We thank the reviewers for their thoughtful criticism of our work and for their suggestions to improve the manuscript. We have incorporated their recommendations into the revised manuscript and believe that this makes it a clearer, more useful resource for others. Line and page numbering in the response lines refer to the revised manuscript.

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. We have corrected additional small typographical errors which we found.

2. Please revise lines 391-398 to avoid previously published text. This has been revised.

3. Keywords: Please provide at least 6 keywords or phrases. We added in an additional term to make it 6 key words.

4. Please expand the Summary to briefly describe the applications of this protocol. This has been added (lines 27-30).

5. Please define all abbreviations before use. This has been corrected (expanded in key words).

6. 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. Please move the discussion about the protocol to the Discussion.

The use of “should” has been removed and “Notes” have been added.

7. 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. Additional clarifications and details have been added to the protocol. The specific points have also been addressed.

8. 1.1: Please list an approximate volume of media to prepare. This has been added as “Notes” in lines 115-116, 122-123, 128-129.

9. 1.2.1: Please provide the composition of LADMAC growth media. If it is purchased, please cite the Table of Materials. This is defined under 1.1.2, lines 118-120.

10. 1.3.4: Please specify the size of the petri dish. This was added in line 166.

11. 2.2: Please specify the concentration of Trypsin-EDTA. This was added in line 181.

12. 4.1 and 4.2, etc.: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a “Note.” This has been edited and tense has been changed in lines 305 and 313.

13. 5.5: What volume of PBS is used to wash? Please specify. This has been added in line 348.

14. Lines 328-335: Please list them as sub-steps. This has been edited in lines 471-478.

15. 7.2, 7.4: Please point out the specific steps that are being repeated here. The compensation correction section has been deleted and incorporated into the text to occur earlier as suggested by a reviewer.

16. 8.4: Please describe how to perform manual compensation. This is now described in sections 6.5-6.12.

17. 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. These parts have been highlighted.

18. 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. Please do not highlight any steps describing anesthetization and euthanasia. This has been noted during highlighting.

19. 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. This has been noted during highlighting.

20. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol. Data from both successful and sub-optimal experiments can be included.

Paragraphs describing the results have been added along with additional figures and descriptions of sub-optimal experiments.

21. Discussion: Please discuss any limitations and future applications of the technique. This has been added.

22. Please remove the embedded figure(s) from the manuscript. This has been removed.

23. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment. This has been edited.

24. References: Please do not abbreviate journal titles. Although the JoVE EndNote style file was used and the “Do not abbreviate journal titles” was selected, abbreviation was still applied by the program. Our apologies for this technical difficulty.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes a method for determining production of ROS in murine-bone marrow-derived macrophages stimulated by their receptors for IgG (FcgR). It uses simultaneously two fluorescent probes: 2',7' DCFH2-DA), which when oxidized to DCF is fluorescent and can be detected in the fluorescein (FL1) channel, and dihydroethidium (DHE) , which is oxidized to hydroxyethidium that is fluorescent at different wavelengths and can be detected in the FL2 channel.

The manuscript is overall well-written and the procedure is clearly explained. I have just minor concerns (below)

Minor Concerns:

1) As the manuscript describes the use of a commercial kit, it is strange that the manufacturer and catalog number is not given in the materials and methods section. Is this because of journal policies? This is indicated in a separate Materials file during the submission.

Also, it would be desirable if the authors comment on the use of different kits and/or using the same fluorescent probes obtained from another manufacturer. This can benefit the readers that may already have in their labs some of the reagents used in the kit. We have performed this and present the results in new Fig 6.

2) For differentiation of macrophages the authors recommend LADMAC conditioned médium. The use of L929 conditioned medium seems to me to be more common in the literature. Have the authors comments for the use of LADMAC medium over the use of L929 medium or the direct addition of M-CSF to the medium? Again, it would be useful to share their experience for users that may have used other sources of M-CSF to differentiate the bone marrow cells.

We, personally, have not done a side-by-side comparison between LADMAC conditioned media, L929 conditioned media, or recombinant cytokines. However, a former colleague who has used both LADMAC and L929 conditioned media reported that better results were obtained with LADMAC supernatants.

