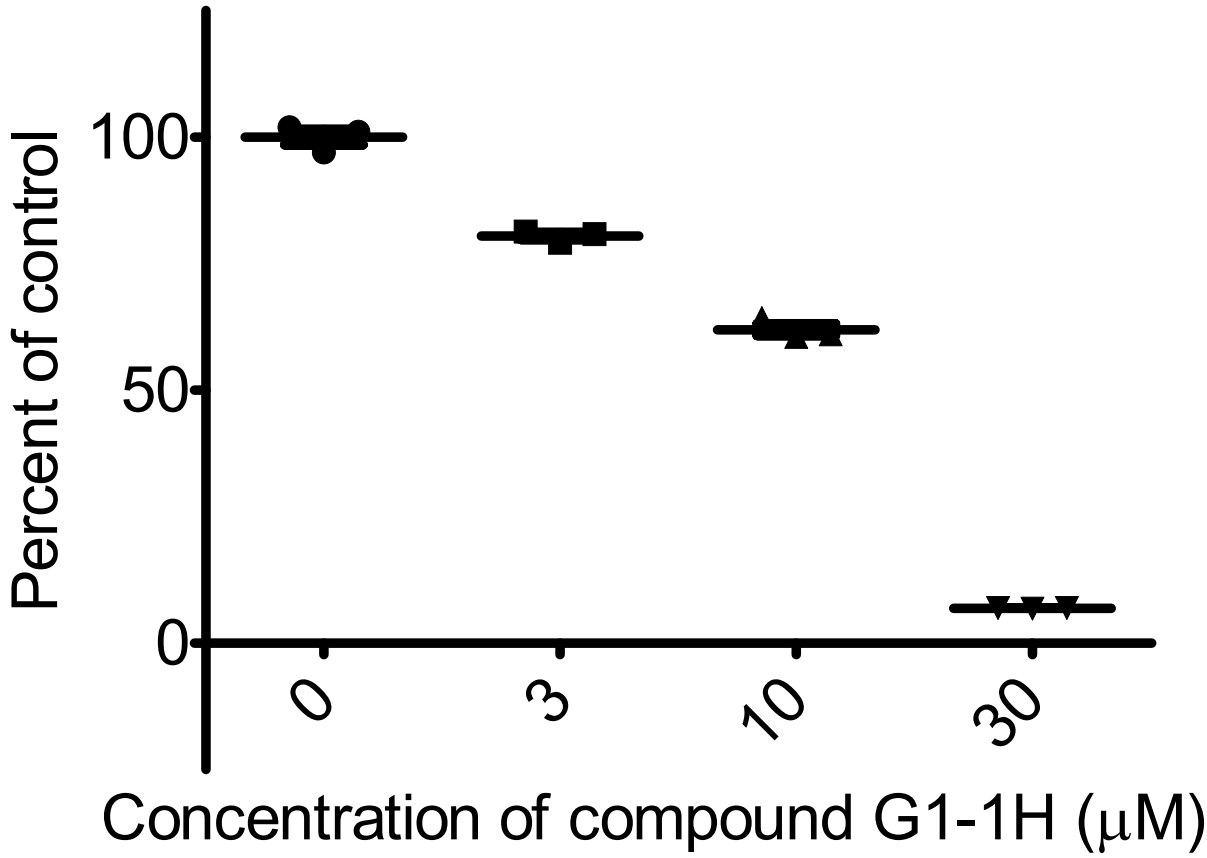

Figure 4

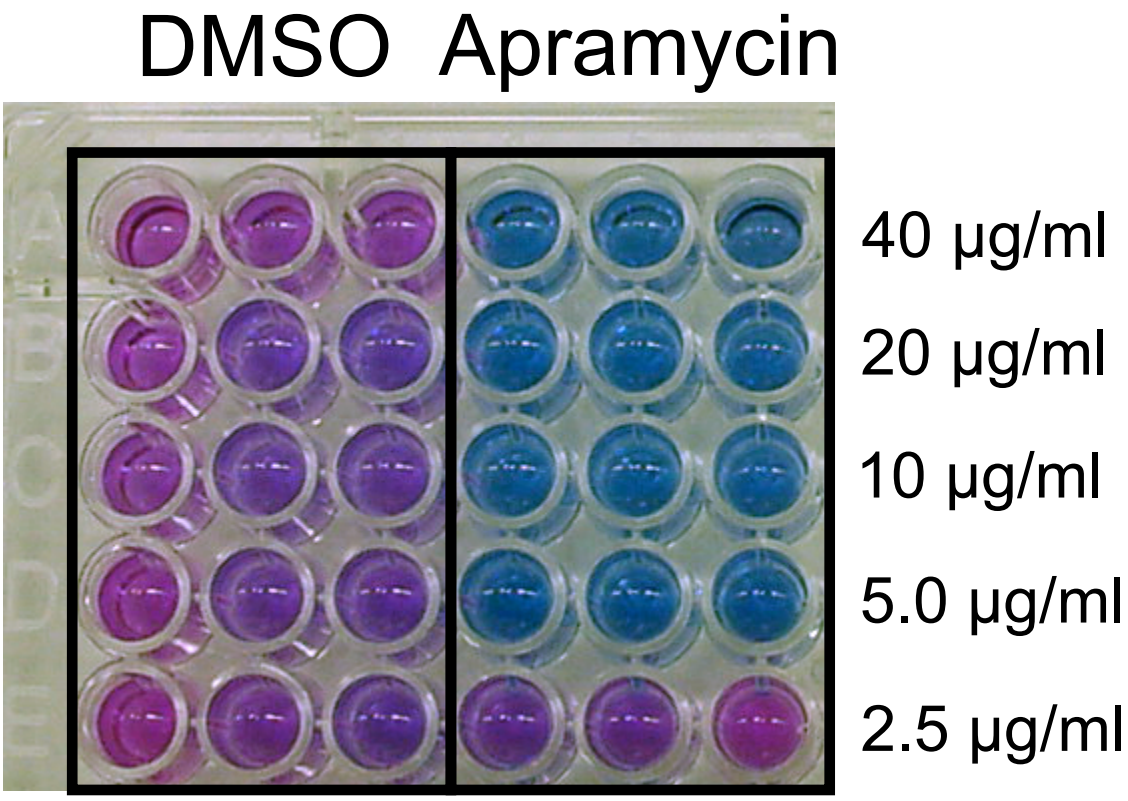

Table 1

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[Rifampicin]	Rep1	Rep2	Rep3	Average	% Reduction
4 µg/ml	184	190	210	195	100
1 µg/ml	244	215	159	206	100
0.1 µg/ml	1037	731	976	915	98
0.01 µg/ml	19200	24400	23919	22506	54
0 µg/ml	39877	49655	57728	49087	0

Table 2

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[G1-1H]	Rep 1 A57C	Rep 2 A57C	Rep 3 A57C	Average A5	% of untreated
30 µM	0.056	0.056	0.055	0.056	7
10 µM	0.518	0.488	0.492	0.499	62
3 µM	0.652	0.638	0.656	0.649	80
0 µM	0.822	0.782	0.815	0.806	100

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment table of materials.xlsx

Name	Company	Catelog Number
RPMI 1640	Sigma-Aldrich	R5886
L-glutamine	Sigma-Aldrich	G7513
Fetal bovine serum (FBS)	Thermo Fisher Scientific	12483020
Middlebrook 7H9	Becton, Dickinson and Company	271210
Tween80	Fisher Scientific	T164
Albumin, Bovine pH7	Affymetrix	10857
Dextrose	Fisher Scientific	BP350
Sodium Chloride	Fisher Scientific	BP358
kanamycin sulfate	Fisher Scientific	BP906
PMA	Sigma-Aldrich	P8139
MTT	Sigma-Aldrich	M2128
N,N-Dimethylformamide (DMF)	Fisher Scientific	D131
1M Hydrocholoric acid (HCl)	Fisher Scientific	351279212
Acetic acid	Fisher Scientific	351269
SDS	Fisher Scientific	BP166
Resazurin	Alfa Aesar	B21187
DMSO	Sigma-Aldrich	D5879
Glycerol	Fisher Scientific	BP229
THP-1	American Type Culture Collection	TIB-202
M. tuberculosis H37Rv		
96-well flat bottom white plate	Corning	3917
95-well flat bottom clear plate	Corning	3595
Transparent plate sealer	Thermo Fisher Scientific	AB-0580
Spectrophotometer	Thermo Fisher Scientific	Biomate 3
Microplate spectrophotometer	Biotek	Epoch
luminometer	Applied Biosystems	Tropix TR717

