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Corresponding Author:	Justine Tigno-Aranjuez UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	Justine.Tigno-Aranjuez@ucf.edu
Order of Authors:	Michael G. Shehat Justine Tigno-Aranjuez
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TITLE:

Flow Cytometric Measurement of ROS Production in Macrophages in Response to FcγR Cross-linking

AUTHORS & AFFILIATIONS:

Michael G. Shehat¹, Justine T. Tigno-Aranjuez¹

¹ Division of Immunity and Pathogenesis, Burnett School of Biomedical Sciences, University of Central Florida, FL, USA

Corresponding Author:

Justine T. Tigno-Aranjuez
justine.tigno-aranjuez@ucf.edu

Email Addresses of Co-authors:

Michael G. Shehat
michael.shehat@knights.ucf.edu

KEYWORDS:

reactive oxygen species (ROS), flow cytometry, Fc gamma receptor (FcγR), Fc receptors, macrophages, respiratory burst

SUMMARY:

This study demonstrates the use of flow cytometry to detect reactive oxygen species (ROS) production resulting from activation of the FcγR. This method can be used to assess changes in the antimicrobial and redox signaling function of phagocytes in response to immune complexes, opsonized microorganisms, or direct FcγR cross-linking.

ABSTRACT:

The oxidative or respiratory burst is used to describe the rapid consumption of oxygen and generation of reactive oxygen species (ROS) by phagocytes in response to various immune stimuli. ROS generated during immune activation exerts potent antimicrobial activity primarily through the ability of ROS to damage DNA and proteins, causing death of microorganisms. Being able to measure ROS production reproducibly and with ease is necessary in order to assess the contribution of various pathways and molecules to this mechanism of host defense. In this paper, we demonstrate the use of fluorescent probes and flow cytometry to detect ROS production. Although widely used, fluorescent measurement of ROS is notoriously problematic, especially with regards to measurement of ROS induced by specific and not mitogenic stimuli. We present a detailed methodology to detect ROS generated as a result of specific FcγR stimulation beginning with macrophage generation, priming, staining, FcγR cross-linking, and ending with flow cytometric analysis.

INTRODUCTION:

Reactive oxygen species (ROS) are reactive molecules or free radicals that are by-products of aerobic respiration (reviewed in ¹). These include the superoxide anion, peroxide, hydrogen peroxide, hydroxyl radical, and hydroxyl ions, among others. Under normal physiologic conditions, ROS are produced mainly by the mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and are rapidly detoxified by various enzymes and proteins such as superoxide dismutase and glutathione. An exaggerated production of ROS or a defect in the ability to remove ROS can result in oxidative stress, whereby reactive oxygen species promote the damage of proteins, lipids, and DNA leading to cellular stress or death and pathological disease states. However, it is currently appreciated that ROS can also act as signaling molecules (redox signaling), and ROS-mediated modification of various molecules and pathway intermediates can influence cellular metabolism, proliferation, survival, inflammatory signaling, and aging². In phagocytic cells, ROS plays an essential role in providing antimicrobial activity during the so-called “respiratory burst”^{1,3-6}. During the response of phagocytes to external stimuli, components of the NADPH oxidase complex (p40^{phox}, p47^{phox}, p67^{phox}) translocate from the cytosol to the phagosomal membrane containing the gp91phox and p22phox subunits, and together with the actions of Rac1/2, form a fully functional NADPH oxidase enzyme complex. The assembled NADPH oxidase then utilizes NADPH to reduce oxygen to superoxide within the phagosomal vacuole. Superoxide anions can directly cause damage or be dismutated into hydrogen peroxide. Both superoxide and hydrogen peroxide can react with other molecules to generate highly reactive hydroxyl radicals. Damage is mediated by reaction of these ROS with iron-sulfur clusters on proteins or by causing base oxidation of DNA, ultimately leading to restricted microbial metabolism or death of the microbe⁵. The importance of the NADPH oxidase enzyme complex and ROS produced during the respiratory burst is illustrated clinically in patients with Chronic Granulomatous Disease (CGD)⁷⁻¹⁰. Individuals with CGD possess mutations in gp91phox, resulting in a lack of ROS production and susceptibility to recurrent infections with bacteria and fungi which are not usually a concern with immunocompetent individuals. Therefore, whether studying oxidative stress, redox signaling, or host defense, being able to measure ROS production in real-time is a useful endeavor.

Multiple assays have been utilized to measure ROS production or the results of oxidative stress¹¹⁻¹³. Among these, one of the most widely used is the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA)¹⁴. This molecule is colorless and lipophilic. Diffusion of DCFH₂-DA across the cell membrane allows it to be acted upon by intracellular esterases, which deacetylates it into DCFH₂, rendering it cell impermeable. The actions of multiple types of ROS (hydrogen peroxide, peroxyxynitrite, hydroxyl radicals, nitric oxide, and peroxy radicals) on DCFH₂ oxidize it into DCF which is fluorescent (reported Ex/Em: 485-500 nm/515-530 nm) and can be detected using a flow cytometer equipped with a standard filter set for fluorescein (FL1 channel). Superoxide does not strongly react with DCFH₂ but can react with another probe dihydroethidium (DHE) to yield the fluorescent product 2-hydroxyethidium (as well as other fluorescent superoxide-independent oxidation products)¹⁵. The fluorescent products of DHE oxidation can be detected using an excitation wavelength of 518 nm and an emission wavelength of 605 nm (FL2 channel). Although relatively simple to use, utilization of these probes for detection of ROS requires knowledge of their limitations and careful incorporation of staining procedures and controls into the specific assay being performed in

order to have valid experimental results and conclusions. The following protocol demonstrates the use of a commercially available kit employing these 2 probes designed to measure ROS by flow cytometry. We stain primed bone marrow-derived macrophages with these probes and induce ROS production through FcγR cross-linking. We present representative data obtained using this protocol and stress appropriate precautions that must be undertaken for successful experimentation.

PROTOCOL:

The protocol for animal handling was approved by the Institutional Animal Care and Use committee (IACUC) of University of Central Florida.

1. Generation of bone marrow derived macrophages (BMDMs)

1.1. Culture media preparation

1.1.1. Prepare D10F base media: To Dulbecco's Modified Eagle Medium (DMEM), add 10% heat inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.05 mM β-mercaptoethanol.

NOTE: Although it is best to use fresh D10F media, D10F base media can be prepared up to two weeks in advance to be used for multiple experiments within a week. For one mouse, around 175 mL of D10F base media is needed (25 mL to flush the bones and 150 mL to make macrophage differentiation media)

1.1.2. Prepare LADMAC growth media: To Eagle's Minimum Essential Medium (EMEM), add 10% heat inactivated FBS and 2 mM L-glutamine. Prepare the media fresh, on the day you plan to start your cultures.

NOTE: One liter of LADMAC growth media will result in approximately (19) 50 mL aliquots of LADMAC-conditioned media.

1.1.3. Prepare complete DMEM media: To DMEM, add 10% heat inactivated FBS and 1x Antibiotic-Antimycotic. Prepare the media fresh, on the day BMDMs will be harvested.

NOTE: For one mouse, around 100 mL of DMEM complete media will be required. This includes media used for priming the cells.

1.2. Preparation of LADMAC conditioned medium

1.2.1. Grow LADMAC cells to confluency in a 10 cm dish using 10 mL of LADMAC growth media (defined in 1.1.2). Incubate cells at 37 °C, 5% CO₂.

1.2.2. At confluency, detach cells by forcefully dispensing media over cells. Passage cells by

adding 1 mL of detached cells into T-175 flasks containing 100 mL of LADMAC growth media. Incubate cells until completely confluent at 37 °C, 5% CO₂ (approximately one week). This way, one 10 cm dish can yield ten T-175 flasks or approximately 1 L of conditioned media.

1.2.3. Once the cells are ready, collect the conditioned media and centrifuge at 350 x *g* for 5 min to pellet unwanted cells. Filter the collected supernatants through a 0.2 µm filter and store 50 mL aliquots at -80 °C. Aliquots of LADMAC-conditioned media can be kept at -80 °C for months with minimal loss of activity.

NOTE: If large experiments are anticipated, prepare large batches of LADMAC-conditioned media at the same time to ensure consistency. If this is not possible, use aliquots derived from the same batch or lot of LADMAC-conditioned media for each experiment.

1.3. Preparation of bone marrow derived macrophages

1.3.1. On the day of the experiment, prepare fresh macrophage differentiation media by adding 25% of LADMAC conditioned media into previously prepared D10F media (1 part LADMAC conditioned media into 3 parts D10F). Do not prepare this media in advance! Only prepare as much media as needed for the BMDM culture.

1.3.2. **Day 1:** Isolate mouse bone marrow cells according to the previously described protocol¹⁶ with a modification to flush the bone marrow from femurs and tibias using D10F base media. Use 2.5 mL of D10F media to flush each end of the (4) bones. Flush bone marrow cells directly into a pre-wet 40 µm cell strainer placed on top of a 50 mL tube. The remaining media (~5 mL) can be used to rinse out the cell strainer.

1.3.3. Centrifuge collected bone marrow cells at 350 x *g* for 5 min. Discard the supernatant and resuspend the cells in a total of 30 mL of macrophage differentiation media (per mouse).

1.3.4. Prepare and label (6) sterile 10 cm Petri dishes. Plate 5 mL of macrophage differentiation media into each into Petri dish. Aliquot 5 mL of resuspended bone marrow cells in macrophage differentiation media into each Petri dish for a total volume of 10 mL per dish. Incubate for 5 days at 37 °C, 5% CO₂.

1.3.5. **Day 5:** Aspirate media from the cells. Wash once with 1-2 mL of PBS and add 10 mL of freshly prepared macrophage differentiation media. Incubate for an additional 3 days.

1.3.6. **Day 8:** Proceed to harvesting BMDMs as described below.

2. Harvesting, seeding and priming of BMDMs

2.1. After 7 days of growing the BMDMs in the macrophage differentiation media, remove the supernatant. Wash adherent macrophages once with 1-2 mL of PBS. Aspirate the PBS.

2.2. Dispense 1 mL of 0.25% Trypsin-EDTA into each macrophage plate and leave them in the 37 °C incubator for 5-10 min with frequent tapping to detach the cells. Dissociate trypsinized macrophages by pipetting up and down using a P1000 pipette and collect macrophages in 50 mL tubes containing 10-15 mL of complete DMEM media. Wash the plate with additional 2 mL complete DMEM media to harvest any remaining macrophages.

CAUTION: Do not leave macrophages in trypsin for more than 10 min.

2.3. Centrifuge the 50 mL tubes at 350 x *g* for 5 min. Discard the supernatant. Gently dissociate the pellet and resuspend the cells in 10 mL of complete DMEM media. Count macrophages using a hemocytometer in the presence of a viability dye such as trypan blue as follows:

2.3.1. For example, mix 90 µL of Trypan blue with 10 µL of cells for a 1:10 dilution.

2.3.2. Of this mixture, load 10 µL into a hemocytometer and count the central square (5 x 5 grid). The total cell count = count x 10⁴ x dilution factor (10) x volume (10 mL).

