Flow Cytometric Measurement Of ROS Production In Macrophages In Response To FcγR Cross-linking

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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.

Keywords

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 aging[2]. In phagocytic cells, ROS plays an essential role in providing antimicrobial activity during the so- called "respiratory burst"[1,3,4,5,6]. 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 microbe[5]. 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)[7,8,9,10]. 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 stress[11,12,13]. Among these, one of the most widely used is the fluorescent probe 2',7' dichlorodihydrofluorescein diacetate (DCFH$_2$-DA)[14]. This molecule is colorless and lipophilic. Diffusion of DCFH$_2$-DA across the cell membrane allows it to be acted upon by intracellular esterases, which deacetylates it into DCFH$_2$, rendering it cell impermeable. The actions of multiple types of ROS (hydrogen peroxide, peroxynitrite, hydroxyl radicals, nitric oxide, and peroxy radicals) on DCFH$_2$ 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 DCFH$_2$ 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.   Culture media preparation

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)

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.

3.   Prepare complete DMEM media: To DMEM, add 10% heat inactivated FBS and 1× 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.

2.   Preparation of LADMAC conditioned medium

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% CO_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% CO_2 (approximately one week). This way, one 10 cm dish can yield ten T-175 flasks or approximately 1 L of conditioned media.

3.   Once the cells are ready, collect the conditioned media and centrifuge at $350 \times g$ for 5 min to pellet unwanted cells. Filter the collected

supernatants through a 0.2 mm 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.

3. **Preparation of bone marrow derived macrophages**

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.

2. **Day 1**: Isolate mouse bone marrow cells according to the previously described protocol[16] 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 mm cell strainer placed on top of a 50 mL tube. The remaining media (~5 mL) can be used to rinse out the cell strainer.

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

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% CO_2.

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.

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

2. Harvesting, seeding and priming of BMDMs

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. 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.

3. Centrifuge the 50 mL tubes at $350 \times g$ 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:

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

    2. Of this mixture, load 10 μL into a hemocytometer and count the central square (5×5 grid). The total cell count = count $\times 10^4 \times$ dilution factor (10) $\times$ volume (10 mL).

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.

5. Incubate the plates overnight at 37 °C, 5% CO_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% CO_2.

7. If desired, take an aliquot of the cells to assess appropriate generation of BMDMs by flow cytometry. Use 1×10^6 cells per test and prepare polystyrene FACs tubes for the following conditions: isotype control, stained with F4/80 (or alternatively, stained with CD11b).

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

9. Aliquot 1×10^6 cells per tube and centrifuge at $750 \times g$ for 5 min.

10. Wash once by decanting or aspirating supernatant and resuspending cells in 2 mL of flow cytometry buffer. Centrifuge at $750 \times g$ for 5 min.

11. Aspirate supernatant and resuspend cells in 100 μL of flow cytometry staining buffer. Add 1 μL of anti-mouse CD16/32 Fc blocking antibody to each tube. Incubate on ice for 5 min.

12. Without washing, add 2 μL of FITC anti-mouse F4/80 or 1 μL 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.

13. Wash cells by adding 2 mL of PBS directly to the cells. Centrifuge at $750 \times g$ for 5 min.

14. Aspirate supernatant and resuspend cells in 150 μL of PBS.

15. Acquire samples on a flow cytometer using a stop condition of 100 μL 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.

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).

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.

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.

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

1. Reconstitute the lyophilized oxidative stress detection reagent in 60 μL 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.

2. Reconstitute the lyophilized superoxide detection reagent in 60 μL 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. Reconstitute the ROS inducer (Pyocyanin) in 20 μL of anhydrous DMF to yield a 50 mM stock solution.

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

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

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

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

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

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

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

NOTE: To prepare smaller volumes of 2× 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 2× detection solution, dilute 1 μL of each probe into 9 μL of DMEM (1:10 intermediate dilution). Dilute 4 μL of this 1:10 intermediate dilution into 1 mL of DMEM.

4.   Assay conditions and controls

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.

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

  3. Calculate the amount of 2× probe solution needed based on the number of mice and conditions (200 μL of the 2× 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 2× probe solution needed is at least 6 mL (30*200 μL).

5. Cell preparation

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

  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 IgG$_1$ 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 IgG$_1$ to treated plates.

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

  3. Incubate the plates for 4 h at 37 °C, 5% CO$_2$.

  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 $750 \times g$ for 5 min. Keep track of which cells were treated with murine anti-BSA IgG$_1$.

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

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

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

    1. From the untreated cells, take 200 μL for tubes labelled "unstimulated", 200 μL for tubes labelled "positive inducer" and 200 μL for tubes labelled "positive inducer + inhibitor"

    2. From the anti-BSA IgG$_1$ treated cells, take 200 μL for tubes labelled "FcγR crosslinking/FcγR XL" and 200 μL for tubes labelled "FcγR XL + inhibitor"

    3. From the untreated cells to be used for compensation controls, take 200 μL for the tube labelled "unstained unstimulated", 200 μL for the "green + inducer" control and 200 μL 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 μL of cells will be needed for an "unstained unstimulated" experimental control for a).