Comments

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Author(s): XINGJI ZHENG, YOSSEF AV-GAY

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Dear Editor. Thank you for yours and the reviewer's comments. As you can see below we took all comments seriously and addressed each of the issues they raised. Two of the reviewers requested inclusion of some additional references. We have accepted this and included the missing references. We have also added contents to discussion section to address their comments. We have provided point to point response to their comments and expanded appropriate sections to address their concerns. We have also made changes to address all the deficiencies you have listed.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes a relatively high throughput methodology for testing compounds for anti-mycobacterial activity.

Major Concerns:

I have issues with the way the 'representative' data is presented, using means and standard errors. Is this three technical replicates rather than biological replicates? The mean and standard error severely mislead the reader as to the actual variability of the data (see legend of Fig. 6 in this post:https://garstats.wordpress.com/2016/05/27/the-percentile-bootstrap/ for an explanation of this and a visual example). If it really is only three data points, then they should be shown as a scatter plot, or if more than 3-6 data points as a box-whisker plot. If this is just technical replicates of one experiment, then the data is essentially meaningless and biological replicates should also be included.

Thank you for your comment. Since mean and SEM are standard statistical measurements of the data we respectfully keep maintaining these as our statistical measures. The mean is designed to reduce inconsistencies between the technical replicates, and SEM is a measure of the confidence on the mean for a small dataset. This is common practice for screening projects as exemplified in following references[1-3]**.** Furthermore, both were used to efficiently identify hit compounds against intracellular *M. tuberculosis*, the mean value is only there to serve as a measure of the compounds activity to compare to an artificial cut-off value. The cut-off values for filtering compound is usually set lower than the ideal value in fear of losing compounds. Therefore, extra repetitions and superior statistical accuracy may only be theoretical improvements. In any case Hit compounds from initial screening are scrutinized in greater detail with follow-up experiments. Therefore, we consider mean and SEM to be more than adequate for the purpose of initial compound screening.

Minor Concerns:

In the introduction, the authors cite the work of Andreu et al (reference 5) who developed a luciferase-based macrophage infection assay similar to that described in this manuscript. Andreu et all compared using the firefly luciferase with the bacterial luciferase, which does not require the addition of substrate. The authors found that the bacterial luciferase assay was cheaper, quicker and less variable. This should be commented on in the discussion, and reference made to their alternative bacterial luciferase construct (Andreu et al, PLOS One. 2010 May 24;5(5):e10777) as it provides an alternative to the assay described for those who wish to take up the proposed methodology but would prefer to remove the requirement for luciferin.

We agree that a brief discussion about various luciferase systems would help the readers to make a more informed decision regard to which system is more suitable for their specific needs.

As the reviewer #1mentioned, bacterial luciferase assay requires no external reagents, and thus bacterial luciferase system would be more suitable for in vivo studies and would further reduce the cost for *in vitro* work. However, the firefly luciferase system has a few benefits over the bacterial system. Bacterial luciferase system is made up of two (or five) proteins, over expression of either extra genes at high levels would likely have a greater impact on Mtb growth rate both in intra- and extracellular environments. As an example, *luxD* is toxic at higher concentrations as mentioned by Andreu *et al.* in the same publication mentioned by reviewer #1. A more complex reaction also has increased susceptibility to signal inhibitions, which would increase number of false positive observations. Lastly, commercially available reagents used for firefly luciferase assay is a complex mixture that contains ATP and DTT (just to name two) in addition to luciferin. The reagent provides the necessary condition for optimal signal generation, whereas the bacterial luciferase system relies on ATP and cofactors inside the bacteria for signal generation. Therefore, the luciferase reagent equalizes the reaction condition across samples. The signal generated from firefly luciferase assay is mainly based on the amount luciferase enzymes produced by Mtb, and thus should offer greater consistency between wells treated by different compounds.

