**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/](https://garstats.wordpress.com/2016/05/27/the-percentile-bootstrap/" \t "_blank) 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](#_ENREF_1)**.** 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](#_ENREF_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](#_ENREF_5). Furthermore, our experience with 384-well system showed very good correlation between luciferase assay and high-content screening results[3](#_ENREF_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).