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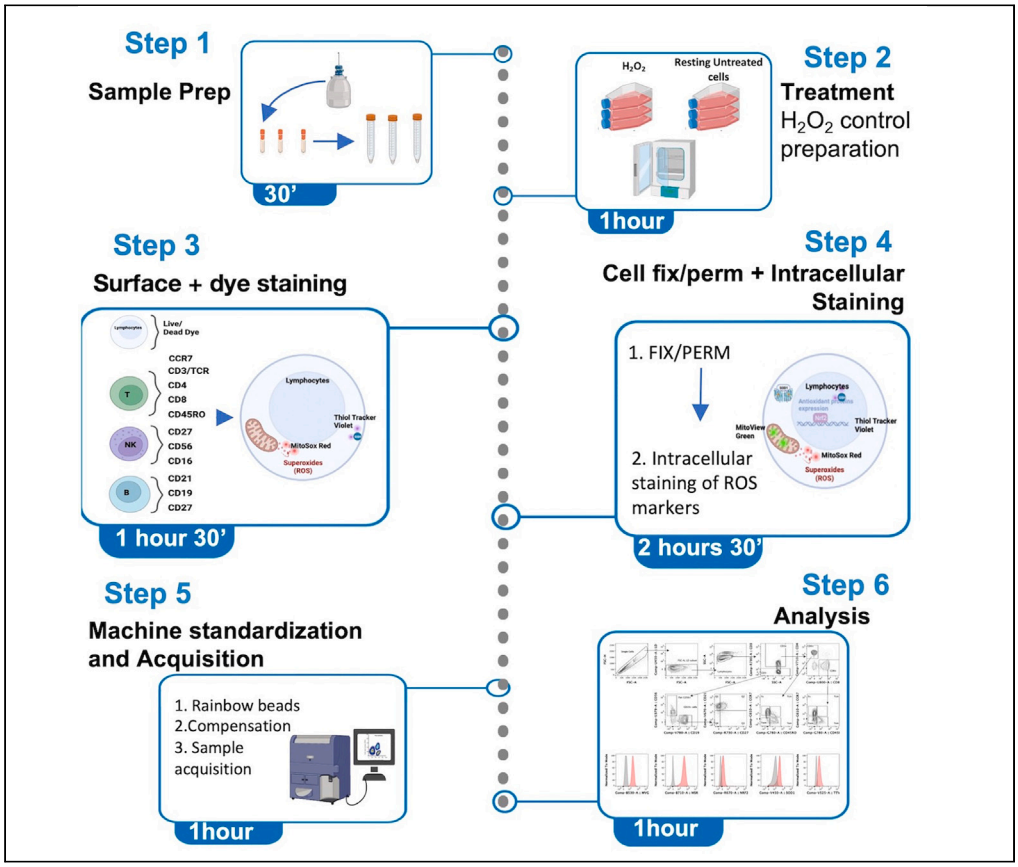
A redox-based characterization of human immune cell subsets by polychromatic flow cytometry



Cellular redox state determinants are traditionally studied using fluorescent microscopy and immunoblot analysis; however, no procedure has been developed for simultaneous measurement in various immune cell subsets. Here, we present a flow cytometry assay for measuring antioxidant defense systems and reactive oxygen species simultaneously in T, B, and natural killer lymphocytes. We describe steps for preparing and treating peripheral blood mononuclear cells, surface and dye staining, cell fixation/permeabilization, and intracellular staining. We then detail machine standardization, acquisition, and analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Optimized PFC panel measuring ROS in T, B, and NK cell subsets

Simultaneous measurement of ROS-related markers by PFC using a staining assay

Simultaneous measurement of reduced glutathione, SOD1, and superoxide anions with PFC

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A redox-based characterization of human immune cell subsets by polychromatic flow cytometry

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SUMMARY

Cellular redox state determinants are traditionally studied using fluorescent microscopy and immunoblot analysis; however, no procedure has been developed for simultaneous measurement in various immune cell subsets. Here, we present a flow cytometry assay for measuring antioxidant defense systems and reactive oxygen species simultaneously in T, B, and natural killer lymphocytes. We describe steps for preparing and treating peripheral blood mononuclear cells, surface and dye staining, cell fixation/permeabilization, and intracellular staining. We then detail machine standardization, acquisition, and analysis.

BEFORE YOU BEGIN

The designed panel dives deep into the heterogeneity of the most abundant immune cell compartments and measure their redox status. We used mAbs specific for CD4 and CD8, to detect helper and cytotoxic T cells, respectively. To identify the different T cell subsets, we used a combination of mAbs specific for the detection of T naïve (TN, CD45RO- CCR7+), T central memory (TCM, CD45RO + CCR7+), T effector memory (TEM, CD45RO + CCR7-) and T terminal effector (TTEM, CD45RO- CCR7-).[1,2] Following the identification of T cells we focus on CD3- cells to analyze B and NK cells. Naïve B cells are defined as CD21+, CD27- while memory B cells are defined as CD21+ CD27+.[3] The two main NK cell subsets are identified as $CD56^{DIM}$ and $CD56^{BRIGHT}$ cells.[4] To detect superoxide anion molecules within the mitochondria, we take advantage of the highly selective red-fluorogenic dye MitoSOX (MSR). As an internal control, we identify the mitochondria using a green-fluorogenic dye (MitoView Green – MVG) that stains mitochondria in live cells and provides normalization with respect to the mitochondrial mass. We also analyze some representative defense systems, harbored in cells against excessive ROS exposure, like superoxide dismutase 1 (SOD1), the nuclear factor erythroid 2-related factor 2 (Nrf2), a regulator of antioxidant responses and plays a key role in the differentiation and functionality of B cells in particular.[5–9] We use a thiol reactive dye to measure the cellular levels of reduced glutathione (GSH), which plays a role in preventing damage to cellular components by ROS.[10,11] To confirm that the panel can detect redox-related changes, appropriate redox-homeostasis modulators should be used as controls.[12,13] In this assay we used Hydrogen Peroxide (H_2O_2) and Menadione to confirm the detection of

superoxide anions, however, H_2O_2 generators like Tert-butyl hydroperoxide (t-BHP) or O_2- inducers like Phenobartbital (PB) can also be used.