8. Keep the tubes on ice until staining and stimulation. During serum starvation and binding of IgG$_1$, 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

1. Prepare 2× positive inducer solution by diluting the Pyocyanin 1:100 in the 2× probe solution (prepared in step 3.9) to obtain a 2× concentration of 500 μM of pyocyanin (final concentration will be 250 μM). Also, dilute pyocyanin 1:100 into each of the "2× probe solution, green only" and "2× 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 2× 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 μL =1.2 mL of 2× positive inducer solution.

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

NOTE: Both conditions labelled with "FcγR XL" and "FcγR XL + inhibitor" will be treated with the 2× 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 μL or 1.2 mL of 2× BSA solution will be needed.

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.

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.

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

6. Incubate the cells for 30 min at 37 °C, 5% CO_2 in dark.

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.

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).

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.

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.

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.

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.

    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.

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.

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 IgG$_1$).

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

    1. For unstimulated cells: add 200 μL of 2$\times$ probe solution without any stimulus to the 200 μL of cell suspension labelled "stained, unstimulated".

    2. For positive controls: add 200 μL of 2$\times$ positive inducer solution (prepared in step 6.1) to 200 μL of cell suspension labelled "positive inducer" or "positive inducer + inhibitor".

    3. For cells stimulated by FcγR cross-linking (specific stimulus): add 200 μL of 2$\times$ 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 $\times$ min where $\times$ is the lag time between acquisition of one sample and the next.

16. Incubate the cells for 30 min at 37 °C, 5% CO$_2$ 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

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.

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

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.

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).

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.

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).

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, peroxynitrite, 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.

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).

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.

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 IgG$_1$ while serum starving while the other two plates will be left untreated.

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.

        1.   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 $Fc\gamma R$

        c.   stained with F4/80 and activated through the $Fc\gamma R$ and treated with ROS inhibitor

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 IgG_1 while serum starving, while the other two plates will be left untreated. Incubate the plates for 4 h at 37 °C, 5% CO_2.

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 $750 \times g$ for 5 min. Keep track of which cells were treated with murine anti-BSA IgG_1.

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

5. Resuspend one of the cell pellets from the plate which did not get anti-BSA IgG_1 in 600 μL of low serum DMEM. These cells will *not* be subjected to cell surface staining. Use these for compensation controls. Aliquot 200 μL 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.

6. Resuspend one of the cell pellets from the plate which did not get anti-BSA IgG_1, in 300 μL of flow cytometry staining buffer (PBS + 0.1% FBS). Aliquot 100 μL of this cell suspension into (2) 5 mL round bottom tubes for staining with Alexa 647 anti-mouse F4/80.

7. Resuspend the cell pellets from the plate which received anti-BSA IgG_1 in 300 μL of flow cytometry staining buffer. Aliquot 100 μL of this cell suspension into (2) 5 mL round bottom tubes for staining with Alexa 647 anti-mouse F4/80.

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

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

1. For cells not receiving anti-BSA IgG$_1$, label tubes with the following: a) no stimulus or b) ROS inducer.

2. For cells which received anti-BSA IgG$_1$, label with the following: a) Fc XL or b) Fc XL + inhibitor.

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.

11. Incubate the cells for 30 min at 37 °C, 5% CO$_2$ in dark. Analyze the samples in the order they were stimulated using a flow cytometer equipped with an autosampler.

12. Perform manual compensation as described in 6.7–6.12 and data analysis as described in section 7.

13. 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.).

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

    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.

15. 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 pretreatment 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).

Discussion

DCFH$_2$-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 mefanamic 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, $DCFH_2$-DA measures multiple ROS species, and so, fluorescence resulting from the green probe cannot be used to discriminate one ROS species from another[14]. 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 HPLC[15]. 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.

Acknowledgments

References

1. Winterbourn CC, Kettle AJ, Hampton MB Reactive Oxygen Species and Neutrophil Function. Annual Review of Biochemistry. 85, 765–792 (2016).