2.4. Adjust the cell suspension to be 1 million/mL in complete DMEM media and plate 4 mL of this cell suspension into each 6 cm dish.

NOTE: A minimum of 3 plates is required per experiment. One plate of cells will be left unstimulated, a second set of cells will be stimulated through cross-linking of FcγRs, and the third plate of cells will be used for all of the additional experimental and flow cytometric controls.

2.5. Incubate the plates overnight at 37 °C, 5% CO₂.

2.6. The following day, aspirate the supernatant, wash cells once with 1-2 mL of PBS. Add 4 mL of complete media containing 100 ng/mL mouse IFN-γ to each plate. Incubate the plates overnight at 37 °C, 5% CO₂.

2.7. If desired, take an aliquot of the cells to assess appropriate generation of BMDMs by flow cytometry. Use 1x10⁶ cells per test and prepare polystyrene FACS tubes for the following conditions: isotype control, stained with F4/80 (or alternatively, stained with CD11b).

2.8. Prepare flow cytometry staining buffer by adding 0.1% FBS into 1x PBS. Use only this amount of FBS so as not to negate the effects of serum-starvation in later steps.

2.9. Aliquot 1 x 10⁶ cells per tube and centrifuge at 750 x *g* for 5 min.

2.10. Wash once by decanting or aspirating supernatant and resuspending cells in 2 mL of flow cytometry buffer. Centrifuge at 750 x *g* for 5 min.

2.11. Aspirate supernatant and resuspend cells in 100 µL of flow cytometry staining buffer. Add

1 μ L of anti-mouse CD16/32 Fc blocking antibody to each tube. Incubate on ice for 5 min.

2.12. Without washing, add 2 μ L of FITC anti-mouse F4/80 or 1 μ L of APC anti-mouse CD11b antibody to the “stained” tubes and a similar amount of the corresponding isotype control antibody to “isotype” tubes. Incubate on ice, in the dark, for 30 min.

2.13. Wash cells by adding 2 mL of PBS directly to the cells. Centrifuge at 750 x g for 5 min.

2.14. Aspirate supernatant and resuspend cells in 150 μ L of PBS.

2.15. Acquire samples on a flow cytometer using a stop condition of 100 μ L or 10,000 events on the gate of interest and ensure that FSC, SSC, FL1 (FITC anti-mouse F4/80) or FL4 (APC anti-mouse CD11b) channels are selected for the parameters to be analyzed.

2.16. Using the flow cytometry software, open a dot plot for FSC (on the x-axis) vs SSC (on the y-axis) and draw a gate around the cells of interest, excluding dead cells and debris (dead cells and debris are much smaller events than the main cell population and appear on the lower left of the plot).

2.17. Gating on the cells of interest, open a histogram plot with FL1 (FITC anti-mouse F4/80) or FL4 (APC anti-mouse CD11b) on the x-axis.

2.18. Run the “isotype” sample. Generate a marker gate so that majority of “isotype” sample events are to the left of the gate (<1% positive). Apply this template to the stained sample file.

2.19. Run the “stained” sample. Successful generation of BMDMs will result in >95% expression of FITC anti-mouse F4/80 or APC anti-mouse CD11b (**Figure 1A**), while incorrect culture conditions may result in sub-optimal generation of macrophages (**Figure 1B**).

NOTE: If generating BMDMs from multiple genotypes, take note of the expression of these markers for each batch of BMDM generated, in the case that this may be grounds for excluding a sample from analysis when ROS generation in response to stimulus is assessed.

3. Reagent and material preparation for ROS measurement

3.1. Reconstitute the lyophilized oxidative stress detection reagent in 60 μ L of anhydrous DMF to yield a 5 mM stock solution. Gently mix before use.

NOTE: It is stated in the ROS-ID kit manual that the shelf life of the reconstituted reagent is about 1 week at -20 °C. Aliquoting the reagent immediately after reconstitution into small light-proof vials minimizes its oxidation and maximizes the shelf life at -20 °C.

3.2. Reconstitute the lyophilized superoxide detection reagent in 60 μ L of anhydrous DMF to

yield a 5 mM stock solution. Gently mix before use.

CAUTION: As stated in the kit manual, treat both detection reagents as possible mutagens, handle each with care, and dispose of properly.

3.3. Reconstitute the ROS inducer (Pyocyanin) in 20 μ L of anhydrous DMF to yield a 50 mM stock solution.

3.4. Reconstitute the ROS Inhibitor (N-acetyl-L-cysteine) in 123 μ L of deionized water to yield a 0.5 M stock concentration.

3.5. Label 5 mL round polystyrene tubes (flow cytometry tubes) with the experimental controls and conditions. (See 4. Assay conditions and controls)

3.6. Prepare low serum DMEM (no phenol red) by adding 0.1% FBS to phenol red-free DMEM.

3.7. Aliquot the anti-BSA antibody into small aliquots (depending on the usage) and store them at -80 °C.

3.8. Prepare stock solution of 100 mg/mL BSA in HBSS (Hanks' balanced salt solution) or PBS. Aliquot into 100 μ L aliquots and store at -20 °C.

3.9. Prepare a 2x solution of the ROS probes (2x probe solution): for each 10 mL of low serum DMEM, add 4 μ L of oxidative stress detection reagent (green dye) and 4 μ L of superoxide detection reagent (orange dye).

3.10. Prepare 2x probe solutions containing **only** oxidative stress detection reagent (2x probe solution, green only), or containing **only** superoxide detection reagent (2x probe solution, orange only). Prepare only the amount of reagent needed for the experiment and always prepare the 2x solution immediately prior to use.

NOTE: To prepare smaller volumes of 2x detection solution, use an intermediate 1:10 dilution of both Green and Orange detection reagents prior to final dilution in DMEM. For example, to prepare 1 mL of a 2x detection solution, dilute 1 μ L of each probe into 9 μ L of DMEM (1:10 intermediate dilution). Dilute 4 μ L of this 1:10 intermediate dilution into 1 mL of DMEM.

4. Assay conditions and controls

4.1. Include the following experimental controls for flow cytometric compensation for each experiment:

- a) Unstained and unstimulated cells.
- b) Cells stained only with the oxidative stress detection reagent (green reagent) and treated with ROS inducer.
- c) Cells stained only with the superoxide detection reagent (orange reagent) and treated

with ROS inducer.

4.2. For each mouse or biological replicate, include the following 6 conditions:

- a) Unstained and unstimulated cells
- b) Stained and unstimulated cells
- c) Stained cells treated with positive inducer
- d) Stained cells treated with positive inducer and ROS inhibitor
- e) Stained cells activated through FcγR cross-linking
- f) Stained cells activated through FcγR cross-linking and treated with ROS inhibitor

4.3. Calculate the amount of 2x probe solution needed based on the number of mice and conditions (200 μL of the 2x probe solution will be used for each condition requiring staining).

NOTE: For an assay using 6 mice, 5 conditions requiring staining with the probes will be needed per mouse. This brings the total number of conditions to 30. Thus, the total amount of 2x probe solution needed is at least 6 mL (30*200 μL).

5. Cell preparation

5.1. After priming the macrophages overnight, aspirate the supernatant. Wash the cells once with PBS.

5.2. Serum starve the cells by replacing media with the same volume of low serum DMEM. For each mouse, one plate will be treated with anti-BSA IgG₁ while serum starving, while the other will be left untreated. Add only low serum DMEM to untreated plates. Add low serum DMEM containing 2.5 μg/mL of murine anti-BSA IgG₁ to treated plates.

NOTE: One additional untreated plate is needed for each experiment for the flow cytometric compensation controls.

5.3. Incubate the plates for 4 h at 37 °C, 5% CO₂.

5.4. After serum starving, harvest the cells by gentle scraping or by using 0.2 mM EDTA in PBS. Collect them in labeled 5 mL round bottom tubes and centrifuge them at 750 x g for 5 min. Keep track of which cells were treated with murine anti-BSA IgG₁.

5.5. Wash cell pellets once with 2 mL of PBS to get rid of any residual anti-BSA from the treated cells.

5.6. Resuspend cell pellet in 600 μL of low serum DMEM.

5.7. From the 600 μL cell suspension, aliquot 200 μL into the pre-labelled 5 mL round bottom tubes as follows:

5.7.1. From the untreated cells, take 200 μ L for tubes labelled “unstimulated”, 200 μ L for tubes labelled “positive inducer” and 200 μ L for tubes labelled “positive inducer + inhibitor”

5.7.2. From the anti-BSA IgG₁ treated cells, take 200 μ L for tubes labelled “Fc γ R crosslinking/Fc γ R XL” and 200 μ L for tubes labelled “Fc γ R XL + inhibitor”

5.7.3. From the untreated cells to be used for compensation controls, take 200 μ L for the tube labelled “unstained unstimulated”, 200 μ L for the “green + inducer” control and 200 μ L for “orange + inducer” control.

NOTE: If the cells from 5.7.1 and 5.7.3 are derived from the same mouse, the same “unstained unstimulated” cells can be used for the experimental and compensation control. If not, an additional 200 μ L of cells will be needed for an “unstained unstimulated” experimental control for a).

5.8. Keep the tubes on ice until staining and stimulation. During serum starvation and binding of IgG₁, prepare the probes, specific stimuli, and inducers as outlined below.

NOTE: If performing the assay for the first time, if no template is available, or no after-the-fact compensation is available on the flow cytometer and corresponding software, stimulate and stain compensation controls and run these on the flow cytometer to perform manual compensation **prior to** addition of stimuli to experimental samples.

6. Performing the assay

6.1. Prepare 2x positive inducer solution by diluting the Pyocyanin 1:100 in the 2x probe solution (prepared in step 3.9) to obtain a 2x concentration of 500 μ M of pyocyanin (final concentration will be 250 μ M). Also, dilute pyocyanin 1:100 into each of the “2x probe solution, green only” and “2x probe solution, orange only” tubes prepared in 3.10.

NOTE: Both conditions labelled with “positive inducer” and “positive inducer + inhibitor” will be treated with the positive inducer. Plan accordingly when calculating the amount of 2x positive inducer solution to prepare. For example: If performing the assay with 3 mice, 6 conditions (3*2) will be treated with positive inducer, so prepare 6*200 μ L = 1.2 mL of 2x positive inducer solution.

6.2. Prepare a 2x BSA solution by diluting the BSA stock solution in 2x probe solution to obtain a concentration of 2 μ g/mL (final concentration will be 1 μ g/mL).

NOTE: Both conditions labelled with “Fc γ R XL” and “Fc γ R XL + inhibitor” will be treated with the 2x BSA solution. Plan accordingly when calculating the amount of solution to prepare. For example: If performing the assay using 3 mice, 6 (3*2) conditions will be treated with BSA solution and so 6*200 μ L or 1.2 mL of 2x BSA solution will be needed.

6.3. Before starting the specific stimulation (FcγR crosslinking), make sure that all the reagents and cells are ready. Place the tubes on ice in the order they will be stimulated.

6.4. For flow cytometers with an autosampler, take note of the time that the cytometer takes to analyze one sample and move on to the next, including any mixing and probe washing steps (for example 3.5 min).