The protocol has been optimized and described using Peripheral Blood Mononuclear cells (PBMCs) from healthy human whole blood. Any human PBMCs either fresh or stored in liquid nitrogen can be used for this protocol. However, in case of different biological sources (peripheral blood, lymph nodes, tissues etc), the panel can be easily adjusted with the addition of an anti-CD45 antibody to specifically select lymphocytes. For the development of the assay, mAbs and dyes have to be titrated for identifying the best concentration that allows us to distinguish the populations of interest.[14] It is highly advised that all antibodies and dyes used are titrated before conducting the assay. Additionally, this protocol has been optimized using 5 mL round bottom polystyrene tubes but one can apply all the following staining steps by using 96-well plates. To do this, simply recalculate the titers of each antibody and dye by using 50 μL staining buffer instead of 100 μL (as done for this experiment) and thus lower buffer volumes. For example, if sample requires 5 μL per 100 μL buffer for FACS tubes, recalculate by dividing by two (therefore the antibody volume will become 2.5 μL in 50 μL of buffer). We recommend setting up the Flow cytometer by completing instrument quality control before beginning. For BD LSR Fortessa X-20, CS&T beads are used. Finally, before beginning, use any Rainbow calibration beads to standardize the acquisitions.[15] Rainbow calibration beads allow the setting of a peak MFI target, and that this target is close to identical every time a new experiment is acquired.[16,17]

Institutional permissions

Sample collection from healthy donors was approved by the ethics committee: REC 17WS0172 at the Experimental Medicine and Rheumatology department, Queen Mary University of London. Healthy volunteers signed informed consent.

Preparation of dye stock solutions and buffers

Timing: 30 min

Refer to materials and equipment for all the recipes that need to be prepared prior to starting.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Nrf2 Antibody A-10, use as , use as 1:40 dilution	Santa Cruz	Cat#sc-365949
APC-H7 Mouse Anti-Human CD3, use as 1:167 dilution	BD Pharmingen	Cat# 560275
BV711 Mouse Anti-Human CD4, use as 1:333 dilution	BD Horizon	Cat#563913
BUV805 Mouse Anti-Human CD8, use as 1:167 dilution	BD Horizon	Cat#612890
PE-CF594 Mouse Anti-Human CCR7 (CD197), use as 1:167 dilution	BD Horizon	Cat#566769
PE-Cy7 Mouse Anti-Human CD45RO, use as 1:40 dilution	BD Pharmingen	Cat#560608
Alexa Fluor 700 Mouse Anti-Human CD27, use as 1:20 dilution	BD Pharmingen	Cat#560611
BV786 Mouse Anti-Human CD19, use as 1:167 dilution	BD Horizon	Cat#563325
BV650 Mouse Anti-Human CD21, use as 1:80 dilution	BD OptiBuild	Cat#740569
BUV395 Mouse Anti-Human CD56, use as 1:167 dilution	BD Horizon	Cat#563554
SOD1/Cu-Zn SOD Antibody, , use as 1:25 dilution	Novus Biologicals	Cat#JF1005
Goat anti-Rabbit IgG (H + L) Secondary Antibody, DyLight 405, , use as 1:1,000 dilution	Thermo Fisher Scientific	Cat#35551
Biological samples		
Cryopreserved human peripheral blood mononuclear cells (PBMCs)	Healthy volunteers	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's phosphate-buffer saline (DPBS) solution without calcium and magnesium	Gibco	Cat#14190-144
RPMI 1640 Medium (RPMI)	Thermo Fisher	Cat#11875093
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher	Cat#15140122
Fetal bovine serum (FBS)	EuroClone	EUS00AY
Distilled cell culture water	Gibco	Cat#A12873-01
Viability Dye - LIVE/DEAD Fixable Blue	Thermo Fisher Scientific	Cat#L34962
ThiolTracker Violet (TTV)	Thermo Fisher Scientific	Cat#T10096
MitoSOX RED	Thermo Fisher Scientific	Cat#M36008
MitoView Green (MVG)	Biotium	Cat#70054
Brilliant stain buffer	BD Horizon	Cat#566349
Stain buffer (BSA)	BD	Cat#554657
Foxp3 / Transcription Factor Fixation/Permeabilization Concentrate and Diluent kit	eBioscience	Cat#00-5521-00
Permeabilization buffer (10×)	eBioscience	Cat#00-8333-56
UltraComp eBeads Plus	Thermo Fisher	Cat#01-3333-42
ArC Reactive Beads	Thermo Fisher	Cat#A10346
DIVA CS&T RUO BEAD 150	BD	Cat#655051
Trypan blue	BioWhittaker	Cat#17-942E
H_2O_2 (3%)	DR MARCUS	02083626000200
Critical commercial assays		
Menadione sodium bisulfite, approx. 95%	Sigma-Aldrich	M5750-25G
MG132	Sigma-Aldrich	M7449
Deposited data		
Raw and analyzed data	This paper	N/A
Software and algorithms		
FACSDiva 8.0.3	BD	659528
FlowJoTM v10 Software	FlowJo was acquired by BD	NA
BioRender: Scientific Image and Illustration Software – (Graphical abstract, Figure 1)	BioRender	NA

MATERIALS AND EQUIPMENT

Complete RPMI 1640 medium

Reagent	Final concentration	Amount
RPMI	N/A	445 mL
FBS	10%	50 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	5 mL
Total		500 mL

Medium is filtered (0.22 μm) and stored at 4°C up to 1 month.

Live/Dead Fixable Blue Stock Solution

Reagent	Final concentration	Amount
LIVE/Dead Fixable Blue	N/A	1 vial
DMSO	N/A	50 μL
Total		50 μL

Vortex well after preparation. Store the reconstituted stock solution for up to 2 weeks at −20°C, protected from light and humidity. Prepare small aliquots to avoid freeze-thaw cycles.

ThiolTracker Stock Solution		
Reagent	Final concentration	Amount
ThiolTracker Violet	10 mM	1 vial
DMSO	N/A	30 μL
Total		30 μL
Vortex well. Prepare small aliquots of stock to avoid multiple freeze-thaw cycles. Store at −20°C up to 1 year.		

MitoSOX RED Stock solution		
Reagent	Final concentration	Amount
MitoSOX RED	5 mM	1 vial
DMSO	N/A	13 μL
Total		13 μL
Vortex well. Prepare small aliquots of stock to avoid multiple freeze-thaw cycles. Store at −20°C up to 6 months		

RPMI with 2% FBS		
Reagent	Final concentration	Amount
RPMI	N/A	97 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	1 mL
FBS	2%	2 mL
Total		100 mL
Prepare fresh each time.		

MitoView Green Stock solution		
Reagent	Final concentration	Amount
MitoView Green 5 M	200 μM	1 vial
DMSO	N/A	400 μL
Total		400 μL
Vortex well. Prepare small aliquots of stock to avoid multiple freeze-thaw cycles. Store at −20°C, stable for a t least 3 years.		

Foxp3 / Transcription Factor Fixation/Permeabilization Concentrate and Diluent kit(1:4) Dilution		
Reagent	Final concentration	Amount
Transcription Factor Fixation/Permeabilization Concentrate	N/A	1 μL
Transcription Factor Fixation/Permeabilization Diluent	N/A	3 μL
Total		4 μL

⚠ CRITICAL: Prepare fresh and in a chemical hood each time. The Transcription Factor Fixation/Permeabilization Concentrate contains formaldehyde and should be used with caution. Acute exposure to formaldehyde is highly irritating to the eyes, nose, and throat while long-term exposure to low levels in the air or on the skin can cause asthma-like respiratory problems, skin irritation and even cancer.