2. Schieber M, Chandel NS ROS function in redox signaling and oxidative stress. Current Biology. 24 (10), R453–462 (2014). [PubMed: 24845678]

3. Robinson JM Reactive oxygen species in phagocytic leukocytes. Histochemistry and Cell Biology. 130 (2), 281–297 (2008). [PubMed: 18597105]

4. Thomas DC The phagocyte respiratory burst: Historical perspectives and recent advances. Immunology Letters. 192, 88–96 (2017). [PubMed: 28864335]

5. Fang FC Antimicrobial actions of reactive oxygen species. MBio. 2 (5) (2011).

6. Iles KE, Forman HJ Macrophage signaling and respiratory burst. Immunologic Research. 26 (1–3), 95–105 (2002). [PubMed: 12403349]

7. Curnutte JT, Whitten DM, Babior BM Defective superoxide production by granulocytes from patients with chronic granulomatous disease. New England Journal of Medicine. 290 (11), 593–597 (1974). [PubMed: 4359964]

8. Good RA et al. Fatal (chronic) granulomatous disease of childhood: a hereditary defect of leukocyte function. Seminars in Hematology. 5(3), 215–254 (1968). [PubMed: 5662845]

9. Holmes B, Page AR, Good RA Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. Journal of Clinical Investigation. 46 (9), 1422–1432 (1967). [PubMed: 6036538]

10. Windhorst DB, Page AR, Holmes B, Quie PG, Good RA The pattern of genetic transmission of the leukocyte defect in fatal granulomatous disease of childhood. Journal of Clinical Investigation. 47 (5), 1026–1034 (1968). [PubMed: 5645849]

11. Dikalov SI, Harrison DG Methods for detection of mitochondrial and cellular reactive oxygen species. Antioxidants & Redox Signaling. 20(2), 372–382 (2014). [PubMed: 22978713]

12. Held P An Introduction to Reactive Oxygen Species: Measurement of ROS in cells. White Paper. (2015).

13. Woolley JF, Stanicka J, Cotter TG Recent advances in reactive oxygen species measurement in biological systems. Trends in Biochemical Sciences. 38 (11), 556–565 (2013). [PubMed: 24120034]

14. Chen X, Zhong Z, Xu Z, Chen L, Wang Y 2',7'-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: Forty years of application and controversy. Free Radical Research. 44 (6), 587–604 (2010). [PubMed: 20370560]

15. Zielonka J, Kalyanaraman B Hydroethidine- and MitoSOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: another inconvenient truth. Free Radical Biology and Medicine. 48 (8), 983–1001 (2010). [PubMed: 20116425]