NOTE: Timing is very critical for this assay. In order for every condition to be well controlled, the stimulation needs to be carried out for the exact time (30 min) for each condition. Stimulate cells in order and incorporate the lag time between sample acquisition by the flow cytometer. For example, if the time needed for the cytometer to analyze one sample and move on to the next is 3.5 min, stimulate cells in the order they will be analyzed every 3.5 min.

6.5. If performing manual compensation at this point, stimulate control tubes to be used for compensation (step 5.7.3). Add 200 μL of “2x probe solution, green only” containing inducer (from 6.1) into the tubes marked “green+inducer” (step 5.7.3). Add 200 μL of “2x probe solution, orange only” containing inducer (step 6.1) into the tubes marked “orange+inducer” (step 5.7.3).

6.6. Incubate the cells for 30 min at 37 °C, 5% CO₂ in dark.

6.7. Using the flow cytometry software, generate and label 3 sample files for the control “unstained, untreated”, “green+inducer”, and “orange+inducer” samples, making sure to indicate the channels/parameters to be analyzed (FSC, SSC, FL1, FL2) and desired stop conditions (100 μL, 3 min, etc.). Generate and label a similar set of files for experimental samples.

6.8. Run the “unstained, untreated” sample. Open a dot plot for FSC (on the x-axis) vs SSC (on the y-axis) and draw a gate around the cells of interest, excluding dead cells and debris (dead cells and debris are much smaller events than the main cell population and appear on the lower left of the plot).

6.9. Using this “cells” gate, open another dot plot of FL1 (x-axis) vs FL2 (y-axis). Draw an initial quadrant gate. Adjust the quadrant gates so that the events appear on the lower left quadrant of the FL1 vs FL2 plot.

6.10. Run the “green+inducer” sample. Adjust the voltage so that the events appear on the lower left and right quadrants of the FL1 vs FL2 plot. Apply this compensation matrix to all 3 sample files.

6.11. Run the “orange+inducer” sample. Adjust the voltage so that the events appear on the upper and lower left quadrants of the FL1 vs FL2 plot. Apply this compensation matrix to all 3 sample files.

6.12. Check each compensation file and ensure that “unstained, untreated” events appear on

the lower left quadrant, “green+inducer” events appear on the lower left and right quadrants, and “orange+inducer” events appear on the upper and lower left quadrants of the FL1 vs FL2 plot. Apply the compensation matrix to all of the experimental sample files.

6.12.1. Ensure that appropriate compensation is applied to all the control sample files before applying the compensation matrix to all of the experimental sample files. Refer to **Figure 2** for representative data showing uncompensated and correctly compensated samples.

NOTE: Many cytometers designate FL1 as the standard FITC/GFP channel (excited by the blue 488 nm laser, and detected with a 530/30 filter set) and FL2 as the standard PE channel (excited by the blue 488 nm laser, and detected with a 585/40 filter set). We use this same convention but caution new flow cytometry users to consult with their flow cytometry core manager to ensure that their cytometer is similarly configured and that the appropriate channels are used to detect these probes. Depending on the model of flow cytometer and accompanying software, it may be possible to perform compensation before or after samples have been acquired. If after-the-fact compensation is possible, the compensation step can be performed after all experimental samples have been acquired by the cytometer. For cytometers with a dynamic range, where PMT voltage adjustments are not needed, a compensation experiment can also be performed on a separate day and the experimental template (including the compensation matrix) can be saved to reduce the time needed to perform manual compensation.

6.13. Once manual compensation has been performed and an experimental template is obtained, start treatment of experimental samples. Before treating with positive inducer or FcγR cell stimulation, mark which tubes get ROS inhibitor. Treat these cells with the ROS inhibitor at least 30 min prior to positive inducer or FcγR stimulation.

6.14. To add the ROS inhibitor, prepare a final concentration of 5 mM by adding 1 μL of the inhibitor to 200 μL of resuspended cells (without anti-BSA IgG₁).

6.15. Treat the cells with the stimulus (or positive inducer) and load the cells with the ROS probes as follows:

6.15.1. For unstimulated cells: add 200 μL of 2x probe solution without any stimulus to the 200 μL of cell suspension labelled “stained, unstimulated”.

6.15.2. For positive controls: add 200 μL of 2x positive inducer solution (prepared in step 6.1) to 200 μL of cell suspension labelled “positive inducer” or “positive inducer + inhibitor”.

6.15.3. For cells stimulated by FcγR cross-linking (specific stimulus): add 200 μL of 2x BSA solution (prepared in 6.2) to 200 μL of cell suspension labelled “FcγR XL” or “FcγR XL + inhibitor”.

NOTE: Remember to incorporate the lag time between sample analysis by adding stimulus every x min where x is the lag time between acquisition of one sample and the next.

6.16. Incubate the cells for 30 min at 37 °C, 5% CO₂ in dark. Analyze the samples in the order they were stimulated using a flow cytometer equipped with an autosampler. Use the analysis templates generated during the initial compensation steps. Do not wash cells prior to analysis.

7. Flow cytometry data analysis and anticipated results

7.1. Within the flow cytometry software, open the previously generated analysis files for the experimental samples (templates were generated in steps 6.7-6.12 and samples were run in step 6.16). Ensure that the compensation matrix from performing manual compensation was correctly applied to the experimental samples.

NOTE: For flow cytometers and flow cytometry software capable of after-the-fact compensation, if the compensation matrix was not previously applied to the experimental sample files, apply it at this point.

7.2. Similar to verifying correct manual compensation, ensure that the controls within the experimental samples behave as expected.

7.2.1. Ensure that the “unstained, untreated” events will appear on the lower left quadrant of the FL1 vs FL2 plot, that the “positive inducer” events show increased fluorescence in the upper left, upper right, and lower right quadrants of the FL1 vs FL2 plot, and that “positive inducer + ROS inhibitor” events show a reduction in fluorescence in upper left, upper right, and lower right quadrants of the FL1 vs FL2 plot compared to the fluorescence observed with the “positive inducer” sample.

7.2.2. If controls within the experimental samples do not show any increased fluorescence, check that all assay steps were performed, verify appropriate generation and priming of bone marrow-derived macrophages (2.7-2.19), and repeat the experiment. If controls within the experimental samples show the expected trends, proceed to analyze experimental samples stimulated through the FcγR (**Figure 3A**).

7.3. Verify that the FcγR-specific stimulus is working appropriately. Run the following samples from a C57BL/6J WT mouse: “unstained, unstimulated”, “stained, unstimulated”, “stained, stimulated through the FcγR”, and “stained, stimulated through the FcγR + ROS inhibitor”. These correspond to samples 4.2a, 4.2b, 4.2e, and 4.2f in section 4, respectively.

7.3.1. Ensure that the “unstained, unstimulated” events will appear on the lower left quadrant of the FL1 vs FL2 plot, that “stained, unstimulated” events will also appear on the lower left quadrant of the FL1 vs FL2 plot, that “stained, stimulated through the FcγR” events show increased fluorescence in the upper left, upper right, and lower right quadrants of the FL1 vs FL2 plot, and that “stained, stimulated through the FcγR + ROS inhibitor” events will show decreased fluorescence in the upper left, upper right, and lower right quadrants of the FL1 vs FL2 plot

compared to the “stained, stimulated through the FcγR” sample (**Figure 3A**).

7.3.2. If these expectations are not met, for example, the “stained, stimulated through the FcγR” sample shows minimal or no increased fluorescence when compared to the “stained, unstimulated” sample, or the “stained, unstimulated” sample already shows markedly increased levels of fluorescence compared to the “unstained, unstimulated” sample, check that all assay steps were performed properly, verify appropriate generation, priming, and handling of BMDMs (2.7-2.19), and repeat the experiment. If these expectations are met, proceed to the analysis of the rest of the experimental samples.

NOTE: Cells producing ROS which react with the green probe (hydrogen peroxide, peroxyxynitrite, hydroxyl radicals, nitric oxide, peroxy radicals, etc.) will appear in the upper right and lower right quadrants of a log FL1 (X-axis) versus a log FL2 (Y-axis) dot plot. Cells producing ROS which react with the orange probe (largely but not exclusively superoxide) will appear in the two upper quadrants of a log FL1 (X-axis) versus a log FL2 (Y-axis) dot plot.

7.4. Generate individual histograms, gated on the cells of interest, for analysis of FL1 and FL2 fluorescence. Using the “unstained, unstimulated” samples, generate a histogram marker such that all “unstained, unstimulated” events appear to the left of this marker. Apply this plot with the marker to the rest of the experimental samples (**Figure 3B**).

7.5. Present the results of the experiment as the percentage of the cells positive for each of the ROS probes or by showing the mean fluorescence intensity (MFI) of the stimulated samples versus control (**Figure 3C**).

8. Cell surface staining in combination with flow cytometric analysis of ROS production (Optional)

NOTE: This step provides a protocol for staining macrophages with a cell-surface marker prior to stimulation of the FcγR and ROS measurement. This may be useful in assessing ROS production in mixed cell populations. It is important to choose an antibody for macrophage surface marker conjugated to an appropriate fluor that does not interfere with the fluorescence from the oxidative stress or superoxide detection reagents. In this protocol, an antibody for mouse F4/80 conjugated to Alexa fluor 647 is used.

8.1 Generate BMDMs, harvest and prime them as described previously (sections 1 and 2).

NOTE: A minimum of three 6 cm plates are needed in order to perform all the experimental controls and conditions as described below. One plate will be treated with anti-BSA IgG₁ while serum starving while the other two plates will be left untreated.

8.1.1. Include 4 experimental controls which will be used for flow cytometric compensation (per experiment):

- a) Unstained and unstimulated cells.

- b) Cells treated with the oxidative stress detection reagent (green reagent) and ROS inducer.
- c) Cells treated with the superoxide detection reagent (orange reagent) and ROS inducer.
- d) Cells stained only with anti-mouse F4/80 conjugated to Alexa 647.

8.1.2. For each mouse or biological replicate, include the following 3 conditions:

- a) stained with F4/80 and left unstimulated
- b) stained with F4/80 and activated through the Fc γ R
- c) stained with F4/80 and activated through the Fc γ R and treated with ROS inhibitor

8.2. After priming the macrophages overnight, aspirate the supernatant. Wash the cells once with 1-2 mL of PBS. Serum starve the cells by replacing the media with the same volume of low serum DMEM. For each mouse, one plate will be treated with anti-BSA IgG₁ while serum starving, while the other two plates will be left untreated. Incubate the plates for 4 h at 37 °C, 5% CO₂.

8.3. After serum starving, harvest the cells by gentle scraping or by using 0.2 mM EDTA in PBS. Collect each plate in labeled 5 mL round bottom tubes and centrifuge them at 750 x *g* for 5 min. Keep track of which cells were treated with murine anti-BSA IgG₁.

8.4. Wash cell pellets once with 1-2 mL of PBS to get rid of any residual anti-BSA from the treated cells.

8.5. Resuspend one of the cell pellets from the plate which did not get anti-BSA IgG₁ in 600 μ L of low serum DMEM. These cells will *not* be subjected to cell surface staining. Use these for compensation controls. Aliquot 200 μ L of this cell suspension into (3) 5 mL round bottom tubes labeled a) unstained, unstimulated b) green oxidative stress reagent + ROS inducer, c) orange superoxide detection reagent + ROS inducer.