3) The ROS signal after FcgR stimulation seems to be rather small. Have the authors primed their cells for longer than 24 hours?. In many labs, IFN priming is carried out for 48 hours. Is the lenght of the priming critical? Could different priming times optimize the amount of ROS produced by the phagosomal NADPH oxidase? We have performed these suggested experiments and, indeed, we do see increased ROS as detected by the orange probe with extended priming time but no differences in ROS detected by the green probe (new Fig 5).

4) Finally, the authors underline the importance of performing the measurements at the exact amount of time (30 minutes) after addition of all reagents, for all samples. They advise to add the necessary reagents to each tube taking into consideration the time that the cytometer takes to analyze one sample and move on to the next. I fully agree with them. And because of this, I consider that the time needed for a proper calibration might be longer, as usually it needs some adjustments to the instrument and re-analyzing the one-color samples. Thus, maybe a note could be included to warn the reader of the possibility of considering more time for the calibrating samples. Thank you for this note. We have re-written the protocol to incorporate the compensation step prior to sample analysis to ensure this will not impact the stimulation times in the cases where no templates or after-the-fact compensation is available.

Reviewer #2:

Manuscript Summary:

In this paper, Shehat et al describe an alternative method to detect ROS production in a single cell level. The protocol is very clear and will be of interest of other readers.

Major Concerns:

The major concern is that this method is based on a kit that authors use to run their ROS quantification. It would be very important to validate how necessary is to use such kit in comparison to other reagents that can be purchased in the market (not belonging to a kit).

Regarding the costs, again, what would be the advantages of using a kit in comparison to use other fluorescent reagents? We have performed a comparison with individually obtained reagents and present the results in new Fig 6. We additionally mention in the text the benefits of using the kit versus individual reagents and, depending on how fast experimentation is expected to take place, suggest use of one or the other.

Also, it would be very relevant if certain populations of cells could be simultaneously stained by other anti-bodies allowing the assessment of ROS production in different cell populations. We have performed this suggestion and added the results as new Fig 7.

Minor Concerns:

None

Reviewer #3: In the manuscript, the authors demonstrate and describe in detail how FcγR-induced ROS production by macrophages can be measured employing fluorescent probes and flow cytometry. The protocol is well written, understandable and allows for easy reproduction by the reader. They provide an endpoint assay for flow-cytometric measurement of production of intracellular ROS. I understand that the assay displays, as stated by the authors, an inter-assay variability, which authors try to resolve by reporting their results as the ratio/percentage of ROS-positive cells, which seems to be a good technical solution for the superoxide detection reagent, where it is clear (Figure 2, bottom row, right panel) that FcγR stimulation results in a portion of cells producing ROS, and a large portion not producing any ROS. Could the authors comment on, from a biological perspective, the fact that rather than all cells producing a certain amount of ROS, which would result in a peak shift, only a fraction of cells seem to respond to the stimulation, despite similar treatment? This is a good point. We have no definitive answer for this except that for many other biological responses we study, where cells are supposedly uniform (even cell lines) and the stimulus is consistent, only a fraction of cells can be seen to respond (for example, degranulation assays, intracellular cytokine experiments where mitogen is applied). The shortcomings could be a result of the technique or a limitation of the approach, where the sensitivity might not be high enough, or the signal is too transient.

As is clear from Figure 2, bottom row, left panel (oxidative stress reagent), it seems like rather than a portion of cells becoming positive, the peak widens, and also shifts to the right, especially as compared to the bottom condition (FcγR stimulation plus ROS inhibitor). Is it possible to represent results in terms of MFI, instead of ROS positivity, normalized to stained but not stimulated cells? Can this aid in decreasing inter-assay variability? Thank you for this suggestion. We now present the data as both percentages and as MFI. Although largely mirroring each other, with regards to the effect of the ROS inhibitor, percentages seem to better reflect what is occurring visually from the dot plots or histograms (especially with the superoxide detection reagent).

All in all, it is I think very important to see how reproducible the assay is, as whole experiments cannot always be performed in one single assay on one day. Could the authors show more than one representative results so that the reader can get a feel of what amount of variation it is to be expected? Yes, this is a very relevant suggestion. We show 3 independently performed experiments (performed on different days) side by side in new Fig 3 and present the mean and SEM values for our results.