Permeabilization Buffer (1×)		
Reagent	Final concentration	Amount
Permeabilization Buffer (10×)	1×	5 mL
Distilled Cell Culture Water	N/A	45 mL
Total		50 mL
Prepare fresh each time.		

Blocking solution		
Reagent	Final concentration	Amount
DPBS	N/A	9.8 mL
FBS	N/A	200 µL
Total		10 mL
Store at 4°C up to 1 week.		

Flow cytometer (BD LSR Fortessa X-20)		
Reagent	Laser (excitation nm)	Emission filter (nm)
Viability Dye - LIVE/DEAD Fixable Blue	UV (355)	450/50
MitoSOX RED (MSR)	Blue (488)	710/50
MitoView Green (MVG)	Blue (488)	530/30
ThiolTracker Violet (TTV)	Violet (405)	525/50
SOD1/Cu-Zn SOD Antibody / Goat anti-Rabbit IgG (H + L) Secondary Antibody, DyLight 405	Violet (405)	450/50
Brilliant Violet 605 anti-human CD16 Antibody	Violet (405)	610/20
BUV395 Mouse Anti-Human CD56	UV (355)	379/28
BV650 Mouse Anti-Human CD21	Violet (405)	670/30
BV786 Mouse Anti-Human CD19	Violet (405)	780/60
Alexa Fluor 700 Mouse Anti-Human CD27	Red (640)	730/45
PE-Cy7 Mouse Anti-Human CD45RO	Yellow/Green (561)	780/60
PE-CF594 Mouse Anti-Human CCR7 (CD197)	Yellow/Green (561)	610/20
BUV805 Mouse Anti-Human CD8	UV (355)	800/40
BV711 Mouse Anti-Human CD4	Violet (405)	710/50
APC-H7 Mouse Anti-Human CD3	Red (640)	780/60
Anti-Nrf2 Antibody A-10	Red (640)	670/30

Alternatives: BD LSR Fortessa X-20 fully equipped with five lasers -blue (488 nm), Red (635 nm), Violet (405 nm), Ultraviolet (355 nm), Yellow/Green (561 nm) - was used in this assay. Any other flow cytometers equipped with the same lasers and similar emission filters can also be used.

Alternatives: In the key resources table, the mAbs used for the detection of the different markers were validated and titrated for this protocol. However, depending on the configuration of the instrument, the fluorochromes of the mAbs can be exchanged, and, if required, any other markers can be incorporated.

STEP-BY-STEP METHOD DETAILS

Thawing PBMCs

Timing: 45 min

This section describes how PBMCs are thawed before starting the staining procedure. This step must be fast to allow good sample recovery.

1. Thawing PBMCs.
   a. Prepare and warm complete RPMI in a water bath at 37°C.
   b. In a biological safety cabinet add 5 mL of warm complete RPMI in a 15 mL tube.
   c. Take PBMC vials from the Liquid Nitrogen tanks and transfer them to the water bath on dry ice.

⚠ CRITICAL: Do this quickly. If the liquid nitrogen is not close to the water bath, cells can be temporarily kept in dry ice before the thawing.

   d. Slightly open the PBMC vials and swivel them in the water bath until no ice crystals are seen.

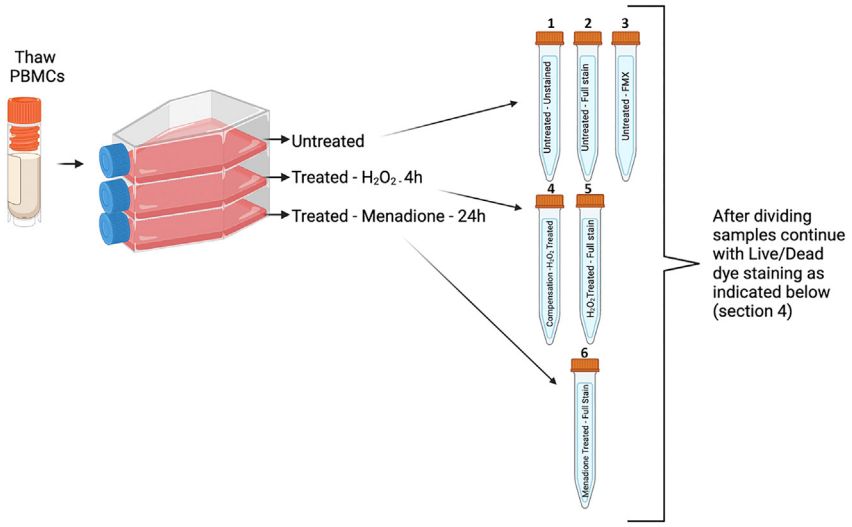


Figure 1. Sample and staining control preparation

After thawing, PBMCs should be treated either with Menadione and/or H_2O_2, while some cells should be kept untreated. Following the treatment, cells should be divided into 15 mL tubes where 1) Untreated cells that will be unstained 2) Untreated but fully stained 3) Untreated but stained only with antibodies and dyes that do not require gating controls 4) H_2O_2 Treated cells that will be used for single stains for compensation of redox dyes 5) H_2O_2 treated cells fully stained 6) menadione treated cells fully stained.

e. Transfer the vials into the biological safety cabinet and add 1 mL of complete warm RPMI to each vial dropwise, resuspending the cell-medium solution very well using a pipette.
f. Transfer the single cell suspension with leukocytes cells in the previously prepared 15 mL tube with the warm medium.

⚠ CRITICAL: Add warm complete RPMI to minimize the risk of osmotic shock dropwise. *However*, don't take too long to complete this process. PBMCs that are stored in liquid nitrogen have cryopreservatives (like 10% DMSO) that can be toxic to the cells once thawed so be relatively fast to avoid cell death.

g. Top up the tube with complete RPMI and wash the cells at 400 g for 10 min at Room Temperature (RT).
h. Discard the supernatant carefully without losing the pellet.
i. Resuspend the cells in appropriate volume and count the number of cells. Consider reaching a concentration suitable for counting (typically 0.5–1 x 10^6 cells/mL). Frozen PBMCs aliquots normally range from 5 × 10^6 to 20 × 10^6 total cells, so this means that a suitable resuspension volume could be from 5 to 20 mL (10 mL as a starting point would be a perfect choice). In case cells are too dense to allow proper counting, dilute them more.

Note: Any cell counter or traditional trypan blue cell counting methods can be conducted.

⚠ CRITICAL: Each washing step may lead to losing cells. In this assay we started with about 5 × 10^6 live cells per donor in order to have 1 × 10^6 cells acquired per donor. This number of cells was more than enough to detect the populations of interest. The starting number of cells can be scaled down to 1 × 10^6 live cells, if necessary.

In vitro H_2O_2 and menadione treatment of human PBMCs to generate redox-positive controls

Timing: 1 h

This section describes how to generate positive controls to confirm that the assay can detect superoxide anion changes. Additionally, resting cells after thawing is also important as thawing can be a stressful process to PBMCs.