We have added this as a paragraph in the discussion after line 392.

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

The manuscript 'System for efficacy and cytotoxicity screening of inhibitors targeting intracellular Mycobacterium tuberculosis' by Zheng and Yossef is describing a luciferase-based assay to monitor the growth of Mycobacterium tuberculosis inside cells. The approach to use luciferase-expressing mycobacteria is not new. A very similar detailed protocol was published in 2010 by Eklund et al (Validation of a medium-throughput method for evaluation of intracellular growth of Mycobacterium tuberculosis. Clin Vaccine Immunol. 2010 Apr;17(4):513-7.) This is not cited in the manuscript.

We appreciate this comment and included the above reference in our revised manuscript. This is discussed in detail as per reviewer #1's comment about bacterial luciferase system.

Others have later published variants of that protocol: the reference number 5 in the manuscript and Ozeki Y et al, PLoS One. 2015 Nov 16;10(11):e0141658. A New Screen for Tuberculosis Drug Candidates Utilizing a Luciferase-Expressing Recombinant Mycobacterium bovis Bacillus Calmette-Guéren.)

Thank you for the comment. Yet this paper described a protocol developed using the vaccine strain of *M. bovis* (BCG) and murine macrophages. As other have shown that compound efficacy in BCG in not necessarily maintained in *M. tuberculosis*[4] and as we discussed murine macrophages are different from human derived macrophages neither of which methods are disease relevant. We have included said points in the first paragraph of the discussion section and clarified our point about the practicality of using different systems in TB drug development. Appropriate references have also been supplied to justify the need for using disease relevant cell types for screening.

JoVE has earlier published a HTS approach that can be used to monitor intracellular growth of mycobacteria (A Microscopic Phenotypic Assay for the Quantification of Intracellular Mycobacteria Adapted for High-throughput/High-content Screening, Christophe. J Queval et al.). On line 97, the authors mention the HTS microscopy-based methods that have previously been described, but they do not cite the paper in the reference list.

This and additional references regarding high-content screen efforts have been added.

A method for testing viability of the cells and the quantification of bacteria from the same samples would be preferable over parallel testing as described. This is possible using for example the Calcein-AM protocol for cell viability in the cells before lysing them and assessing bacterial numbers. From the flow chart one could conclude that the viability testing is done on non-infected cells, which is irrational.

Thank you for the comment. To clarify, testing toxicity on uninfected cell is very important as in the actual disease most of the host cells are NOT infected with Mtb. We agree that assessing the toxicity on infected cells is invaluable to drug screening, and currently there is no simple way of obtaining toxicity and bacterial viability in a high throughput manner without using high-content screening methods. This is why we recommend performing high-content screening on hit compounds from luciferase assay as mentioned in the discussion section. Calcein-AM method is capable of providing toxicity using fluorescence; however it is not well suited for high throughput since the reagents are costly. High-content screening would better serve the purpose.

The assay is not thoroughly characterized. What about the linearity of the luciferase-method? How do CFU correlate with RLU?

This has been added to the discussion section after line 419. Luciferase assay is a measure of luciferase activity inside Mtb. The luciferase activity inside each well is a function of enzyme copy number and the number of bacteria (more or less measured by CFU), and the enzyme copy number is affected by various biological processes that controls protein production and degradation. These processes can be affected by the compounds being tested, as well as how the macrophage is being affected by test compounds. In short, the relationship between CFU and RLU is not always linear. Inhibition of the luciferase signal indicates inhibition of bacterial count and/or health status. In any case, significant inhibition of overall signal, no matter the underline cause other than actual inhibition of luciferase enzyme, indicates reduction in *M. tuberculosis* fitness inside host cells. Therefore, these compounds would be of interest regardless of the relationship between CFU and RLU from the screening method development standpoint.

A previous publication has examined relationship between CFU and RLU for different luciferase systems[5]. Furthermore, our experience with 384-well system showed very good correlation between luciferase assay and high-content screening results[3].

