



Evaluation of Mitochondria Content and Function in Live Cells by Multicolor Flow Cytometric Analysis

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Abstract

To evaluate how a cell responds to the external stimuli, treatment, or alteration of the microenvironment, the quantity and quality of mitochondria are commonly used as readouts. However, it is challenging to apply mitochondrial analysis to the samples that are composed of mixed cell populations originating from tissues or when multiple cell populations are of interest, using methods such as Western blot, electron microscopy, or extracellular flux analysis.

Flow cytometry is a technique allowing the detection of individual cell status and its identity simultaneously when used in combination with surface markers. Here we describe how to combine mitochondria-specific dyes or the dyes targeting the superoxide produced by mitochondria with surface marker staining to measure the mitochondrial content and activity in live cells by flow cytometry. This method can be applied to all types of cells in suspension and is particularly useful for analysis of samples composed of heterogeneous cell populations.

Key words Flow cytometry, FACS, Mitochondria, Quantification, Reactive oxygen species

1 Introduction

Mitochondria is a double-membrane-bound organelle that plays a critical role in the generation of energy in most eukaryotic organisms [1]. Recently a surging number of studies aim to uncover the additional involvement of mitochondria in cellular functions, e.g., differentiation or effector actions. Assays such as Western blot, PCR-based measurement of mitochondrial DNA (mtDNA) copy numbers, electron and immunofluorescent microscopy, or extracellular flux analysis [2–4] are commonly applied to quantify the mitochondria amount or function. Western blot is the standard technique to evaluate the expression and modification of the target protein in the purified mitochondria. With the isolation of both mitochondrial DNA (mtDNA) and nuclear DNA, the quantitative PCR-based assay has been established to measure mtDNA copy

number, which is a critical component of overall mitochondrial health. Electron and immunofluorescent microscopy are powerful tools to visualize the mitochondria morphology and spatial distribution in the cells. The newly developed extracellular flux analysis is specialized to measure the oxygen consumption rate and quantify mitochondrial respiration in living cells [5]. Take the study on mitochondrial protein, dynamin-related protein 1 (Drp1), as an example, by combining the Western blot and electron microscopy techniques, it is observed that the nutrient deprivation induces the dephosphorylation of Drp1 Serine 616 and 637 which subsequently leads to mitochondrial tabulation and elongation [6]. Numerous recent studies use extracellular flux analysis to evaluate the mitochondria activity via measuring the oxygen consumption rate—the rate of reserve capacity/spare respiratory capacity (SRC) is an indicator of mitochondria status which meaning the higher SRC it detects, the more ATP is supplied by mitochondria oxidative phosphorylation [7].

Although these abovementioned methods have all been instrumental in understanding the mitochondria biology, they are limited to analyze the homogenous cell population only. Compared to these classical methods, flow cytometry is a sensitive and time-efficient technique. In combination with surface marker staining, this method allows the detection of individual cell status from the heterogeneous cell population. Multiple mitochondria-specific and mitochondrial superoxide-detection fluorescent probes have been developed, enabling these probes to use in conjunction with surface markers. The application of these mitochondria-specific probes in flow cytometry overcomes the cell homogeneity requirement in classical methods and shortens the processing time, and, most importantly, provides broader applicability.

The measurement of mitochondria mass or membrane potential has been essential to determine the status of intracellular ion homeostasis, energy metabolism, or as an indicator for cell stress/survival in eukaryotic cells [8, 9]. MitoTracker™ Green FM has been used as a measurement of mitochondrial mass—non-fluorescent in aqueous solutions; it accumulates in the lipid environment of mitochondria and becomes fluorescent regardless of mitochondrial membrane potential [10]. It can be visualized under a microscope or quantified by flow cytometric analysis in the intact cells without the isolation of mitochondria. Based on its unique chemical properties, one can assume that the dye's fluorescent intensity correlates with the mitochondria mass in the cells.

Because mitochondria inner membrane is negatively charged, mitochondria-specific dyes are often cationic lipophilic dyes, e.g., JC-1 dye [11], rhodamine 123 [12], and tetramethylrhodamine [13], as well as thiol-reactive chloromethyl groups, including MitoTracker™ Red and MitoTracker™ Orange [14]. They are positively charged compounds that can be transported passively across

the mitochondria membranes and accumulated within the mitochondria. When conjugated with different fluorescent chemicals, these membrane potential-dependent dyes are useful indicators of the mitochondria activity. Mitochondria activity can also be reflected by oxidative stress, one of the most important indexes of the cell metabolic status. By measuring the level of reactive oxygen species (ROS) produced [15], one can evaluate the active effector response and the metabolic change of the cell [16]. Cellular ROS level has multiple implications: high oxidative stress in the cell could lead to the activation of oxidative stress responding pathway or cell death [17], while it is also a sign of active inflammatory response in the macrophages [15, 18]. However, ROS are extremely versatile, making it a difficult parameter to measure. Although chromatography, mass spectrometry, or electrochemical sensors are capable of sensitive and accurate ROS detection, these methods require specialized equipment and the support of a well-established core facility. Several assays [19] have recently been developed to detect the intracellular cell ROS, including the fluorescence-dependent and chemiluminescence-derived methods. These techniques rely on the cell-permeable chemicals that react directly to the ROS and generating radical intermediates, which give rise to fluorescence, e.g., dihydroethidium (DHE) stain has been used to detect mitochondrial superoxide. Combining with flow cytometry, one now can apply cell-permeable fluorescent probes like CellROX™ for intracellular reactive oxygen species or MitoSOX™ for mitochondrial superoxide [20] to perform analysis of ROS on complex cell populations.