2. Treating PBMCs
   a. Once counted, resuspend the cells in RPMI 2% FCS at a concentration of 1 × 10^6 cells/mL and split the suspension into 3 flasks, tubes, or plates.
   b. Label two flasks as the oxidative controls.
   c. In one control allow the H_2O_2 reagent to reach a 50 μM final concentration and the Menadione control to also reach a 50 μM final concentration.
   d. Keep the other flask untreated and place both flasks in an incubator for 1 h at 37°C.

   Note: To find the optimal H_2O_2 and Menadione concentration and time of treatment for your experiment and type of cells, conduct appropriate test experiments. Here we tested a variety of concentrations and times and found that a 1 h treatment and a 50 μM concentration of H_2O_2 was enough to demonstrate a redox related shift after staining with redox related markers. We start from a H_2O_2 stock concentration of 0.9 M (3 wt%), thus we take 1 μL of this stock solution, dilute it 1:9000 in RPMI 2% FCS to generate an intermediate dilution of 100 μM and accordingly dilute 1:2 with the cell suspension to reach a final concentration of 50 μM. For Menadione, we start from a stock concentration of 100 mM and accordingly dilute 1:500 with the cell suspension to reach a final concentration of 50 μM.

Sample and staining control preparation

Timing: 10 min

This section of the protocol lists the samples and the controls necessary for the assay. Together with the full stained samples, additional staining controls have to be prepared. A Fluorescence Minus One (FMO) or a Fluorescence Minus X (FMX) control is a tube of cells labeled with all but one of more fluorophores respectively. In multi-color immunofluorescent investigations, FMO or FMX controls are employed to define the cut-off point between background fluorescence and positive populations during analysis. In this assay, we used an FMX control labeled for all the marker with the exception of MVG, MSG, Nrf2, TTV, SOD1.

3. Preparing Controls.
   a. Collect treated and untreated cells in 15 mL tubes.
   b. From the untreated sample take about 0.5 × 10^6 cells, place them into a separate 15 mL tube and label it as a FMX tube. The other cells (about 2 × 10^6 cells in our case) will be used for the full staining, to detect the redox status in the population of interest.
   c. From the treated sample, keep aside about 0.5 × 10^6 cells to use for compensation controls (see step 12).

   Note: The other treated cells (about 2 × 10^6 cells in our case) will be used for the full staining in treated sample, as positive controls of the redox markers in the population of interest. Please refer to the illustration in Figure 1 to clarify this step.

Live/dead staining

Timing: 40 min

This section of the protocol describes the staining procedure for excluding dead cells from the analysis.

Table 1. CCR7 mix

Marker and Clone	Fluorophore	Volume (for 100 μL cell suspension)	Dilution
CCR7 (2-L1-A)	PE-CF594	0.6 μL	1:167
Brilliant Stain Buffer	N/A	99.4 μL	NA
Total		100 μL	NA

4. Live/dead staining.
   a. Top up all the samples with complete RPMI to wash the cells.
   b. Spin at 400 g for 10 min at RT.
   c. Prepare Viability mix: 1:1000 dilution of LIVE/Dead Fixable Blue (1 μL LIVE/Dead Fixable Blue stock solution and 999 μL of DPBS).
   d. Discard supernatant carefully not disrupting the pellet.

   Note: Use a pipette to remove ALL the residual supernatant

   e. Stain pelleted cells with 100 μL of viability mix (diluted Live/Dead Fixable Staining Solution) directly in the 15 mL tubes.
   f. Vortex cell pellet with viability mix and stain for 15 min at RT protected from light.
   g. Wash cells with 2 mL of washing/staining buffer (like BSA) and centrifuge at 400 g for 5 min.

⚠ CRITICAL: Live/Dead staining is essential as to most flow cytometry-based investigations. Use it to exclude debris and dead cells from analysis especially after optimal H_2O_2 treatment. Viability dyes are sensitive to light and can lose their fluorescence, so make sure to keep the dyes very well protected from light.

Cell staining assay

Timing: 4 h

This section of the protocol describes the staining procedure of immune cells subsets and redox markers using Dyes and Monoclonal antibodies.

5. CCR7 staining.
   a. Discard supernatant and transfer the cells into FACS tubes.
   b. Prepare CCR7 mix as indicated in Table 1, by using the brilliant stain buffer.

   Note: The brilliant stain buffer solution is added to mixtures of specific fluorescent reagents. It was made to go along with polychromatic flow cytometry tests that make use of staining agents coupled to BD Horizon Brilliant fluorescent polymer dyes.

   Note: The preparation provided here is for 1 tube. The volume has to be multiplied by the number of tubes: for one sample you would have to make the surface mix times 3 (fully stained untreated sample, FMX untreated sample, fully stained treated sample). In case of a higher amount of samples, it is suggested to prepare a master mix with at least 10% extra volume (e.g., if 10 tubes are required, calculate on the basis of 11), so that even the last tube is filled properly avoiding the effect of sample loss when dosing.

   The mAbs have to be titrated for identifying the best concentration that allows to distinguish the populations of interest.
   c. Stain both treated, untreated and FMX control cells with 100 μL cell suspension of CCR7 mixture for 20 min at 37°C, protected from light.

Table 2. Surface mix

Marker and Clone	Fluorophore	Volume (for 100 µL cell suspension per sample)	Dilution
CD3 (SK7)	APC-H7	0.6 µL	1:167
CD4 (L200)	BV711	0.3 µL	1:333
CD8 (SK1)	BUV805	0.6 µL	1:167
CD45RO (UCHL1)	PE-Cy™7	2.5 µL	1:40
CD19 (SJ25C1)	BV786	0.6 µL	1:167
CD56 (NCAM16.2)	BUV395	0.6 µL	1:167
CD27 (M-T271)	Alexa Fluor 700	5 µL	1:20
CD21 (B-ly4)	BV650	1.25 µL	1:80
Brilliant Stain Buffer	N/A	88.55 µL	NA
Total		**100 µL**	**NA**

The preparations provided here is for 1 tube. The volume has to be multiplied by the number of tubes: for one sample you would have to make the surface mix times 3 (fully stained untreated sample, FMX untreated sample, fully stained treated sample). In case of a higher number of samples, it is suggested to prepare a master mix with at least 10% extra volume (e.g., if 10 tubes are required, calculate on the basis of 11), so that even the last tube is filled properly avoiding the effect of sample loss when dosing.
The mAbs have to be titrated for identifying the best concentration that allows to distinguish the populations of interest.

⚠ CRITICAL: CCR7 is a molecule better detected at 37°C due to plasma membrane recovery, hence why it is vital to stain CCR7 separately at 37°C.[18]

Note: An incubator can be used to keep cells at 37°C, during the CCR7 staining.