8.6. Resuspend one of the cell pellets from the plate which did not get anti-BSA IgG₁ in 300 μ L of flow cytometry staining buffer (PBS + 0.1% FBS). Aliquot 100 μ L of this cell suspension into (2) 5 mL round bottom tubes for staining with Alexa 647 anti-mouse F4/80.

8.7. Resuspend the cell pellets from the plate which received anti-BSA IgG₁ in 300 μ L of flow cytometry staining buffer. Aliquot 100 μ L of this cell suspension into (2) 5 mL round bottom tubes for staining with Alexa 647 anti-mouse F4/80.

8.8. Stain the cells, which were aliquoted in 8.6 and 8.7, with 5 μ L of Alexa 647 anti-mouse F4/80 for 30 min on ice, in the dark.

8.9. After staining, wash cells by adding 2 mL of PBS and centrifuge at 750 x *g*, aspirate the supernatant, and resuspend each tube in 200 μ L of low serum DMEM without phenol red. Set aside one untreated tube to serve as singly stained FL4 control. Prepare probes, inducer, Fc γ R stimulus, and ROS inhibitors as indicated in sections 3.9, 3.10, 6.1, 6.2, and 6.14.

8.9.1. For cells not receiving anti-BSA IgG₁, label tubes with the following: a) no stimulus or b) ROS inducer.

8.9.2. For cells which received anti-BSA IgG₁, label with the following: a) Fc XL or b) Fc XL + inhibitor.

8.10. Stimulate the samples to be used for compensation according to their respective treatments as indicated in 6.5-6.6, in the order that they will be read on the flow cytometer, incorporating the lag time in between acquisition of one sample and the next.

8.11. Incubate the cells for 30 min at 37 °C, 5% CO₂ in dark. Analyze the samples in the order they were stimulated using a flow cytometer equipped with an autosampler.

8.12. Perform manual compensation as described in 6.7-6.12 and data analysis as described in section 7.

8.12. Using the flow cytometry software, generate and label 4 sample files for the control “unstained, untreated”, “green + inducer”, “orange + inducer” and “F4/80 stained” samples, making sure to indicate the channels/parameters to be analyzed (FSC, SSC, FL1, FL2, FL4) and desired stop conditions (100 µL, 3 min, etc.).

8.13. Run the samples and generate dot plots to perform manual compensation.

8.13.1. For cell surface staining, generate two additional plots: FL1 (x-axis) vs FL4 (y-axis) and FL2 (x-axis) vs FL4 (y-axis). Adjust the voltages to ensure that appropriate compensation is applied as shown in **Figure 7A**. Once all compensation has been properly performed, apply the compensation matrix to all of the experimental sample files.

8.14. Once manual compensation has been performed and an experimental template is obtained, start treatment of experimental samples as indicated in 6.13-6.15.3 and acquire samples on a flow cytometer as described in 6.16.

REPRESENTATIVE RESULTS:

Using the protocol outlined within, we present representative data demonstrating flow cytometric detection of ROS production resulting from stimulation of WT C57BL/6J BMDMs through the FcγR. As expected, we observe minimal changes in FL1 or FL2 fluorescence above background levels in unstimulated cells (**Figure 3A**, compare “stained, unstimulated” vs “unstained, unstimulated” dot plots). We observe a marked increase in FL1 and FL2 fluorescence when cells are stimulated with FcγR cross-linking agent (**Figure 3A**, compare “stained and stimulated via Fc cross-linking” samples vs “stained, unstimulated” dot plots). Lastly, when cells were treated with ROS inhibitor prior to FcγR cross-linking, this increased fluorescence is brought back to basal levels (**Figure 3A**, compare “stained and treated with ROS inhibitor and stimulated via Fc cross-linking” vs “stained and stimulated via Fc cross-linking” dot plots). This is also evident when data is presented as a histogram for each channel (**Figure 3B**) or when data is presented as

a percentage of cells positive for either the green or orange ROS probes (**Figure 3C**). A similar trend is also apparent when data is presented as MFI, although the reduction in orange fluorescence with pre-treatment with ROS inhibitor is not captured as well when presented as MFI versus as a percentage (**Figure 3C**). We also present the results of 3 independent experiments performed on different days (**Figure 3**, Experiment 1, 2, and 3). The average values and corresponding standard error of the mean are indicated in the graphs (**Figure 3C**).

We also present unsuccessful experimentation, where sub-optimal ROS production as a result of FcγR stimulation was observed (**Figure 4**). A minimal increase in FL1 and FL2 fluorescence was detected when comparing “stained and stimulated via Fc cross-linking” samples vs “stained, unstimulated” samples (**Figure 4A,B,C**). This is presented alongside a successful experiment to highlight the large differences between the expected percentages or MFI increases and the observed values in the unsuccessful experiment.

The current protocol utilizes a 24 h priming step. When comparing a 24 h versus a 48 h priming time, we observed no marked difference in the percentage of cells positive for the green, oxidative stress reagent (**Figure 5A**, top histograms and **Figure 5B** green probe, % positive). However, increasing the priming time to 48 h *did* increase the percentage of cells positive for the orange fluorescence (**Figure 5A**, lower histograms and **Figure 5B** orange probe, % positive). This was similarly reflected when data was presented as MFI. This suggests that for optimal detection of all ROS species, a 48 h priming time may be more ideal.

Given that, due to the cost or the time needed for experimentation, use of a kit to perform this assay may not be an option. For this reason, we also tested similar components to those provided in the kit and purchased these from standard vendors (Thermofisher, EMD Millipore, Cayman). We find that using individually procured components and the same experimental protocol for cell loading and FcγR stimulation, we can recapitulate many of the same findings we observed using the kit (**Figure 6A,B**). However, although increases in fluorescence were apparent with stimulation, a higher level of ROS production was observed using the kit. This may indicate that use of individually procured components may be feasible but would need to be further optimized for this specific assay.

Lastly, we demonstrate that it is also possible to combine cell-surface staining with these ROS probes. We use a known macrophage marker, F4/80, conjugated to Alexa 647 and perform cell surface staining prior to treatment with ROS inhibitor, ROS inducer, or specific stimuli to induce ROS production. We demonstrate in **Figure 7B** that macrophages respond as expected when treated with FcγR crosslinking agent and FcγR crosslinking agent + ROS inhibitor (**Figure 7B**, orange vs green dot plots). Furthermore, we can observe increased orange or green fluorescence specifically generated by the F4/80 labeled cells upon treatment with FcγR crosslinking agent and reduced with pre-treatment with ROS inhibitor (**Figure 7B**, F4/80 vs green and F4/80 vs orange dot plots).

FIGURE AND TABLE LEGENDS:

Figure 1: Flow cytometric assessment of appropriate generation of BMDMs. Wild type BMDMs

were generated and were left either unstained or stained with FITC anti-mouse F4/80 or Alexa 647 anti-mouse CD11b. FSC (x-axis) vs SSC (y-axis) plots were generated and macrophages (BMDMs) were gated to exclude dead cells and debris. Using a plot of FSC-H(x-axis) vs FSC-A (y-axis), a singlet gate was generated. Gating on singlets, histograms were generated to show cells stained with either FITC F4/80 or APC CD11b in comparison to the isotype stained control. **A)** Correct BMDM differentiation with more than 95% of cells staining positive for CD11b or F4/80. **B)** Incorrect BMDM differentiation where less than 95% of cells are staining positive for F4/80 and 2 peaks are present for CD11b.

Figure 2. Performing compensation when using green and orange ROS probes. Compensation will require unstained untreated cells, cells stained with green ROS probe and treated with ROS inducer, and cells stained with orange ROS probe and treated with ROS inducer. Dot plots for unstained untreated cells are used to determine quadrant gates. Data for cells singly stained with either green ROS probe or orange ROS probe and treated with ROS inducer are shown prior to, and after, compensation was applied. The compensation matrix is then applied to all subsequent experimental samples.

Figure 3. Measurement of ROS in response to specific FcγR stimulation using green and orange ROS probes and assessment of assay reproducibility. Wild-type bone marrow-derived macrophages (BMDMs) were generated, primed, and were left either unstained or stained with a cocktail of green and orange ROS probes. Stained BMDMs were either left untreated, stimulated through their FcγRs using murine anti-BSA IgG₁ + BSA for 30 min, or treated with ROS inhibitor prior to stimulation via FcγR cross-linking. **A)** Dot plots, showing an increase in the percentages of cells in the upper left, upper right, and lower right quadrants upon specific FcγR stimulation, which is reduced in the presence of ROS inhibitor. **B)** Histograms of each fluorescence channel, showing the marker gate to determine cells positive for each probe. **C)** Presentation of the data as a percentage of cells positive for each probe or as an increase in MFI. Three independent, representative experiments are presented. The mean for the 3 experiments and standard errors of the mean are shown as lines within the graphs. Fc XL, Fc cross-linking; NAC, N-acetyl-L-cysteine.

Figure 4. Examples of successful and suboptimal FcγR stimulation. Wild-type bone marrow-derived macrophages (BMDMs) were generated, primed, and were left either unstained or stained with a cocktail of green and orange ROS probes. Stained BMDMs were either left untreated, stimulated through their FcγRs using murine anti-BSA IgG₁ + BSA for 30 min, or treated with ROS inhibitor prior to stimulation via FcγR cross-linking. Representative results for successful and suboptimal stimulation are shown as A) dot plots, B) histograms, or C) the percentage of cells positive for each probe or as an increase in MFI. Successful stimulation shows increased fluorescence in the upper left, upper right and lower right quadrants of the FL1 vs FL2 plot and increased MFI and percentage of positive cells stained with each probe upon FcγR stimulation. Suboptimal stimulation shows minimal increase in MFI or percentage of positive cells. Fc XL, Fc cross-linking; NAC, N-acetyl-L-cysteine.

Figure 5. Effect of priming time on ROS generation upon FcγR stimulation. BMDMs were generated, primed for either 24 or 48 h, and were left either unstained or stained with a cocktail of green and orange ROS probes. Stained BMDMs were either left untreated, stimulated through their FcγRs using murine anti-BSA IgG₁ + BSA for 30 min, or treated with ROS inhibitor prior to stimulation via FcγR cross-linking. **A)** Histograms for the fluorescence induced by each probe upon stimulation of macrophages primed for either 24 or 48 h. **B)** Percentage of cells positive for each probe upon stimulation of macrophages primed for either 24 or 48 h. Priming macrophages for 48 h resulted in an increase in percent of cells positive for orange fluorescence (or an increase in the MFI for the FL2 channel) compared to priming the macrophages for 24 h. The mean for the 3 experiments and standard errors of the mean are shown as lines within the graphs. Fc XL, Fc cross-linking; NAC, N-acetyl-L-cysteine.

