**Dear Dr. Steindel and reviewers,**

**I would like to thank each of you for your insightful comments and concerns. Detailed below are the changes made in response to each comment individually. Your comments will be copied verbatim in black, and our response will be in blue.**

**Editorial comments:**  
Changes to be made by the author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been carefully proofread for spelling and grammar mistakes, and all

identified issues have been corrected.  
2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Copyright permission email is attached and Figures are cited in the Figure Legend  
3. Figures: Please include a scale bar for images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend. Please also define error bars in the figure legend.

Scale bars have been added to each microscope image, and scale has been defined within

the figure legends  
4. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

Figures are uploaded individually.

5. Table of Equipment and Materials: Please revise to include the name, company, and catalog number of all relevant materials. Please provide lot numbers and RRIDs of antibodies, if available. Please sort the items in alphabetical order according to the Name of Material/ Equipment.

The Table of Equipment and Materials has been updated to include all requested

information.  
6. Please revise the title to avoid punctuation.

The title has been revised to exclude punctuation.  
7. Please provide an email address for each author.

Email addresses for each author have been included in the manuscript.  
8. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis).

Reference numbers have been changed to superscript following proper placement as per

editorial suggestion.  
9. Line 30: The references should be numbered in order of appearance; 11 and 12 should be 3 and 4.

References were re-numbered in order of appearance.

10. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Centrifuge speeds were converted to centrifugal force (x g) rather than rpm.  
11. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Sigma-Aldrich, MP Biomedicals, Cedarline Laboratories, Life Technologies, Thermo Fisher, etc.

All commercial language was removed from the manuscript.  
12. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Ethics statement was added to manuscript before numbered protocol steps.  
13. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Language throughout the protocol was revised to include only imperative tense

instructions.  
14. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Protocol steps were divided to reduce the number of actions per step.  
15. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Detail within protocol steps has been increased to avoid any potential confusion of those

wishing to replicate these procedures.  
16. 1.1: Please specify the euthanasia method and the surgical instruments used to expose the trachea.

Requested details have been included.  
17. 1.2: What volume of DMEM with 10 % non-heat inactivated FBS is used? Please specify the temperature for culturing primary alveolar macrophages. Please specify the volume/weight of FITC-beads added.

Requested details have been included.  
18. 1.3: What volume of PBS is used to wash?

Requested details have been included.

19. 1.4: Please describe how to quantify phagocytosis and how to calculate percentage positivity and mean fluorescence intensity.

Requested details have been included.  
20. 2.1: Please specify incubation temperature.

Requested details have been included.  
21. 4.1: Please specify growth conditions.

Requested details have been included.  
22. 4.2: Please specify the dose of ketamine-xylazine. Please mention how proper anesthetization is confirmed.

Requested details have been included.  
23. 4.3: Please describe how to collect the bronchoalveolar lavage fluid (BALF). Please specify centrifugation conditions.

Protocol has been edited to refer the reader to steps 1.1-1.6, which detail exact procedure

for BALF collection and macrophage pelleting.  
24. 5.1: Please mention how the mice are anesthetized.

Protocol has been edited to refer the reader to step 4.2 for anesthetization procedure.  
25. Please include single-line spaces between all paragraphs, headings, steps, etc.

Manuscript has been edited to use single spacing throughout.  
26. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The steps that should be visualized are highlighted.   
27. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

These instructions were considered when highlighting the protocol for the video.  
28. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

These instructions were considered when highlighting the protocol for the video.  
29. Discussion: Please also discuss the significance with respect to existing methods and any future applications of the technique.  
30. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below:  
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

References were edited to use this format.  
  
**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
The focus of this manuscript is supposed to be a 'comprehensive methodology' (per the title) to determine phagocytic uptake by alveolar macrophages. Not only does the manuscript not reflect a comprehensive methodology, it describes methodology that does not rigorously measure phagocytosis.  
We would like to thank the reviewer for careful and thorough reading of this manuscript and for the thoughtful comments and constructive suggestions, which help to improve the quality of this manuscript.  
Major Concerns:

1) The use of 'comprehensive' in the title and in the manuscript is misleading since there are other, and better, methods to quantitatively measure phagocytic uptake, particularly of bacteria. For example, the gentamicin protection assay and even microscopic assays that distinguish the plasma membranes of the cells.

The title has been edited to remove the term ‘comprehensive’. Relevant references are cited.

2) Important: the described assays do NOT distinguish between internalized bacteria or beads and those that are cell-surface associated. The assays described assess total cell-associated particulates, not internalized. No rigorous methodology is described to distinguish between associated and internalized beads (Section 1) or fluorescent particles or bacteria (Sections 3, 4).