6. Surface staining.
   a. After staining cells with CCR7, wash the cells with 2 mL of PBS and centrifuge at 400 g for 5 min at room temperature.
   b. Discard supernatant carefully without disrupting the pellet and prepare a mix for all the other surface Abs using Table 2.
   c. Stain each dry pellet with 100 µL of surface marker staining mixture to create a 100 µL cell suspension per sample.
   d. Stain surface markers for 20 min at RT protected from light.
7. Redox markers staining.
   a. Prepare the ThiolTracker & MitoSOX RED Dye Mix as indicated in Table 3.
   b. Once surface markers are stained wash with 1 mL of PBS and centrifuge at 400 g for 5 min at room temperature.
   c. Discard supernatant being careful not to disrupt the pellet.
   d. Stain dry pellets, except the FMX tube, with 100 µL of ThiolTracker & MitoSOX RED Dye Mix immediately. Resuspend the FMX dry pellet in 100 µL RPMI with 2% FBS.
   e. Incubate cells for 15 min at 37°C protected from light.
   f. Wash with 1 mL of PBS and centrifuge at 400 g for 5 min at room temperature.
8. Fixation.
   a. Discard supernatant being careful not to disrupt the pellet.
   b. Add 1 mL of diluted (1:4) Foxp3 / Transcription Factor Fixation/Permeabilization Concentrate (refer to materials and equipment for further instructions on how to prepare the fixative).
   c. Fix for 30 min at 4°C protected from light.

Note: Remember to add the fixative in a chemical hood due to the presence of formaldehyde.

9. Intracellular staining.
   a. Prepare MVG staining concentration as indicated in Table 4.
   b. After 30 min of fixation, wash cells with 2 mL of 1× Perm Buffer (refer to materials and equipment).

Table 3. ThiolTracker & MitoSOX RED Dye Mix

Reagent - stock solution concentration	Final concentration	Volume (for 1 mL mix)	Dilution
ThiolTracker Violet (TTV) - 10 mM	10 μM	1 μL	1:1000
MitoSOX RED (MSR) - 5 mM	5 μM	1 μL	1:1000
RPMI with 2% FBS	N/A	998 μL	NA
Total		**1 mL**	**NA**

The required volume of the mix is 100 μL per tube. For one sample you would have to make the redox markers staining times 2 (fully stained untreated sample, fully stained treated sample). Here we prepared 1 mL of Mix, enough for 9 tubes with 10% of extra volume.
Vortex well and prepare fresh before using.

c. Spin at 500 g for 5 min at room temperature.
d. Wash cells again with 2 mL of 1× Perm Buffer.
e. Spin at 500 g for 5 min at room temperature.
f. Decant supernatant and stain all tubes except the FMX one with 100 μL of MVG per sample. Resuspend the FMX dry pellet in 100 μL 1× Perm Buffer.
g. Stain cells for 15 min at RT protected from light.
h. Prepare Nrf2 intracellular mix as indicated in Table 5.
i. After MVG incubation, wash cells with 2 mL of 1× Perm Buffer and spin at 500 g for 5 min at room temperature.
j. Repeat washing step.
k. Decant supernatant and resuspend all samples except the FMX tube with 100 μL of intracellular Nrf2 mix per sample.
l. Resuspend the FMX dry pellet in 100 μL 1× Perm Buffer.
m. Stain for 30 min at RT protected from light.
n. Wash cells with 2 mL of 1× Perm Buffer and spin at 500 g for 5 min at room temperature.
o. Repeat washing step.
p. Use 1 mL blocking solution to perform a blocking step.

Alternatives: use PBS with 2% FBS instead of BSA

q. Perform blocking for 30 min at RT protected from light.

Note: The blocking step here is to avoid any unspecific binding of the SOD1 staining that follows.

r. Prepare SOD1 primary intracellular mix as indicated in Table 6.
s. Wash cells with 2 mL of 1× Perm Buffer and spin at 500 g for 5 min.
t. Stain all tubes, except the FMX one, with 100 μL primary antibody mix.
u. Resuspend the FMX dry pellet in 100 μL 1× Perm Buffer.
v. Incubate al samples 30 min at RT.
w. Prepare the secondary Antibody Mixture Targeting SOD1 as indicated in Table 7.
x. Wash cells with 2 mL of 1× Perm Buffer and spin at 500 g for 5 min at room temperature.

Table 4. MitoView Green Mixture

Reagent - stock solution concentration	Final concentration	Volume	Dilution
MitoView Green - 200 μM	50 nM	1 μL	1:4000
1× Perm Buffer	N/A	3999 μL	NA
Total		**4 mL**	**NA**

The required volume of the mix is 100 μL per tube. For one sample you would have to make the redox markers staining times 2 (fully stained untreated sample, fully stained treated sample). Here we prepared 4 mL of Mix because of the high dilution factor of the stock solution.

Table 5. Nrf2 Intracellular staining mix

Marker and Clone	Fluorophore	Final dilution	Volume (for 100 μL cell suspension)
Nrf2 (A-10)	APC	1:40	2.5 μL
1× Perm Buffer	N/A	N/A	97.5 μL
Total			100 μL

The required volume of the mix is 100 μL per tube. The final volume has to be multiplied by the number of tubes. For one sample you would have to make the redox markers staining times 2 (untreated sample full staining, treated sample full staining). In case of a higher amount of samples, it is suggested to prepare a master mix with at least 10% extra volume (e.g., if 10 tubes are required, calculate on the basis of 11), so that even the last tube is filled properly avoiding the effect of sample loss when dosing.

y. Decant supernatant and repeat washing step.
z. Stain all samples with 100 μL Goat anti-Rabbit IgG (H + L) Secondary Antibody each sample for 30 min at RT.

Note: The FMX control sample has to be stained with secondary Antibody Mixture Targeting SOD1 to prove the specificity of the primary staining.

aa. Wash cells with 2 mL of 1× Perm Buffer and spin at 500 g for 5 min at room temperature.
bb. Decant supernatant and repeat washing step.
cc. Re-suspend cells with 200 μL of BD Stabilizing Fixative.
dd. Samples are ready for acquisition.

Note: Store at 4°C until ready to acquire. Acquire within 4 h after staining completion.

Preparation of compensation controls

Timing: 40 min

Compensation controls for flow cytometry are very important. Compensation is a mathematical calculation that removes unwanted fluorescence signal that is coming into a primary channel and overlapping in a secondary channel. Here we use both compensation beads and single stained cells to calculate compensation.

Note: the compensation controls can be prepared the same day of the staining, in any moment prior of the acquisition (before, during or after the samples staining).

10. Compensation controls for fluorophore conjugated antibodies.
    a. Prepare 1 FACS tube for each fluorophore conjugated antibody and prepare also an unstained tube.
    b. Vortex well UltraComp eBeads (CatalogNo. 01-3333-42) for 2 min and allow them to reach RT.

Table 6. Primary SOD1 Antibody Mixture

Marker and Clone	Fluorophore	Final dilution	Volume (for 100 μL cell suspension)
SOD1/Cu-Zn SOD Antibody	N/A	1:25	4 μL
1× Perm Buffer	N/A	N/A	96 μL
Total			100 μL

The required volume of the mix is 100 μL per tube. The final volume has to be multiplied by the number of tubes. For one sample you would have to make the redox markers staining times 2 (untreated sample full staining, treated sample full staining). In case of a higher amount of samples, it is suggested to prepare a master mix with at least 10% extra volume (e.g., if 10 tubes are required, calculate on the basis of 11), so that even the last tube is filled properly avoiding the effect of sample loss when dosing.