Figure 6. Flow cytometric ROS measurement upon FcγR cross-linking using reagents from different vendors. BMDMs were generated, primed for 24 h, and were left either unstained or stained with a cocktail of oxidative stress and superoxide detection probes. Stained BMDMs were either left untreated, stimulated with ROS inducer, stimulated through their FcγRs using murine anti-BSA IgG₁ + BSA for 30 min, or treated with ROS inhibitor prior to stimulation via FcγR cross-linking. Probes, ROS inducer and ROS inhibitor were used either from the kit or were purchased separately from different vendors and used at a similar concentration. **A)** Dot plots showing a side-by-side comparison of results obtained using kit and non-kit components. **B)** Histograms showing a side-by-side comparison of results obtained using kit and non-kit components. Fc XL, Fc cross-linking; NAC, N-acetyl-L-cysteine.

Figure 7. Combining cell-surface staining with flow cytometric measurement of ROS production upon FcγR cross-linking. **A)** Dot plots for the various fluorescent channels using unstained or singly stained compensation controls. Wild-type BMDMs were generated, primed for 24 h, and were left either unstained, stained with Alexa 647 anti-mouse F4/80 only, stained with green ROS probe only and treated with ROS inducer, or stained with orange ROS probe only and treated with ROS inducer. Dot plots for unstained untreated cells are used to determine quadrant gates. Plots demonstrate expected results if channels are correctly compensated. The compensation matrix was then applied to all subsequent experimental samples. **B)** Wild-type BMDMs were generated, primed for 24 h and stained with Alexa 647 anti-mouse F4/80. Afterward, BMDMs were either left unstimulated, stimulated through their FcγRs using murine anti-BSA IgG₁ + BSA for 30 min, or treated with ROS inhibitor prior to stimulation via FcγR cross-linking. Dot plots demonstrate that green or orange fluorescence specifically produced by F4/80+ cells can be detected.

DISCUSSION:

DCFH₂-DA and DHE-based detection of ROS is a widely-used technique^{14,15}. Ease of use and the adaptability of these ROS probes for kinetic microplate formats, fluorescence microscopy or flow cytometric analysis has contributed to their popularity. However, in our studies of FcγR-mediated macrophage functions, there did not seem to be a standard protocol for performing this assay for flow cytometric analysis of FcγR cross-linked cells. Given the reactive nature of the analytes

being assessed, we have found that timing is of crucial importance in making sure that reproducibility is achieved. Although we have also performed kinetic microplate assays, there can be a wide variability in the intensity of fluorescence from experiment to experiment, making it difficult to aggregate biological replicates for statistical analysis. Flow cytometric use of these probes allows for quantification of “ROS positive” cells which resolves some of these issues but is not without complications. This is especially true when using a flow cytometer equipped with an autosampler. In this case, analysis of a large number of samples will impact the amount of time the cells are exposed to various stimuli. To mitigate this, we have incorporated the lag time in between sample analysis into the protocol to make sure that the analysis is performed on cells stimulated for a similar amount of time.

Another important factor in flow cytometric ROS analysis is the inclusion of appropriate controls. It cannot be overstated that every experiment must have (at least) all the experimental and compensation controls we list in this protocol. These are important to ensure the validity of each experiment as well as to be able to justifiably exclude samples if needed. Some examples of this include if macrophages did not differentiate/mature appropriately and exhibited much lower ROS production even with inducer treatment. Another case could be inappropriate activation of macrophages even prior to FcγR cross-linking, resulting in a high basal ROS signal. Use of ROS inhibitors in conjunction with specific FcγR cross-linking showing disappearance of fluorescent signals is another important control we have often found lacking in other studies performing similar assays. The ROS inhibitor used in this study, N-acetyl-L-cysteine, is regarded as a universal ROS inhibitor. However, if one is interested in ROS produced by specific enzymes, other specific ROS inhibitors can be included in the assay as well. Some examples include mefenamic acid (cyclooxygenase-dependent ROS inhibitor), apocynin (NADPH oxidase-dependent ROS inhibitor), or allopurinol (a xanthine oxidase-dependent ROS inhibitor).

It is additionally crucial to understand what is actually being measured in this readout. As previously mentioned, DCFH₂-DA measures multiple ROS species, and so, fluorescence resulting from the green probe cannot be used to discriminate one ROS species from another¹⁴. Likewise, the orange probe (likely DHE), although often cited as superoxide specific, can produce both superoxide-specific and superoxide-independent oxidation products, which cannot be distinguished without the use of additional techniques such as HPLC¹⁵. However, for the purposes of measuring “total ROS” or “induced ROS” during the respiratory burst, use of these probes alongside the appropriate controls, may be acceptable. Many new fluorescent based ROS sensors have emerged or are being developed^{11,13}. Some, such as the redox-sensitive fluorescent proteins, have the advantage of a dynamic measurement of ROS production due to their reversible oxidation. However, these would require genetic manipulation of cells, which may not always be feasible or desired. In many cases, fluorescent probe-based ROS detection is still a valid and useful tool, as long as the experiment is standardized, well-controlled, and the conclusions derived from such experiments are not overstated.

The benefits to using this commercially available ROS kit is that all necessary reagents including ROS inducers, scavengers, and titrated probes are included and “ready-to-use”. If the period of experimentation is anticipated to be brief (completed within a week), this kit can provide a more

cost-effective method for performing flow cytometry-based ROS detection without the need to buy or optimize each specific component individually. We additionally demonstrate using this kit with a specific agonist (FcγR stimulation) and demonstrate reproducibility across experiments; we assess effects of priming times on ROS generation; we compare using similar probes, inhibitors, and inducers from different companies; we provide examples of unsuccessful experimentation; and lastly, we combine cell-surface staining with use of these ROS probes. This would potentially allow for simultaneous detection of ROS from different cell types within a mixed population, requiring less sample and reagents. For example, this might be particularly useful when differentiating between macrophage and neutrophil-derived ROS, the main cell types capable of responding to FcγR ligation. For multiple experiments which have to be performed with long intervening periods in between, use of a kit might not be as ideal. Multiple aliquots of the probes are not provided and once reconstituted, the manufacturer only recommends that reagents be used within a week (as ROS probes are highly reactive). If it is anticipated that this period is incompatible with experimentation, we recommend that individual reagents rather than a kit be purchased separately and reconstituted only during the day of experimentation. As we demonstrate here, further optimization of the individually purchased components may additionally be needed prior to the assay. Overall, we hope that this work provides a useful resource for researchers using flow cytometry to reproducibly measure ROS generation.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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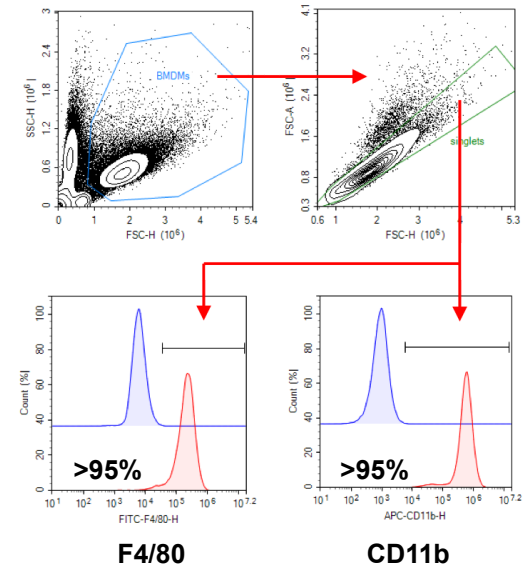
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893

A. Figure 1 **Correct BMDM differentiation**



B. Figure 1 **Incorrect BMDM differentiation** [Click here to access/download,Figure,Figure 1 BMDM differentiation.ai](#)

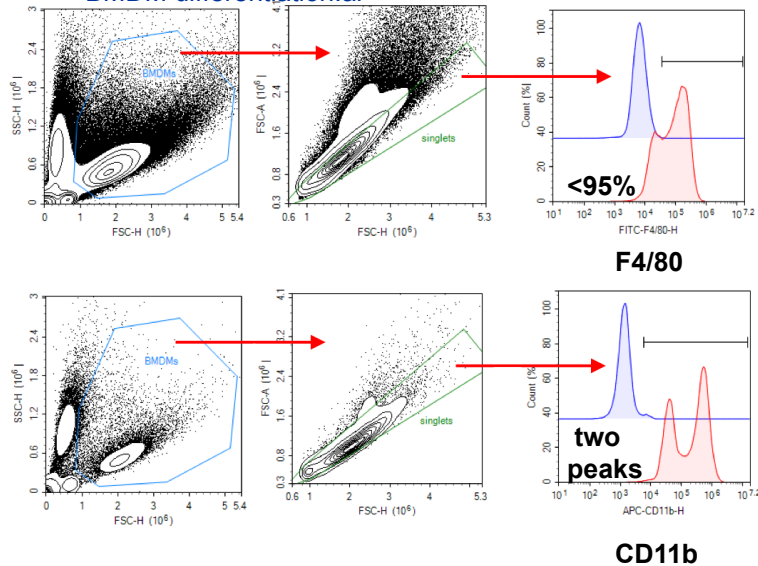


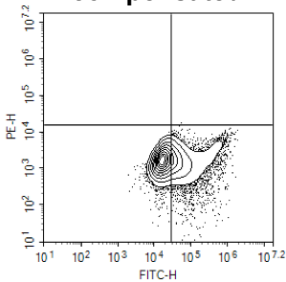
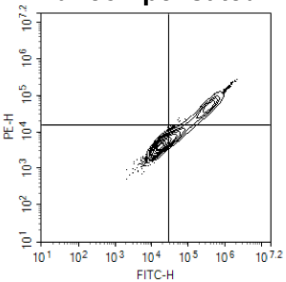
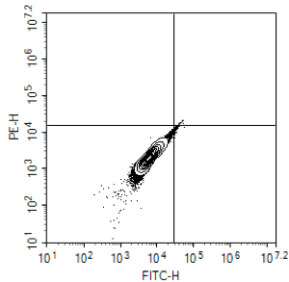
Figure 2

green probe + inducer
uncompensated

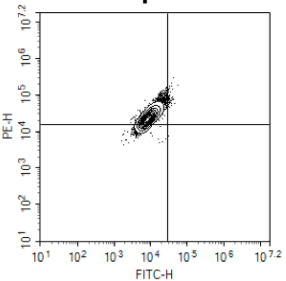
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green probe + inducer
compensated