16. Swamydas M, Lionakis MS Isolation, purification and labeling of mouse bone marrow neutrophils for functional studies and adoptive transfer experiments. Journal of Visualized Experiments. (77), e50586(2013). [PubMed: 23892876]
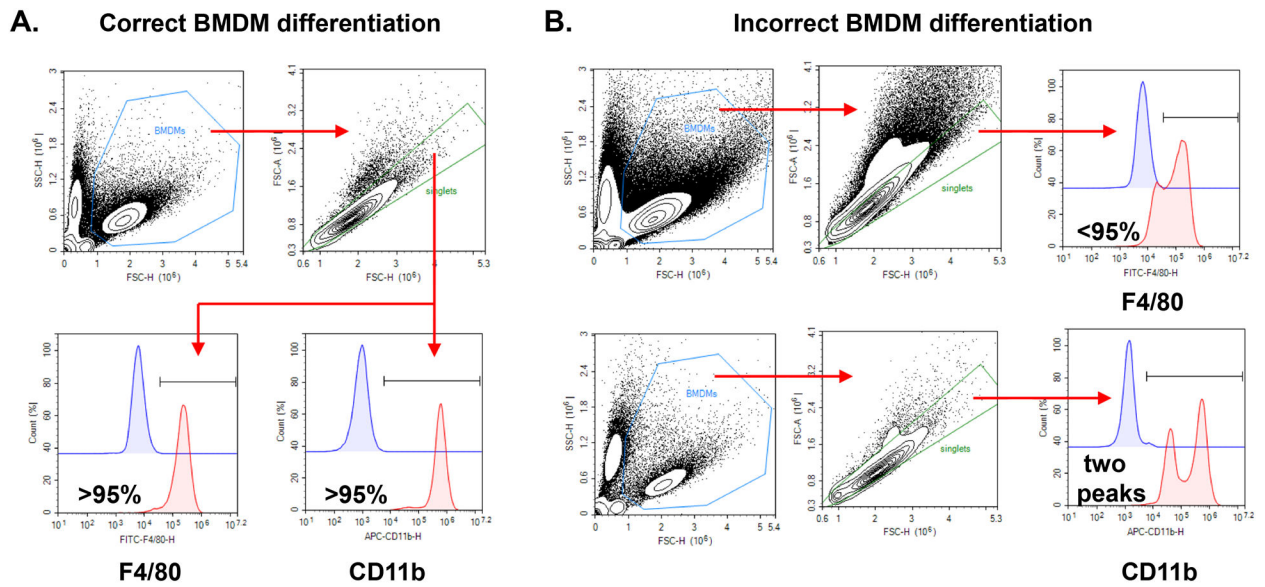
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Figure 1: Flow cytometric assessment of appropriate generation of BMDMs.
Wild type BMDMs were generated and were left either unstained or stained with FITC anti-mouse F4/80 or Alexa 647 anti-mouse CD11b. FSC (x-axis) vs SSC (y-axis) plots were generated 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.
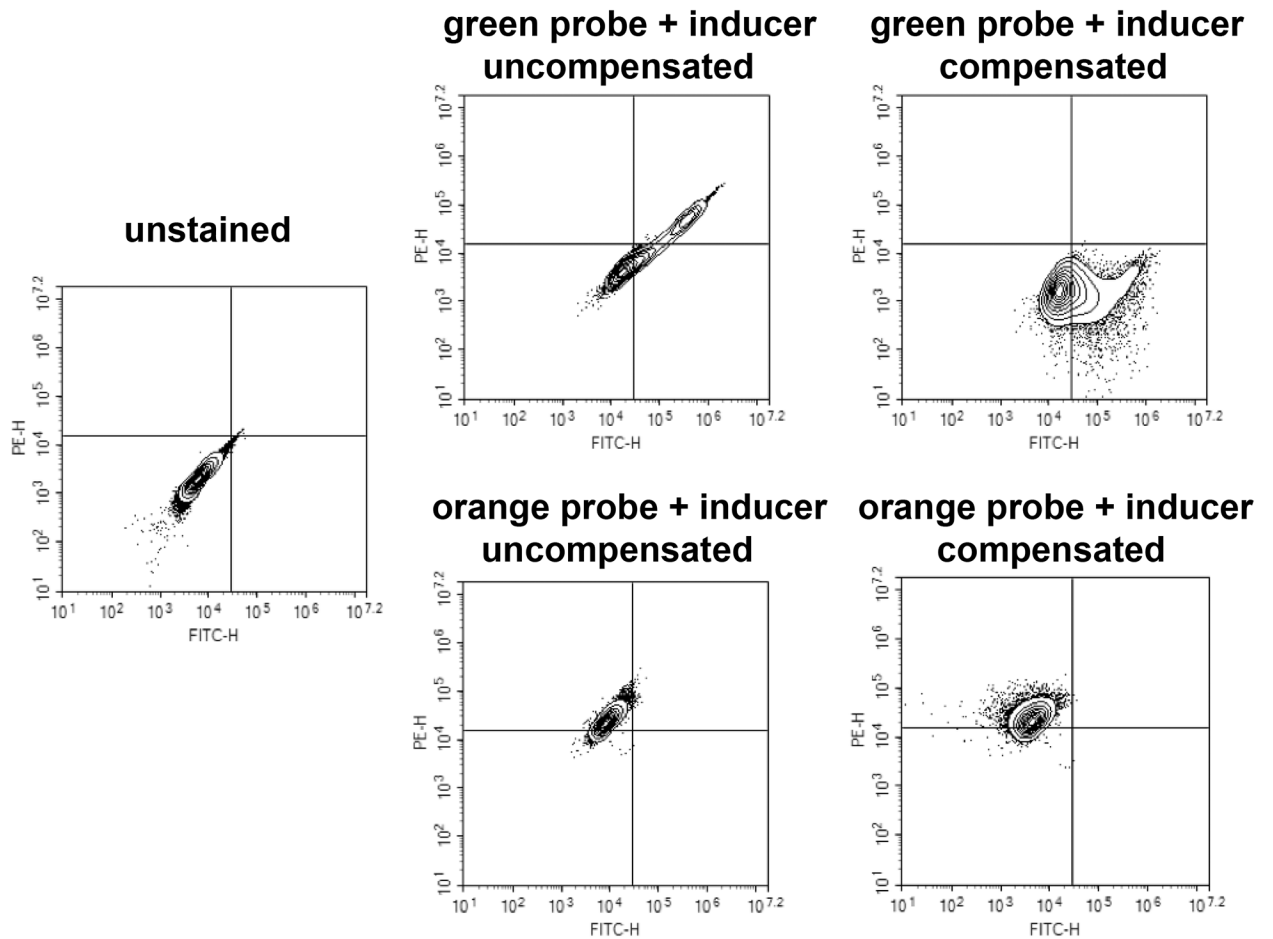