Table 7. Secondary Antibody Mixture Targeting SOD1

Marker and Clone	Fluorophore	Final dilution	Volume (for 100 μL cell suspension)
Goat anti-Rabbit IgG (H + L) Secondary Antibody,	DyLight 405	1:1000	1 μL
1× Perm Buffer	N/A	N/A	999 μL
Total			1 mL

The required volume of the mix is 100 μL per tube. For one sample you would have to make the redox markers staining times 2 (untreated sample full staining, treated sample full staining). Here we prepared 1 mL of Mix, enough for 9 tubes with 10% of extra volume.

c. Add one drop of UltraComp eBeads in each tube for each fluorophore conjugated antibody. Add one drop of UltraComp eBeads also in the unstained tube.
d. Use at least 1 μL of fluorophore conjugated antibody to stain the beads. If the volume of fluorophore conjugated antibody used per 100 μL cell suspension is higher than 1 μL use the same amount used per cell suspension. Refer to Tables 1, 2, and 7 for verifying the amount of antibody.
e. Vortex the FACS tubes and incubate at RT for 15 min protected from light.
f. Wash beads in 2 mL of BSA and spin at 500 g for 5 min at room temperature.
g. Resuspend in 300 μL of BSA.
h. Store at 4°C–8°C until samples are ready to be acquired.

11. Compensation controls for Viability Dye - LIVE/DEAD Fixable Blue.
a. Prepare and label one FACS tube for the Viability Dye and one FACS tube for the respective unstained compensation control.
b. Gently vortex ArC Amine Reactive Compensation Bead Kit components for 30 s to completely resuspend before use.
c. Add 1 drop of ArC Amine Reactive (+) to the FACS tube for the Viability Dye.
d. Add 1 drop of ArC Amine Reactive (-) to the FACS tube for the unstained compensation control.
e. Add 1 μL of undiluted dye to the FACS tube for the Viability Dye.
f. Vortex the FACS tubes and incubate at RT for 15 min protected from light.
g. Wash beads in 2 mL of BSA and spin at 500 g for 5 min at room temperature.
h. Resuspend in 300 μL of BSA.
i. Store at 4°C–8°C protected from light until samples are ready to be acquired.

12. Compensation controls for TTV, MVG, and MSR.
a. Divide the 0.5 × 10^6 cells into three separate FACS tubes. Designate one tube for each dye (TTV, MVG, and MSR).
b. Wash cells in each tube by adding 1 mL and spin at 500 g at room temperature.
c. First stain cells for TTV for a final concentration of 10 μM and the tube for MSR with 5 μM (dilute in RPMI with 2% FBS (as done for Table 3 for the full stain).
d. Incubate cells for 15 min at 37°C protected from light.
e. Wash with 1 mL of PBS and centrifuge at 500 g for 5 min at room temperature.
f. Discard supernatant being careful not to disrupt the pellet Add 1 mL of diluted (1:4) Foxp3 / Transcription Factor Fixation/Permeabilization Concentrate (refer to materials and equipment for further instructions on how to prepare the fixative).
g. Fix for 30 min at 4°C protected from light – do this also for the cells in the tube for MVG.
h. Wash cells with 2 mL of 1× Perm Buffer and spin at 500 g for 5 min at room temperature.
i. Decant supernatant and repeat washing step.
j. Resuspend the TTV and MSR tubes with 200 μL of BD Stabilizing Fixative. These compensation controls are ready for acquisition.
k. Stain the cells designated for MVG with a 50 nM concentration as prepared for the full stain in Table 4 (use perm buffer as a dilutant).
l. Stain cells for 15 min at RT protected from light.
m. Wash cells with 2 mL of 1× Perm Buffer and spin at 500 g for 5 min at room temperature.

n. Decant supernatant and repeat washing step.
o. Resuspend MVG tube with 200 µL of BD Stabilizing Fixative. All compensation controls are now ready for acquisition.

Note: Remember to add and prepare the fixative in a chemical hood due to the presence of formaldehyde.

⚠ CRITICAL: It is important to check the quality of the compensation controls: make sure the positive peaks are well resolved and have higher signal compared to that one observed in the fully stained samples.

EXPECTED OUTCOMES

This protocol allows to clearly identify the main populations of lymphocytes: CD4+ CD3+ T helper, CD8+ CD3+ cytotoxic T cells, CD19+ B cells, CD56+ NK cells. By gating on the total CD3+ cells, both CD4+ T helper and CD8+ cytotoxic T cells we can look deep in the T cell differentiation subsets: T_n (CD45RO- CCR7+), T_{cm} (CD45RO + CCR7+), T_{em} (CD45RO + CCR7-), T_{tem} (CD45RO- CCR7-). Likewise, within the CD19+ B cells we can identify Naïve B cells (CD21+ CD27-) and memory B cells (CD21+ CD27+). Other than CD19+ cells, amongst the CD3- cells we can also dive into defining NK cells (CD56+) (Figure 2A). All redox related marker of interest can be clearly identified amongst the total lymphocytes. The FMX controls allow the identification of positive signals from the background (Figure 2B). The increase in the MFI with H_2O_2 and menadione controls is specific for MSR, but not for MVG, resulting in an increase in the MSR/MVG ratio and demonstrating the specificity of the staining in the detection of oxidative events (Figures 3A and 3B). Thiol Tracker Violet measures instead reduced glutathione, so you would expect to see a concentration-dependent decrease in its fluorescence peak intensity when an oxidizing agent like H_2O_2 or menadione is added (Figure 3C).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are expressed as median fluorescence intensity (MFI), because "median" is considered a much more robust statistic than "arithmetic mean". In the case of not-normal distributed data (like flow data often are), median is less influenced by skew or outliers and works well on a log scale. The Median Fluorescence Intensity (MFI) of TTV, Nrf2 and SOD1 is thus used to study the redox status of the population of interest. For the analysis of MSR, it is important to normalize the MFI of MSR with the MFI of MVG. MVG is strategically included as an internal control as it detects mitochondrial mass overall, independently of the status of the organelles themselves or of the cells, and the dye localizes mitochondria regardless of mitochondrial membrane potential. Therefore, MVG can be used to normalize the MSR median fluorescence intensity that instead detects mainly mitochondrial ROS, specifically anion superoxide. Therefore, by calculating the ratio between MSR and MVG MFI, the mitochondrial ROS would be normalized for the mitochondrial mass, meaning that the residual relative differences among samples (comparing the ratios) would be only ROS-dependent (and not due to a random shift of the mitochondrial mass or to a differential dye- uptake/metabolism by the cells) (Figure 3A). Two-tailed Student's t-test was used for comparing normalized MSR values of H_2O_2 and Menadione treated against untreated samples. We could detect significant difference between the two groups where p-values of <0.05 are considered statistically significant (Figure 3B).