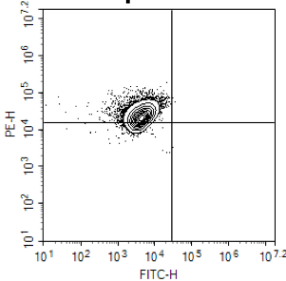
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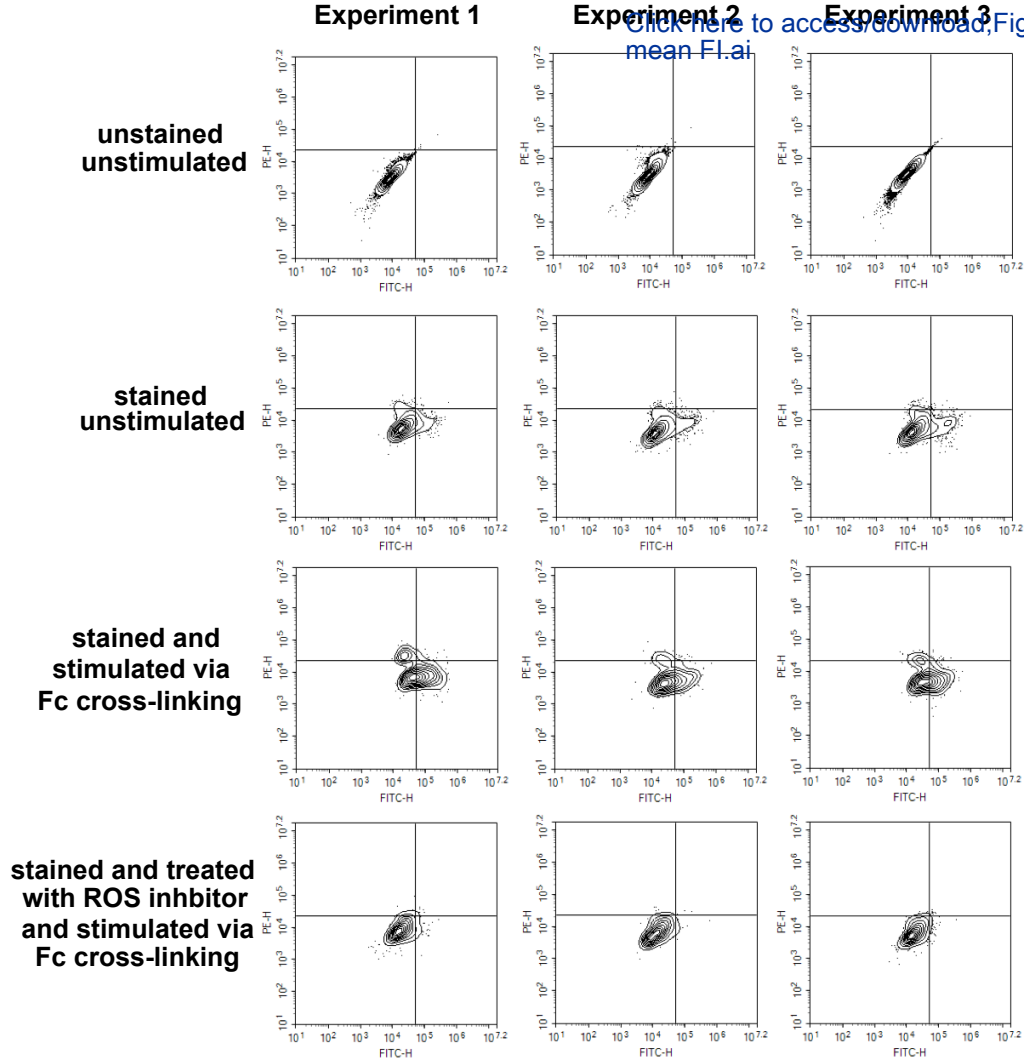
orange probe + inducer
uncompensated



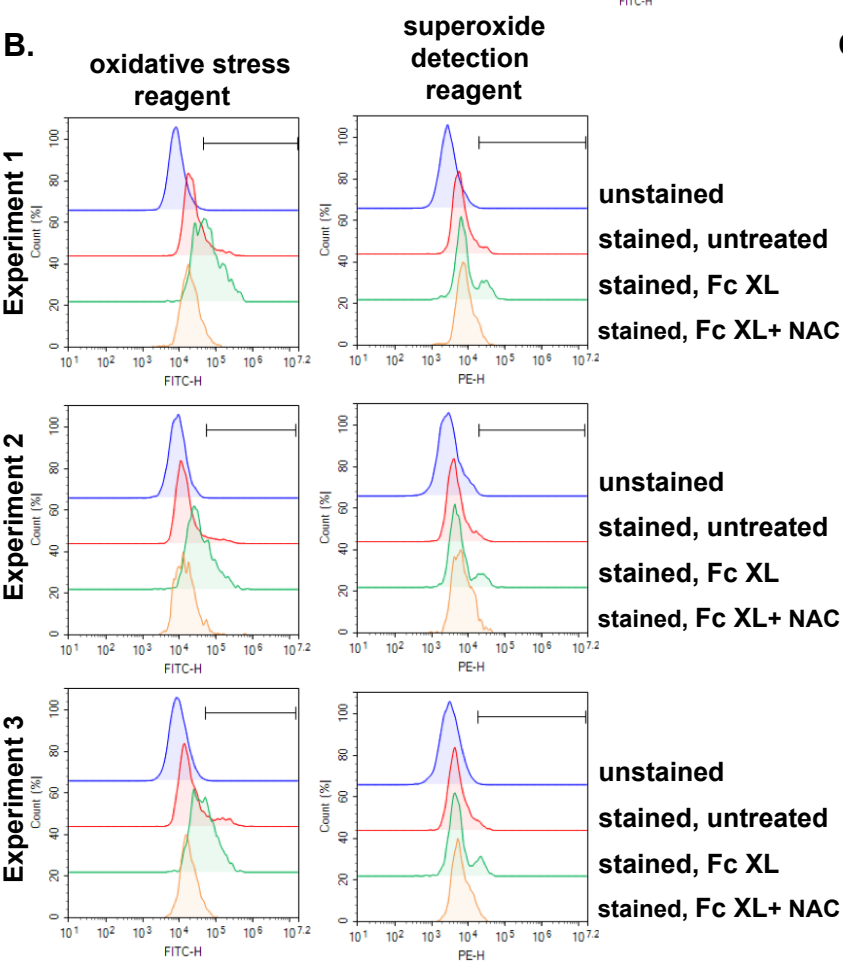
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A. Figure 3



B.



C.

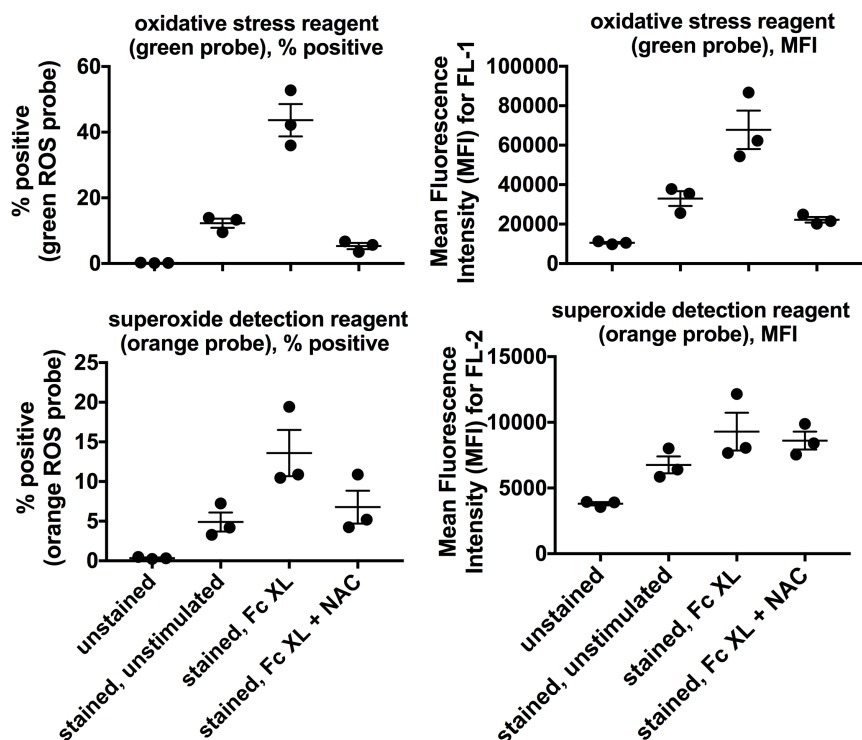


Figure 4

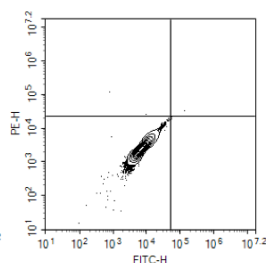
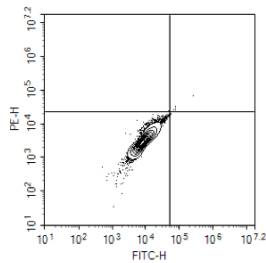
B.

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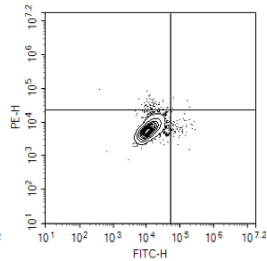
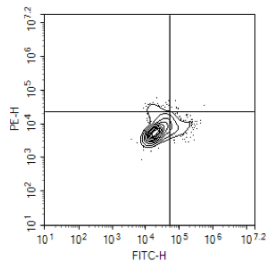
Successful stimulation

Suboptimal stimulation

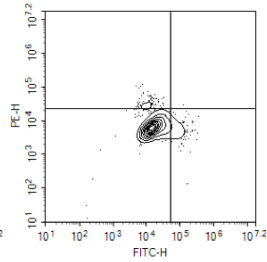
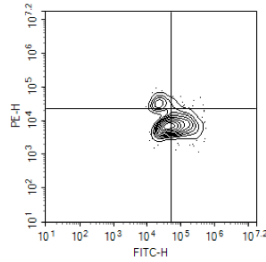
**unstained
unstimulated**



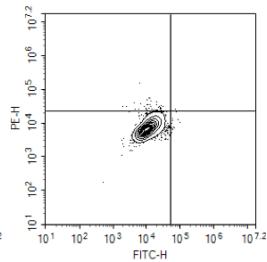
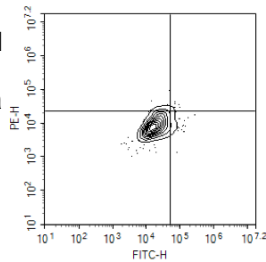
**stained
unstimulated**



**stained and
stimulated via
Fc cross-linking**

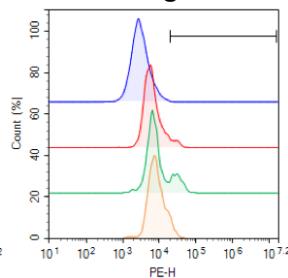
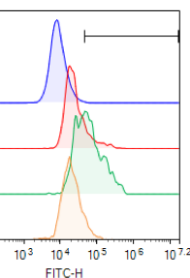


**stained and treated
with ROS inhibitor
and stimulated via
Fc cross-linking**

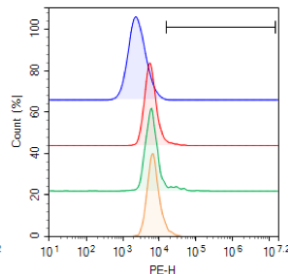
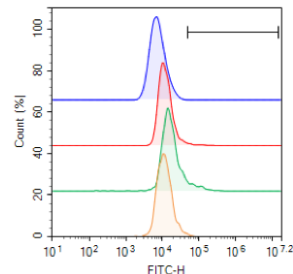


Successful stimulation

Suboptimal stimulation



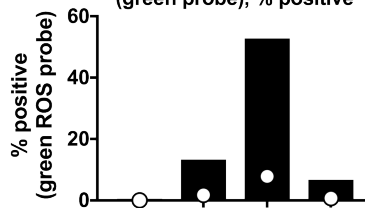
**unstained
stained, untreated
stained, Fc XL
stained, Fc XL+ NAC**



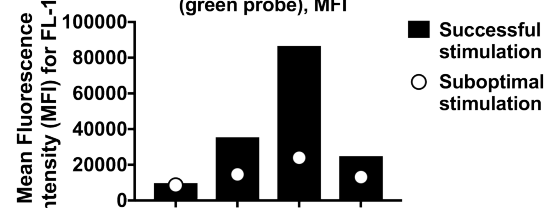
**unstained
stained, untreated
stained, Fc XL
stained, Fc XL+ NAC**

C.