Figure 2. Performing compensation when using green and orange ROS probes.
Compensation will require unstained untreated cells, cells stained with green ROS probe and treated with ROS inducer, and cells stained with orange ROS probe and treated with ROS inducer. Dot plots for unstained untreated cells 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.
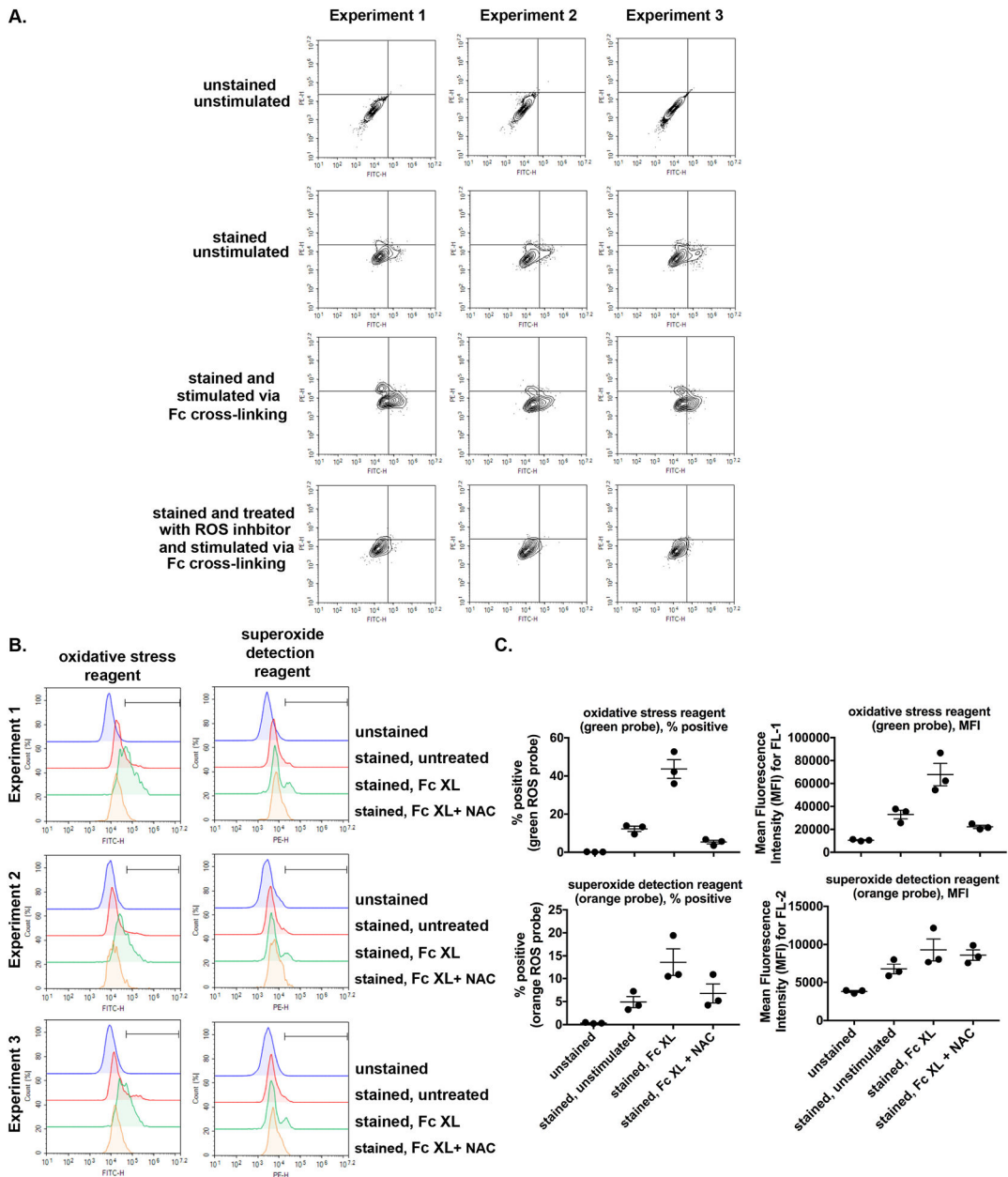
Figure 3. Measurement of ROS in response to specific FcγR stimulation using green and orange ROS probes and assessment of assay reproducibility.
Wild-type bone marrow-derived macrophages (BMDMs) were generated, primed, and were left either unstained or stained with a cocktail of green and orange ROS probes. Stained