LIMITATIONS

While this approach allows for the standardized measurement and simultaneous comparison of redox-related markers, it does not allow an absolute quantification of cellular levels of ROS molecules or redox-related markers, as this would imply usage of standard reference samples and controlled experimental conditions (e.g., nitrogen/oxygen monitoring and continuous pH evaluation).

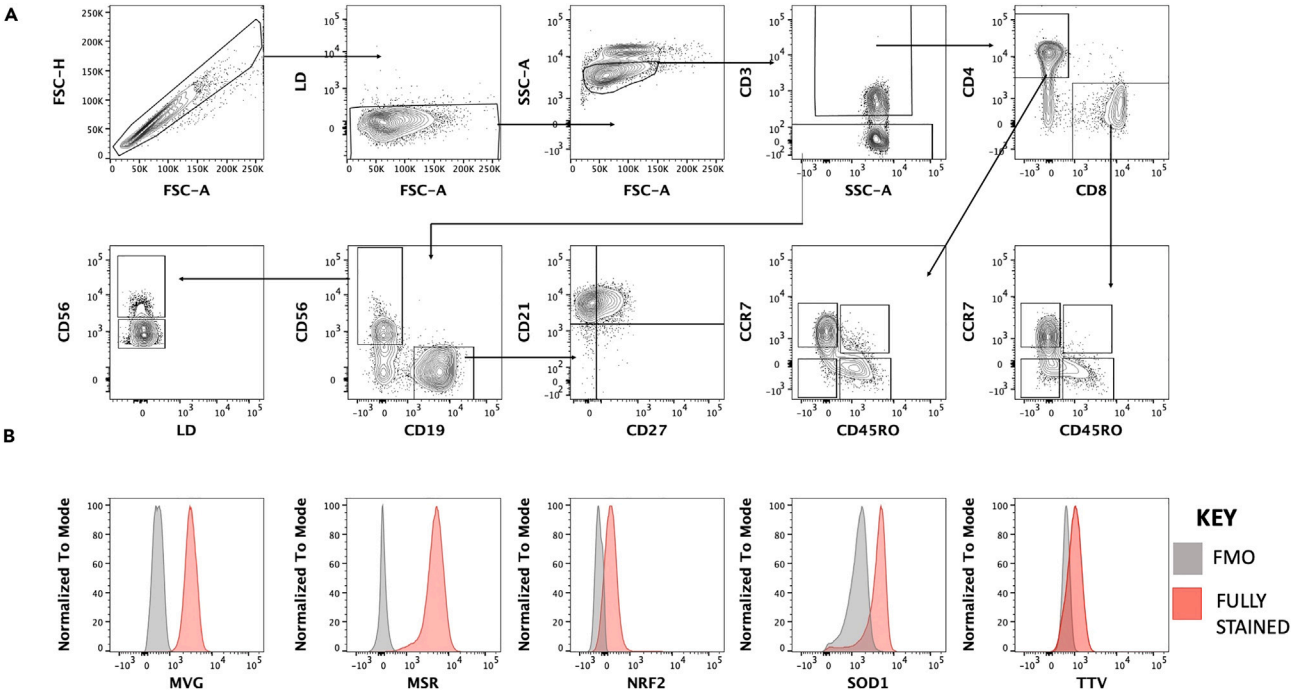


Figure 2. Gating Strategy for flow cytometry analysis of redox makers on human immune cell subsets

(A) Gating strategy to detect T, B and NK cell subsets. Singlets are defined by gating FSC-A against FSC-H. Live cells are identified by gating on viability dye (LD) negative cells . Morphological parameters FSC-A and SSC-A then define the Lymphocyte gate. T cells are identified as CD3+ cells. On the CD3+ cells, CD4 and CD8 markers allow to identify the major T cell populations (CD4+ helper T cells and CD8+ Cytotoxic T cells). In both CD4+ and CD8+ compartment, T naïve-like (NL, CCR7+CD45RO-), T central memory (CM, CCR7+CD45RO+), T effector Memory (EM, CCR7- CD45RO+), T terminal effector memory (TE, CCR7-CD45RO-) are defined. On CD3- cells, B cell are defined as CD19+. On CD19+ cells, B Naïve are defined as CD21+,CD27- (Q1), and B cell Memory are defined as CD21+,CD27+ (Q2). On CD3- cells, total NK cells are defined as CD56+ cells. From the CD56+ population we can move further into defining CD56BRIGHTand CD56DIM.

(B) Redox markers represented by histograms. Compensated redox related markers on the total gate of lymphocytes with the red peaks defining the fully stained (MVG - MitoView Green, MSR - MitoSOX Red, Nrf2 - nuclear factor erythroid 2–related factor 2, SOD1 - superoxide dismutase 1, TTV -ThiolTracker Violet) and the gray peak defines their FMO controls.

TROUBLESHOOTING

Problem 1

Issues with compensation.

Potential solution

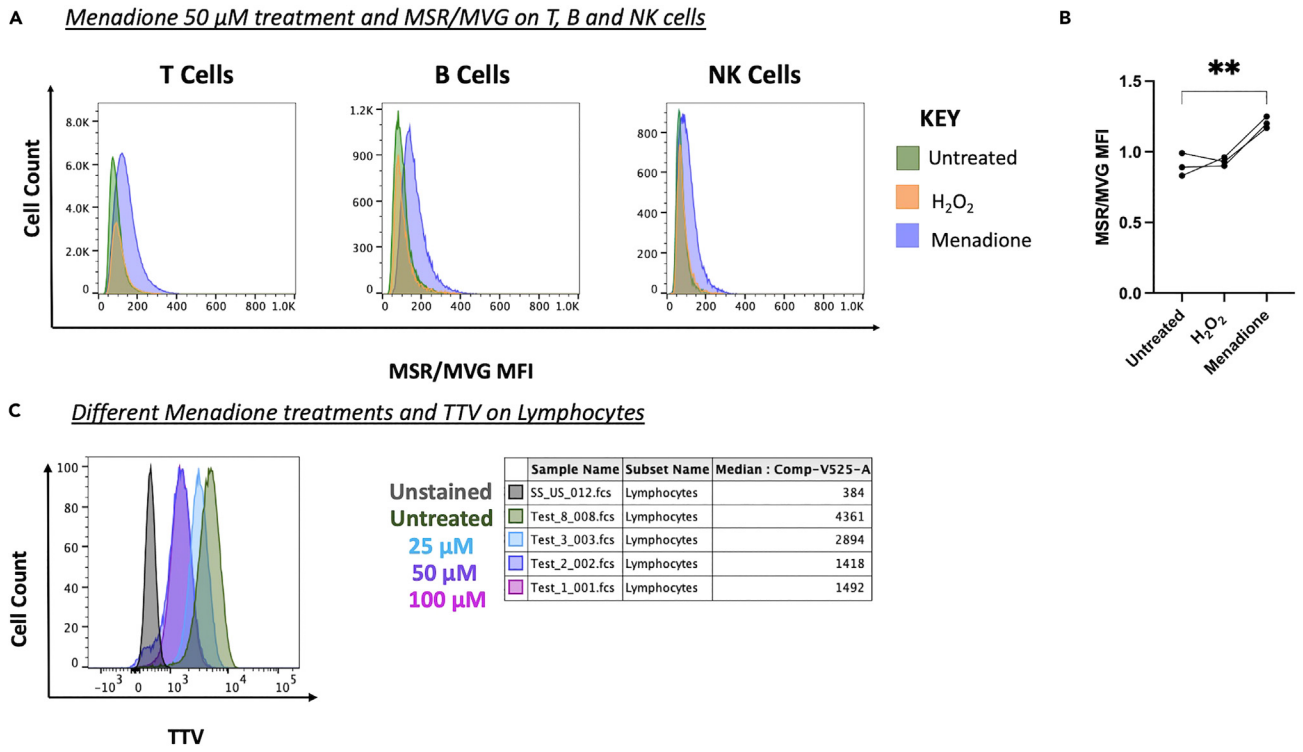
If you notice that there are issues with compensation controls, first perform additional washing step and check instrument performance. If the issue persists, re-prepare the compensation tubes and re-run them using the same machine settings used to acquire the samples. For cell-based compensation tubes, it is suggested to use a different donor (biological source). Moreover, cell based comps can be used also for fluorophore-conjugated antibodies.