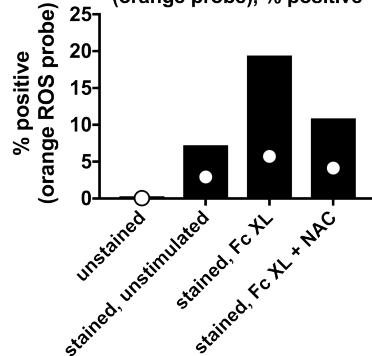
**oxidative stress reagent
(green probe), % positive**



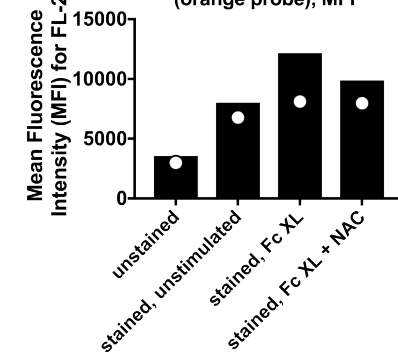
**oxidative stress reagent
(green probe), MFI**



**superoxide detection reagent
(orange probe), % positive**



**superoxide detection reagent
(orange probe), MFI**



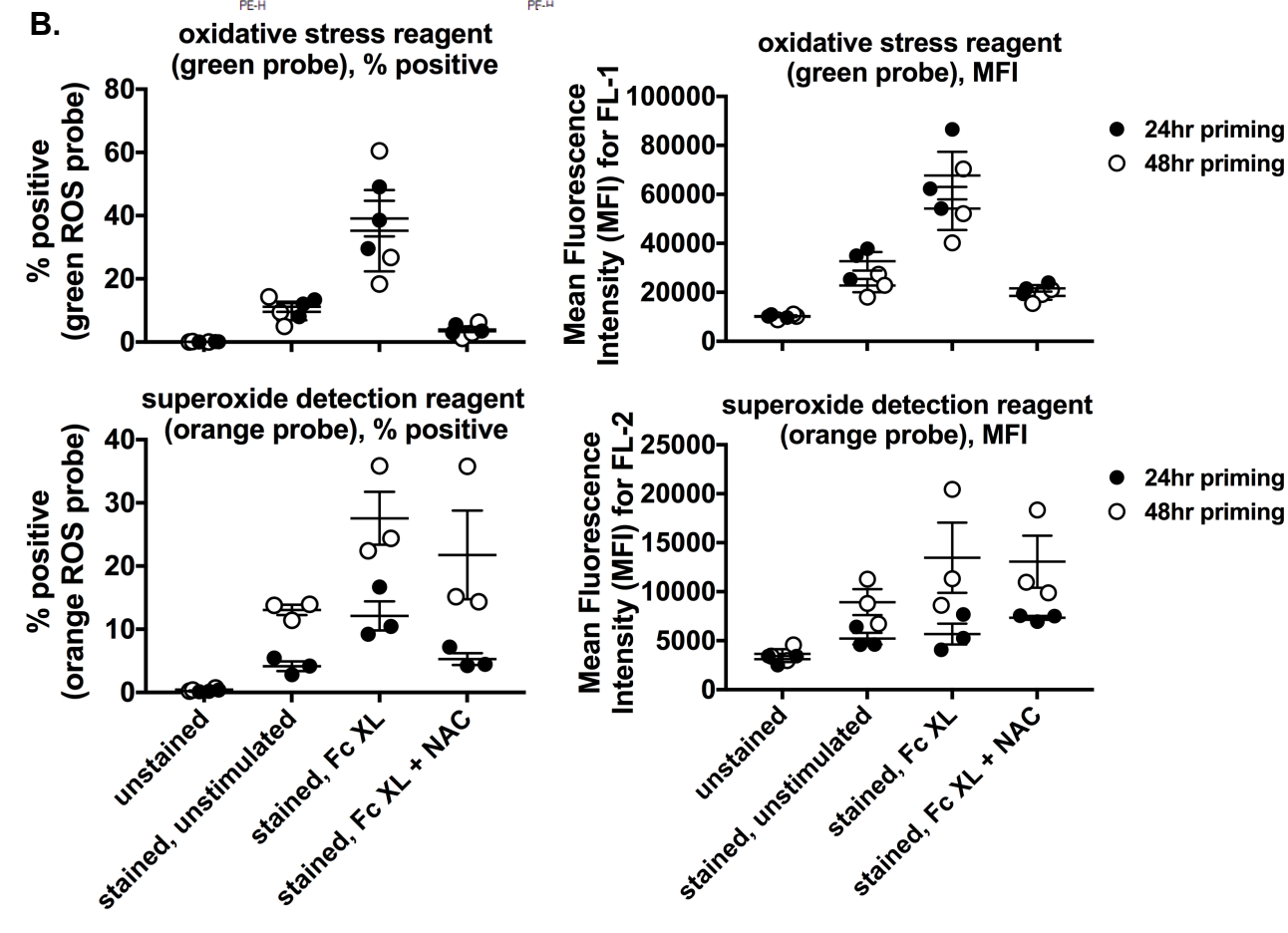
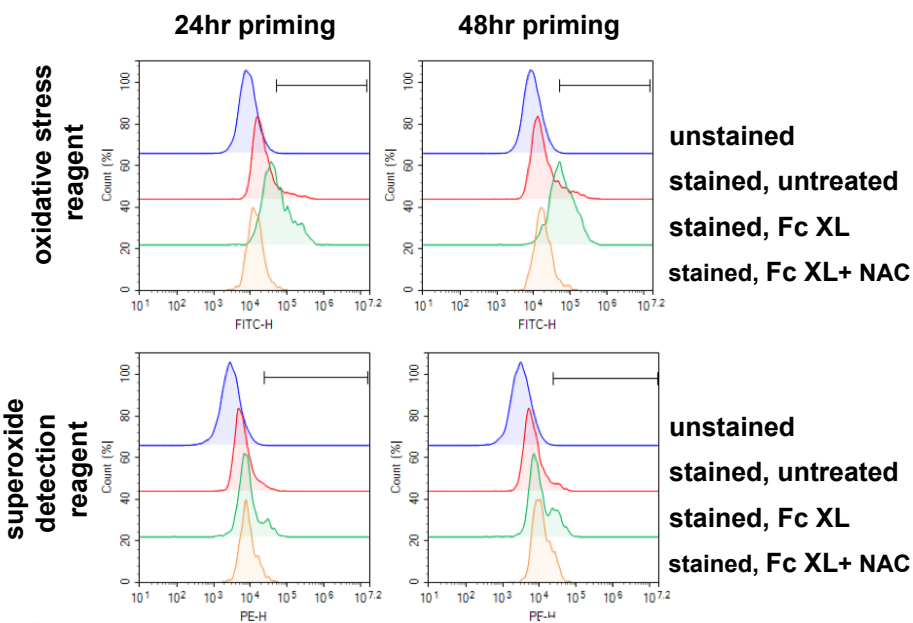
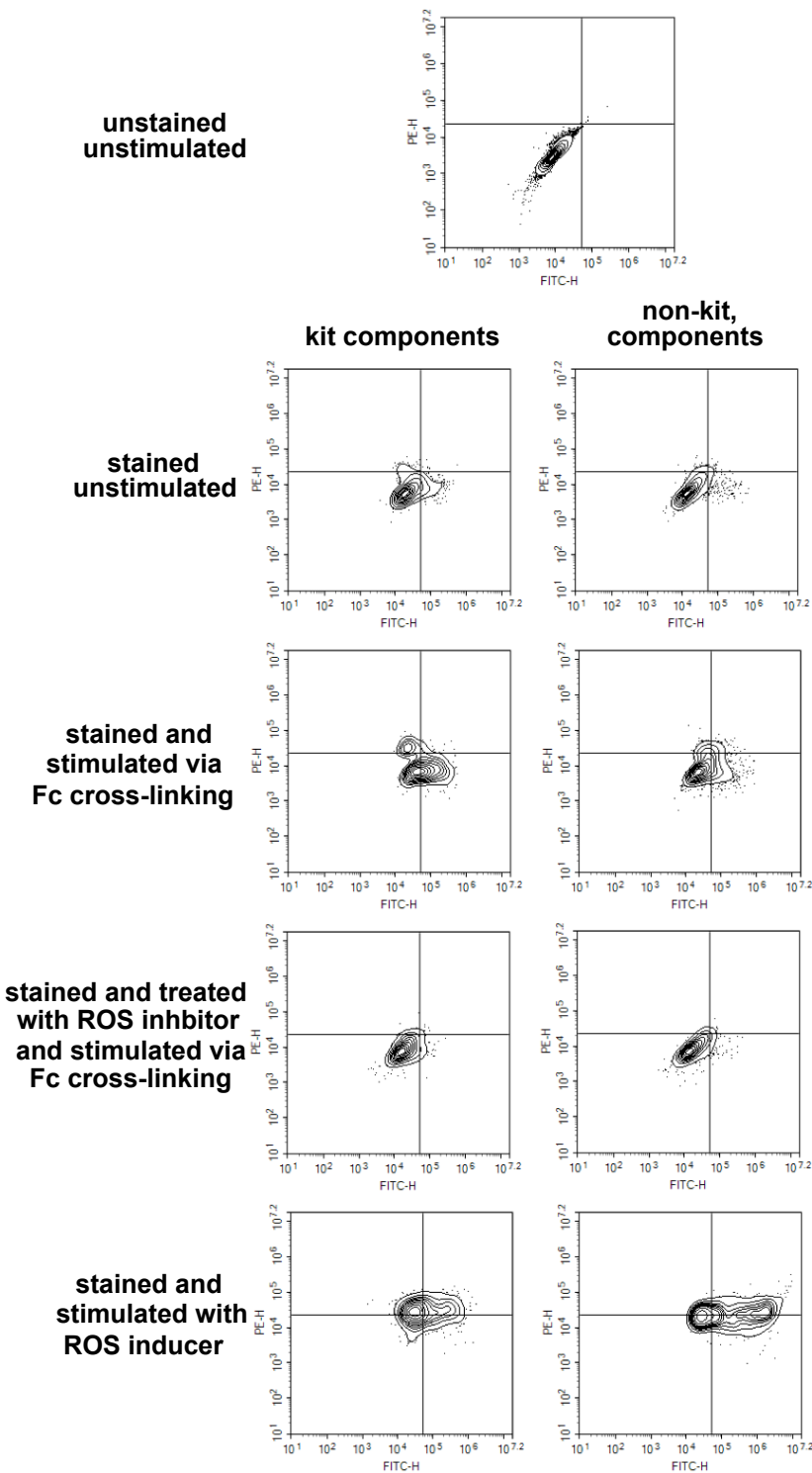