How do the authors assess inter- and intra-assay variability?

As of now they merely mentioned this as 'It is normal for base luminescent levels to vary between experiments' (line 229-30).
The manuscript (text, data and presentation of data) do not reach a minimum level of scientific stringency.

Intra-assay variability is accounted for with technical replications. Normalizing data using untreated sample is able to account for variations between biological replicates. It is unclear to us what else the reviewer is looking for.

Major Concerns:
N/A

Minor Concerns:
What was the source of the plasmid?

The source of the plasmid is from reference #12 as mentioned in line 140.

The word 'bug' for a bacterium should not be used in scientific texts.

The word “bug” has been replaced.

The 96-well plate format is medium-throughput but not high throughput. Compound libraries

High throughput commonly refers to 96-well plates and up, and more importantly it refers to compatibility with automation equipment. Although definition may change as equipment design improves over time. We believe it is justified to keep “high” throughput since there is a physical limitation in miniaturization of cell-based assays.

THP-1 is a cell line and cannot be said to be 'ex vivo'. Ex vivo means that primary cell/tissue material is extracted from a living organism and experiments are performed outside the body.

This method was developed using THP-1 cell line. Yet the same method has been used with human peripheral blood monocytic cells (PBMCs) as illustrated in reference #10. However, we will concede that screening using THP-1 is not truely *ex vivo*, and it is not feasible to screen large libraries using PBMCs. We have made changes throughout the text and refer to THP-1 infection as “intracellular”, and extracellular *M. tuberculosis* experiments as “in-broth” as recommended by a later comment.

The rationale for the resazurin assay is unclear. Why cannot the luciferase assay be used for determining growth in broth?

Luciferase assay can indeed be used for in broth growth determination; however we opted for resazurin method as our desire to strive for greater fiscal responsibility (AKA cheaper). We have opted to leave this out as to not mention cost reduction excessively.

Also, the THP-1/mycobacteria assay is in vitro, so instead the word 'in broth' could be used to discriminate from the 'intracellular' assay.

This point is addressed for the comment regarding "*ex vivo*" usage.

On line 229: the luciferase is not a measure of mycobacterial metabolism.

This point is addressed for the comment regarding RLU vs CFU.

The text contains many grammatical errors and typos.

We have made many corrections to address these.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
The manuscript by Jain and Av-Gay describes a marvelous simple system for screening for compounds that are active in Mycobacterium tuberculosis macrophages. The protocol is exceptionally well written and clear and should b easy to use for many groups. The data provided is straightforward and compelling. It is clear that this would be a valuable protocol for many to use in the tuberculosis research community but also has applicability in other systems. I whole-hearted recommenced they produce this video. The manuscript is fine as written.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

We hope that we have addressed the comments and suggestions made by the editor and first two reviewers. Please feel free to contact us if any clarification is needed.

Sincerely,

Xingji Zheng

Dr. Yossef Av-gay

1 Eklund, D. *et al.* Validation of a medium-throughput method for evaluation of intracellular growth of Mycobacterium tuberculosis. *Clin Vaccine Immunol* **17**, 513-517, doi:10.1128/CVI.00446-09 (2010).

2 Lam, K. K. *et al.* Nitazoxanide stimulates autophagy and inhibits mTORC1 signaling and intracellular proliferation of Mycobacterium tuberculosis. *PLoS Pathog* **8**, e1002691, doi:10.1371/journal.ppat.1002691 (2012).

3 Sorrentino, F. *et al.* Development of an Intracellular Screen for New Compounds Able To Inhibit Mycobacterium tuberculosis Growth in Human Macrophages. *Antimicrob Agents Chemother* **60**, 640-645, doi:10.1128/AAC.01920-15 (2015).

4 Ballell, L. *et al.* Fueling open-source drug discovery: 177 small-molecule leads against tuberculosis. *ChemMedChem* **8**, 313-321, doi:10.1002/cmdc.201200428 (2013).

5 Andreu, N. *et al.* Optimisation of bioluminescent reporters for use with mycobacteria. *PLoS One* **5**, e10777, doi:10.1371/journal.pone.0010777 (2010).