Where it is sufficient to remove all extracellular fluorescent material, the protocol

outlines the use of multiple PBS washes (Sections 1, 3). Where PBS washes would be

ineffective, the protocol details the use of ACK lysis buffer to remove extracellular

SRBCs (Section 2).

3) The description of alveolar macrophage isolation is deficient, as is a description of why MH-S cell may (or might not) be used in place of alveolar macrophages.

The macrophage isolation procedure has been edited to include more detail.

Use of MH-S cells is beneficial due to potential for genetic modulation, as is mentioned

in the third discussion paragraph.  
4) Section 5 does not belong in the protocol, it is not specific to alveolar macrophages nor does it rigorously describe phagocytosis. It more likely describes PMN-mediated killing by a variety of mechanisms.

Although a previous report suggests that PMNs are major innate immune cells ﬁghting *P. aeruginosa* infection, we believe that TRIM72, which is used for a comparison purpose in this protocol, does not regulate PMN function, because it is not expressed in neutrophils. As AM phagocytosis of invading pathogens occurred earlier than any signiﬁcant amount of neutrophil recruitment to the airspace, we believe the observed bacterial clearance is due to AMs and regulated by TRIM72.  
5) The figures are poor. The representative figure of zymosan (Fig.2 ) is hard to see and not informative, the same is true of Fig. 3A, and the circles/squares of Fig. 4 are not accurately described in the legend with respect to the figure.

Quality of specified figures has been improved. Figure 4’s circles and squares are now

accurately described in the legend.  
6) TRIM72 was not introduced in the text but is featured in the results. The role and inclusion of TRIM72 is unnecessary to this methods proposal.

The inclusion of TRIM72 was for demonstration purposes. Our lab has used this protocol

to study the effects of TRIM72 on macrophage phagocytosis, so this data was available to

use as a representation of the functionality of these methods.  
7) The statistical tests in this manuscript are not identified.

Statistical analysis section was added to the manuscript.  
  
Minor Concerns:  
1) P. aeruginosa should be italicized throughout the text.

Each mention of *P. aeruginosa* has been italicized.   
  
  
**Reviewer #2:**  
Manuscript Summary  
This is an interesting protocol.

We appreciate the positive feedback from the reviewer.  
  
Minor Concerns:  
please change the title in assessing murine alveolar macrophages phagocytosis...  
This is more informative and human alveolar macrophages are different from the same murine cells.

The title has been edited to specify murine alveolar macrophages.  
I would also underline the importance of using the appropriate controls for FITC-beads considering that the autofluorescence of the alveolar macrophages is in the same spectrum of FITC.

We have used non-treated AMs as unstained cells or controls in flow cytometry.   
  
**Reviewer #3:**   
Manuscript Summary:   
In this manuscript the authors present a method for analyzing alveolar macrophage phagocytic function and bacterial clearance from the lung. This method involves isolation of macrophages and treatment with fluorescent beads to determine phagocytic capacity. They also provide methods to differentiate among PRR, CR and FcγR mediated phagocytosis.  
We greatly appreciate the positive feedback from the reviewer. We thank the reviewer for thorough reading of this manuscript.   
  
Major Concerns:  
None  
  
Minor Concerns:  
While this is a comprehensive method to evaluate phagocytic function of alveolar macrophages, there are other methods that are not discussed here (e.g., pHrodo).

The relevant methods are cited.   
**Reviewer #4:**  
Manuscript Summary:  
Manuscript be Nagre et al claims to provide comprehensive methodology of assessing alveolar macrophage phagocytosis, however I have not found anything in that manuscript that was not published previously, moreover techniques described are quite standard and commonly used in the laboratories across the world.

In addition although authors aim to 'report methods for comprehensive analysis of the phagocytic function of AMs using in vitro and in vivo assays, and experimental strategies to differentiate pattern recognition receptor-, complement receptor- and Fc gamma receptor-mediated phagocytosis', they somewhat interchangeably use primary murine alveolar macrophages and MH-S cell line.

Use of MH-S cells is beneficial due to potential for genetic modulation, as is mentioned

in the third discussion paragraph.

Also there is no mention of the controls which should be done in each of the protocols.

In Flow cytometry experiment, unstained AMs were used as controls. We have used TRIM72 genetic modification for a comparison purpose, and we used comparison between WT, TRIM72KO and TRIM72OE.

Not clear why 'Characterization of *P. aeruginosa* pneumonia model' was included in the manuscript.

*P. aeruginosa* model is included to bring more insight into *in vivo* protocol. Although *P. aeruginosa* GFP clearance highlights the methodology for *in vivo* phagocytosis, we believe, including an assay to explain *P. aeruginosa* bacterial clearance mechanism in pneumonia mouse model will be an added advantage.

In compliance with data protection regulations, please contact the publication office if you would like to have your personal information removed from the database.