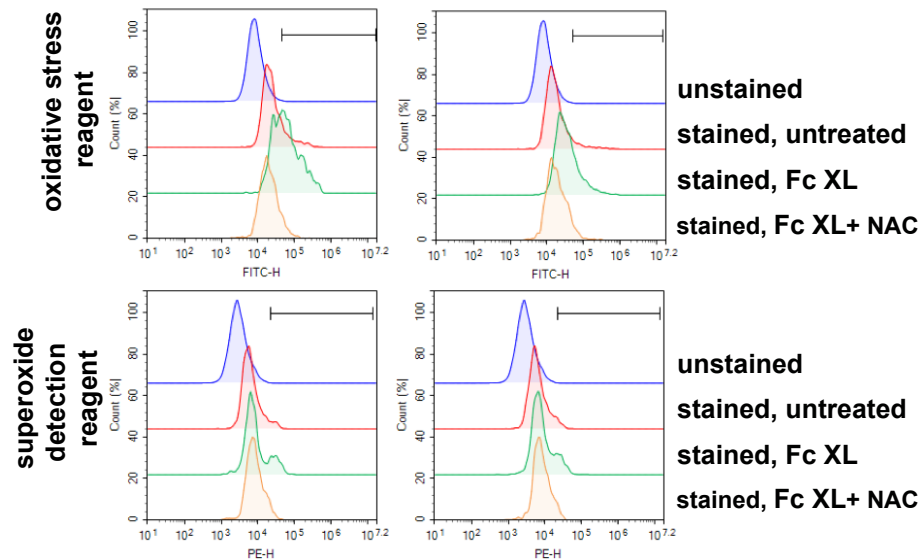
Figure 6

A.

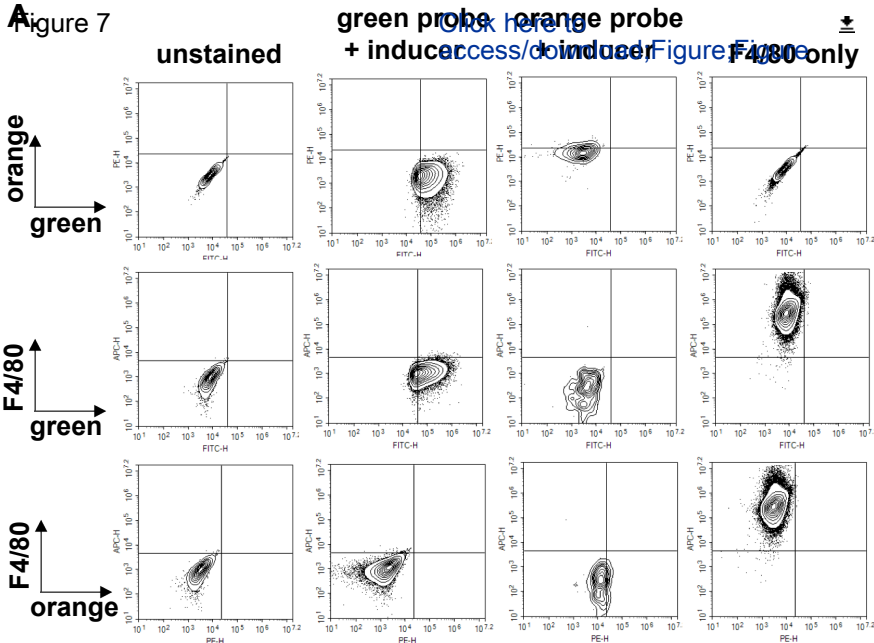


B.

[Click here to access/download;Figure-Figure 6 kit vs individual.ai](#)



A Figure 7



B.

**stained
unstimulated**

**stained and
stimulated via
Fc cross-linking**

**stained and treated
with ROS inhibitor
and stimulated via
Fc cross-linking**

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Anti-BSA IgG1	Innovative Research	IBSA9E2C2	
Alexa Fluor 647 Rat IgG2b, κ			
Isotype Ctrl Antibody	BioLegend	400626	
Anti-mouse CD16/32	BioLegend	101302	
Anti-mouse F4/80 antibody conjugated to Alexa Fluor 647	BD Biosciences	565853	
Anti-mouse F4/80 antibody conjugated to FITC	BioLegend	123108	
Anti-mouse/human CD11b antibodyconjugated to Alexa Fluor 647	BioLegend	101218	
beta-mercaptoethanol (BME)	Sigma	M3148-100ml	
Bovine Serum Albumin (BSA) FractionV	Fisher	BP1600-100	
C57BL/6J	Jackson labs	Stock No.000664	
CM-H2DCFDA	Molecular Probes	C6827	Can be a substitute for oxidative stress detection reagent
Dihydroethidium (DHE)	Molecular Probes	D11347	Can be a substitute for superoxide detection reagent in th
DMEM 1x	Corning	10-013-CV	
DMEM no phenol red	Gibco	31053-028	
DMF Anhydrous	Acros Organics	61094-1000	
Fetal Bovine Serum (FBS)	VWR	97068-085	
FITC Rat IgG2a, κ Isotype Ctrl Antibody	BioLegend	400506	
HEPES (1M)	Gibco	15630-080	
L glutamine	Gibco	25030-081	

LADMAC cells	ATCC	CRL-2420	
MEM	Corning	10-010-CV	
mouse IFN-g	GoldBio	1360-06-100	
N-Acetyl-L-cysteine	EMD Milipore	106425	Can be a substitute for ROS inhibitor/scavenger in the Enz
Novocyte flow cytometer with autosampler	Acea	2060R	
Pyocyanin (ROS inducer)	Cayman chemical	10009594	Can be a substitute for inducer in the Enzo kit
ROS-ID total ROS/superoxide detection kit	ENZO	ENZ-51010	
Sodium pyruvate (100mM)	Gibco	11360-070	
Trypsin-EDTA (0.25%)	Gibco	25200-056	

in the Enzo kit

e Enzo kit

o kit



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tel. 617.945.9051
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Author(s):	Michael G. Shehat and Justine T. Tigno-Aranjuez

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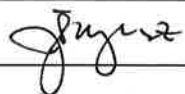
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A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Justine T. Tigno-Aranjuez	
Department:	Burnett School of Biomedical Sciences, Division of Immunity and Pathogenesis	
Institution:	University of Central Florida	
Title:	Flow Cytometric Measurement of ROS Production in Macrophages in Response to FcγR Cross-linking	
Signature:		Date: 9/28/18

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December 17, 2018

To the Editors of JoVE:

We thank you for your prompt review of the manuscript. Please find below the editorial revisions which have been requested and our response to address each of them. The line numbers correspond to the revised (unmarked, untracked) document. We hope you find this version of the manuscript clearer and free of formatting errors.

Sincerely,



Justine Tigno-Aranjuez

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have additionally corrected the following typographical errors as indicated below.

Line 350: “to” replaced with “into”.

Line 391: extra space removed.

Line 487: uppercase corrected to lowercase.

Line 612: step 3.10 has been added.

Line 627: step 6.13 has been corrected to 6.12.

Line 694: “FcγR” corrected to “FcγR”.

Line 696: “FcγR” corrected to “FcγR”.

Line 718: extra space removed.

Line 835: extra space removed.

2. Please do not highlight notes/cautions for filming.

Highlighting of notes was removed from lines: 182, 196 and 477.

3. The highlighted protocol steps are over the 2.75 page limit. Please highlight fewer steps for filming.

Fewer steps have been highlighted as requested.

4. The Summary is over the 50 word limit.

This has been corrected and the summary now is 49 words.

Line 24: The words "fluorescent probes" has been removed from line 24.

Line 25: "as a result of stimulation of macrophages through the FcγR" has been reworded to "resulting from activation of the FcγR".

Line 27: The word "and" has been changed to "or"

Line 27: The word "Fcγ receptor" has been replaced with "FcγR".

5. Please use a single space between numerical values and their units.

This has been corrected in lines 79, 84, 85, 104, 105, 110, 111, 114, 117, 120, 123, 128, 129, 132, 133, 134, 137, 138, 148, 154, 155, 158, 159, 161, 162, 163, 164, 166, 174, 176, 177, 178, 179, 184, 185, 188, 190, 191, 193, 200, 202, 203, 204, 210, 213, 215, 216, 218, 219, 221, 223, 225, 227, 229, 242, 244, 254, 255, 261, 262, 267, 270, 271, 273, 276, 281, 282, 284, 285, 288, 289, 293, 295, 297, 298, 318, 319, 322, 323, 333, 338, 340, 341, 345, 348, 350, 352, 353, 354, 355, 356, 357, 362, 375, 376, 377, 378, 381, 383, 385, 386, 391, 407, 408, 411, 416, 445, 446, 460, 462, 463, 468, 471, 474, 475, 480, 563, 580, 582, 584, 585, 589, 592, 594, 598, 599, 602, 603, 602, 603, 606, 607, 609, 610, 624, 633, 672, 675, 678, 706, 707, 722, 735, 747, 761, and 778.

6. Please use standard SI unit symbols and prefixes such as μL, mL, L, g, m, etc., and h, min, s for time units.

This has been corrected in lines 128, 137, 154, 155, 161, 176, 177, 191, 203, 213, 216, 223, 225, 229, 254, 261, 281, 282, 284, 285, 297, 298, 319, 323, 383, 416, 460, 462, 463, 468, 471, 472, 474, 475, 478, 585, 589, 633, 672, 675, 678, 722, 735, 745, 747, 749, 750, 751, 752, 758, 761, 770, 776, and 778.

7. Please split some long steps into two or more sub-steps so that each step contains 2-3 actions and is less than 4 lines.

This has been corrected as indicated below.

Line 103, section (1.1.1.): Additional details were put in a separate note.

Line 136, section (1.2.3): Additional details were put in a separate note.

Line 184, section (2.3): The long steps were split into two sub-steps.

Line 284, section (3.9): The long steps were split into 2 steps (3.9 and 3.10).

Line 365, section (5.8): Additional details were put in a separate note.

Line 435, section (6.12): Additional details were put in a separate note.

Line 486, section (7.1): Additional details were put in a separate note.

Line 495, section (7.2): Additional details were put in a separate note.

Line 511, section (7.3): Additional details were put in a separate note.

8. Step 7.2, 7.3: Please ensure that all text is written in the imperative tense.

Line 495, section (7.2), text was rephrased to be written in imperative tense and additional details were put in a separate note.

Line 511, section (7.3), text was rephrased to be written in imperative tense and additional details were put in a separate note.

9. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

The registered symbol was removed from row 2, 5 and 7 in the table of materials.

The trademark symbol was removed from row 20 in the table of materials.

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 (FcγR). 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 FcγR 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.