BMDMs were either left untreated, stimulated through their FcγRs using murine anti-BSA IgG$_1$ + 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.
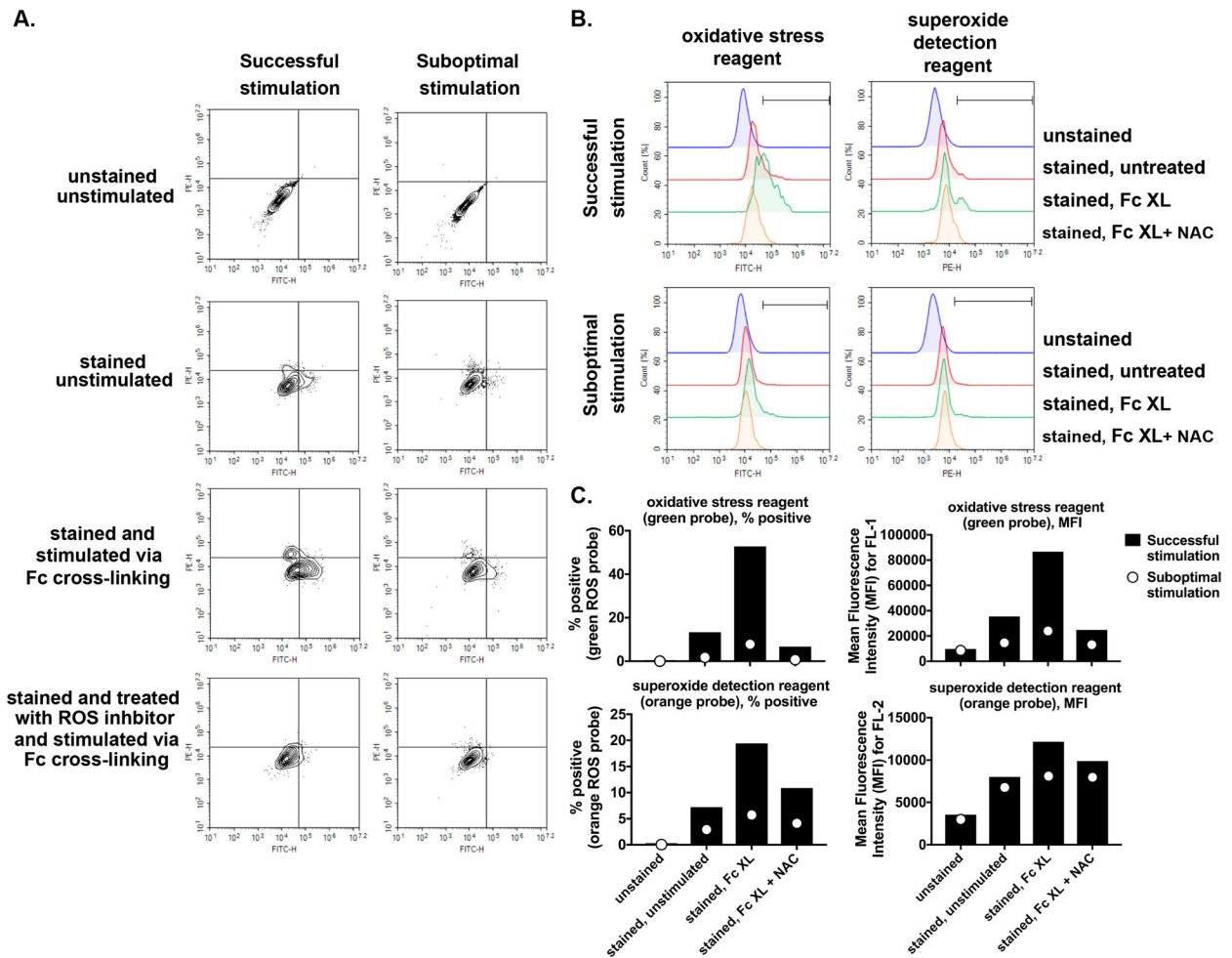
Figure 4. Examples of successful and suboptimal FcγR stimulation.
Wild-type bone marrow-derived macrophages (BMDMs) were generated, primed, and were left either unstained or stained with a cocktail of green and orange ROS probes. Stained BMDMs were either left untreated, stimulated through their FcγRs using murine anti-BSA IgG$_1$ + 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.
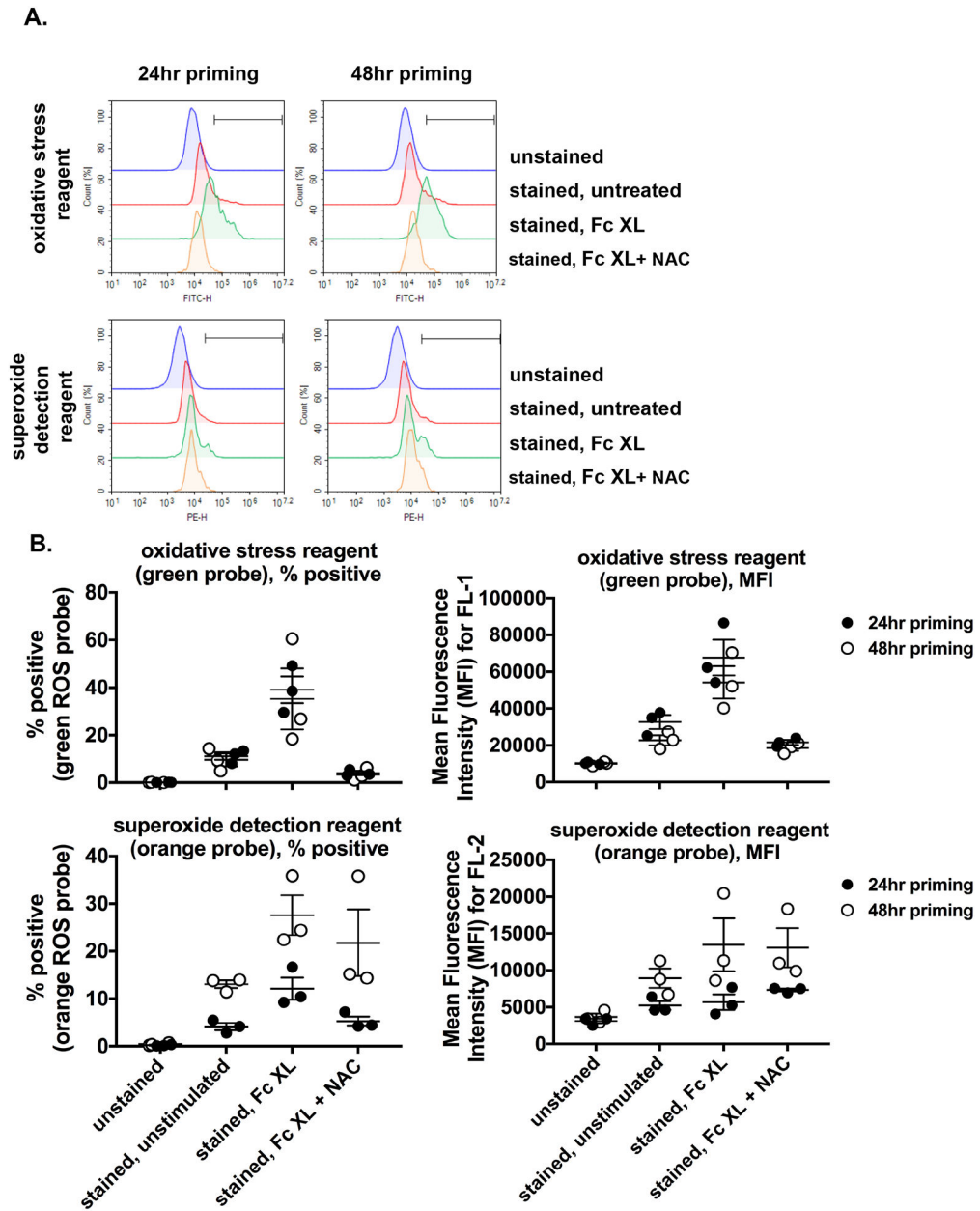
Figure 5. Effect of priming time on ROS generation upon FcγR stimulation.