Problem 2

Clumps in sample.

Potential solution

PBMCs are sensitive to thawing, thus it is possible that some samples are not of the best quality. If clumps are found in the sample while resuspending, pass the cell suspension through filtered FACS tubes before acquisition (For example: Corning Falcon Round-Bottom Polystyrene Tubes with Cell Strainer Snap Cap, 5 mL - Catalog No.08-771-23). It is important that no clumps are passed through



	Sample Name	Subset Name	Median : Comp-V525-A
	SS_US_012.fcs	Lymphocytes	384
	Test_8_008.fcs	Lymphocytes	4361
	Test_3_003.fcs	Lymphocytes	2894
	Test_2_002.fcs	Lymphocytes	1418
	Test_1_001.fcs	Lymphocytes	1492

Figure 3. Positive controls

(A) Histograms showing the ratios amongst MSR and MVG in T, B and NK cells. Results from PBMCs treated with H2O2 (orange) and Menadione (purple) and untreated (green) are presented.

(B) Ratio of MSR and MVG MFI values of three different healthy donors in each condition on total lymphocytes. Menadione demonstrates a significant increase in the ratio of MSR/MVG when compared to the untreated (P = 0.0044).

(C) Histograms showing TTV levels in total Lymphocytes. Results from PBMCs untreated or treated with different concentrations of menadione are presented (20 µM – light blue, 50 µM – purple, 100 µM – fuchsia).

the flow cytometer as this may block it. One can also consider adding EDTA to staining/blocking buffers to help avoid cell clumps.

Problem 3

No shift with H_2O_2 control.

Potential solution

It is possible that you may see no H_2O_2 shift or one that is no expected for each sample. This can be expected as the chemical reaction is fragile and may not yield the expected result. To combat this, it is important to test for the appropriate H_2O_2 concentration needed for the cell type of interest and use better oxidative controls like Menadione (Figure 3). You should also check the effectiveness of your H_2O_2, as redox reagents tend to be air sensitive and repeated opening/closing cycles should be minimized. In case of doubts, replace all the reagents with fresh ones.

Problem 4

Difficulties in visualizing Nrf2 as it can be hardly visible in a steady-state.

Potential solution

It may be difficult to detect Nrf2 in its steady-state form. Even though an FMO can help define the positive and the negative gates for Nrf2 detection, it may be beneficial to treat cells with an inhibitor of proteasomal degradation like MG132. In Figure 4, we show the effects of two different

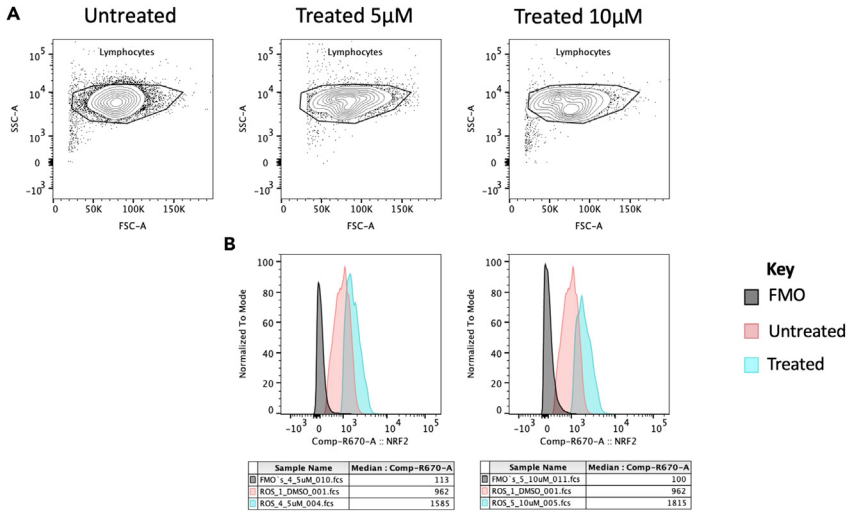


Figure 4. Visualizing Nrf2 by proteasomal degradation inhibition

PBMCs were treated for 24 h with 5 µM and 10 µM of inhibitor MG132 of proteasomal degradation.

(A) Morphology of lymphocytes untreated and treated with MG132.

(B) Histogram overlays of MFI of Nrf2 on total lymphocytes in treated and untreated samples. The gray peak represents total lymphocytes treated with MG132 but not stained for Nrf2_APC while the blue peak represent PBMCs that have been treated wither with 5 µM (left histogram) or 10 µM (right histogram). As controls, we also include untreated (DMSO) cells in red. MFI values for each peak are represented in the table below each histogram plot.

concentrations (5 µM and 10 µM) of MG132 by displaying the MFI of Nrf2. Indeed treatment with MG132 does increment Nrf2 detection levels after 24 h (Figure 4B). Specifically, the 10 µM concentration of MG132 can almost increase the expression of Nrf2 by two fold when compared to the untreated (DMSO equivalent).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alessandra Roberto (aroberto@flowmetric.com)

Materials availability

This study did not generate new unique materials or reagents.

Data and code availability

This study does not have data or codes available.

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AUTHOR CONTRIBUTIONS

C.P. has developed and collected the data for this report with the input of M.B. and A.R. M.B. initiated the assay and designed the protocol outline. C.P. wrote the manuscript with support from M.B. and A.R. I.M. helped with the instrument setting and maintenance. C.P. and M.B. performed revisions. E.P. provided part of the human PBMC samples. All authors discussed the results and contributed to the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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