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 IgG$_1$ + 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.

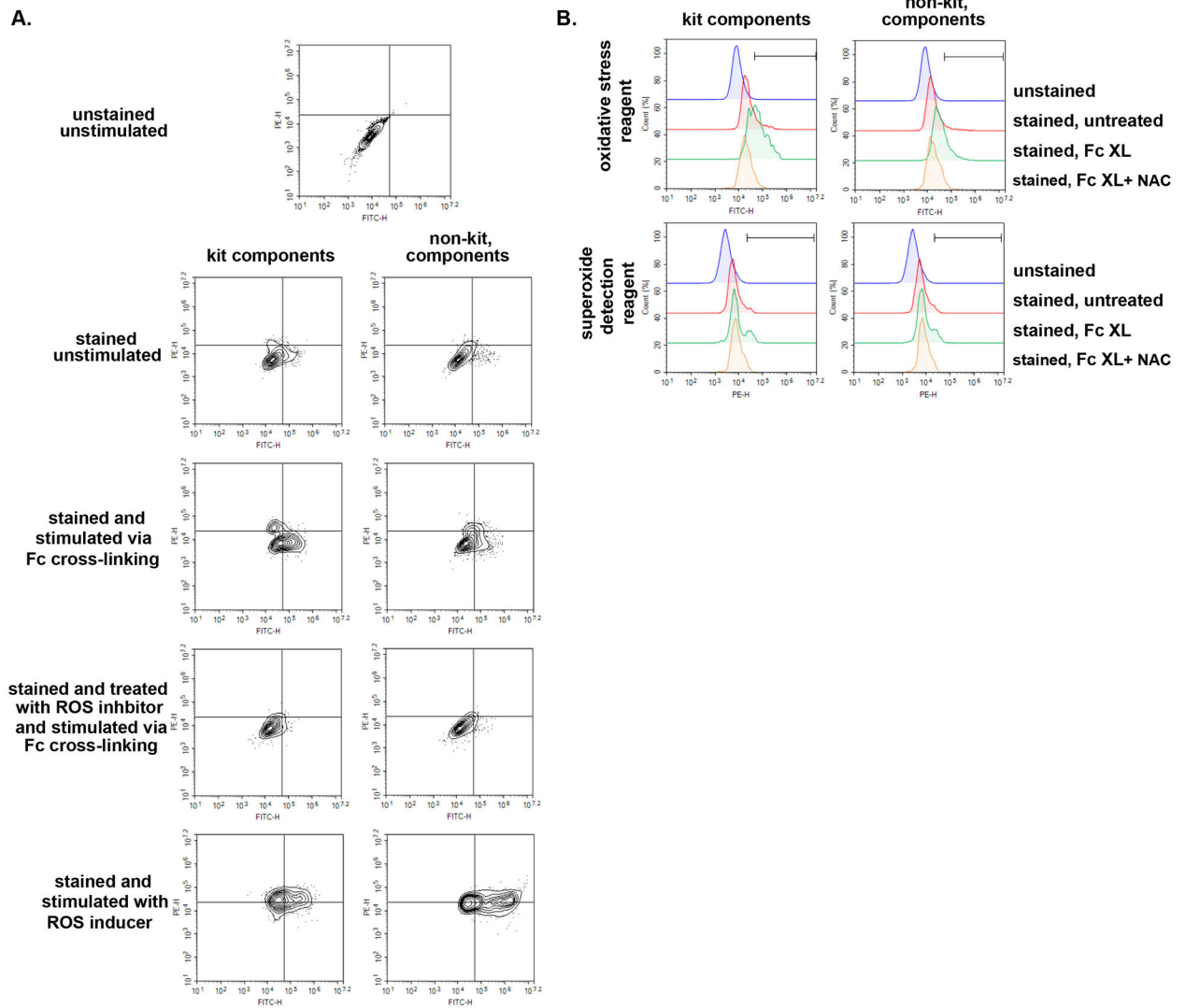**Figure 6. Flow cytometric ROS measurement upon FcγR cross-linking using reagents from different vendors.**

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 IgG$_1$ + 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.
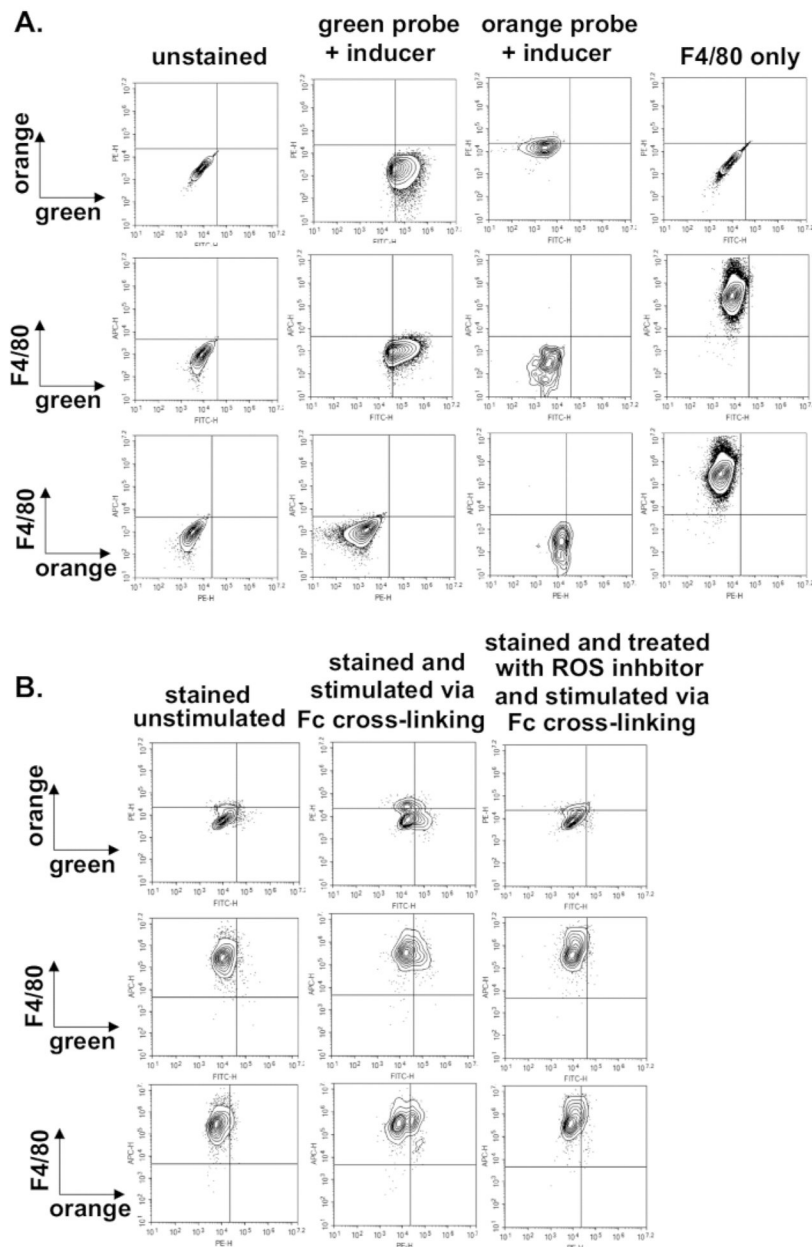
Figure 7. Combining cell-surface staining with flow cytometric measurement of ROS production upon FcγR cross-linking.

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 IgG$_1$ + 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.