The current protocol is a demonstration of how to combine surface marker staining and mitochondria- or ROS-specific dyes to measure the mitochondrial activities of the cells of interest within the heterogeneous cell population. It is worth noting that different staining procedures often are necessary to optimize the efficiency of different organelle-specific dye detection. We applied this technique to examine the mitochondria mass, membrane potential, total ROS, and mitochondrial superoxide in tissue macrophages (Mφs). Depending on its biological functions, each organ forms its unique microenvironment. Even cells from the same lineage may behave distinctively when residing in different microenvironments or under stress. Immune cells, for example, maintain a versatile metabolic program adapting to their habitats and to satisfy the biosynthetic needs upon encountering antigens. By establishing an assay that spontaneously detects the cell identity and mitochondrial activities, it allows us to compare the immune cell status of different organs and, more importantly, the function and physiological role of the immune cells in homeostatic or disease settings [21]. We found that compared to splenic Mφs (Fig. 1), the peritoneal cavity Mφs harbored more mitochondria (Fig. 2a) and had higher mitochondrial activities (Fig. 2b). Moreover, the elevated

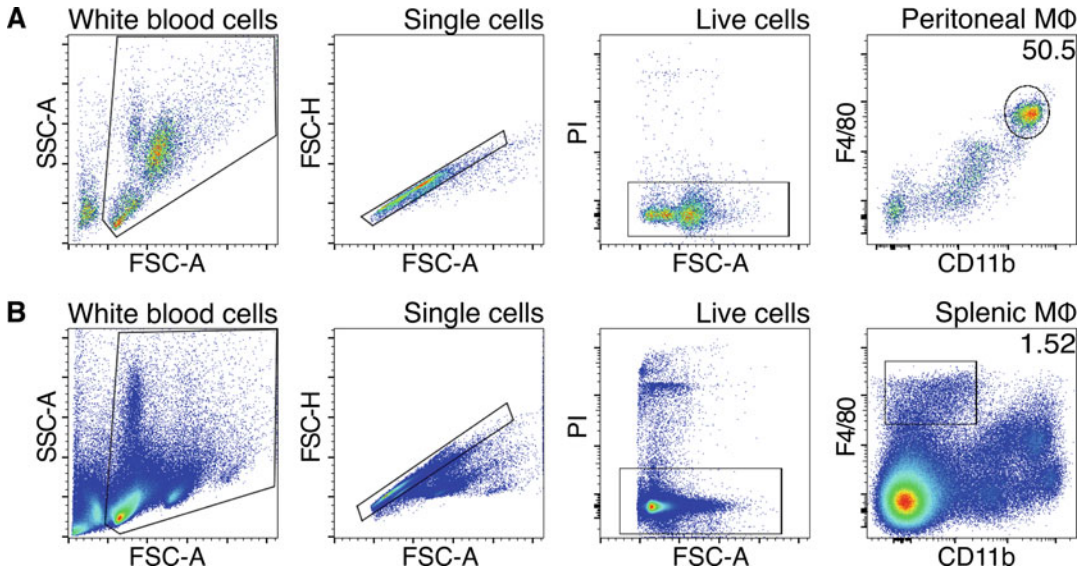


Fig. 1 Gating strategy of peritoneal and splenic macrophages. Cells were pre-gated on FSC/SSC and PI⁻ to obtain live singlets. Total peritoneal cells (**a**) and splenocytes (**b**) from 5.5 weeks old mice were stained with F4/80 and CD11b. Macrophages were defined as F4/80⁺ CD11b⁺ population. The results are representative of three independent experiments

mitochondria activity in peritoneal cavity MΦs was also reflected in their ROS production capacity (Fig. 3). Together, these data suggested that tissue MΦs can adjust their metabolic profile according to the residing microenvironment.

2 Material

2.1 Preparation of the Single-Cell Suspension

1. Dulbecco's Modified Eagle Medium (DMEM).
2. Serum-free DMEM: DMEM, supplemented with 44 mM NaHCO₃, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% MEM nonessential amino acids.
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄.
4. 5 mL syringe.
5. 24G needle.
6. 15 mL centrifuge tube.
7. 6 cm petri dish.
8. Ammonium-Chloride-Potassium (ACK) lysing buffer: 155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA.
9. Nylon mesh with pore size 75 μm.

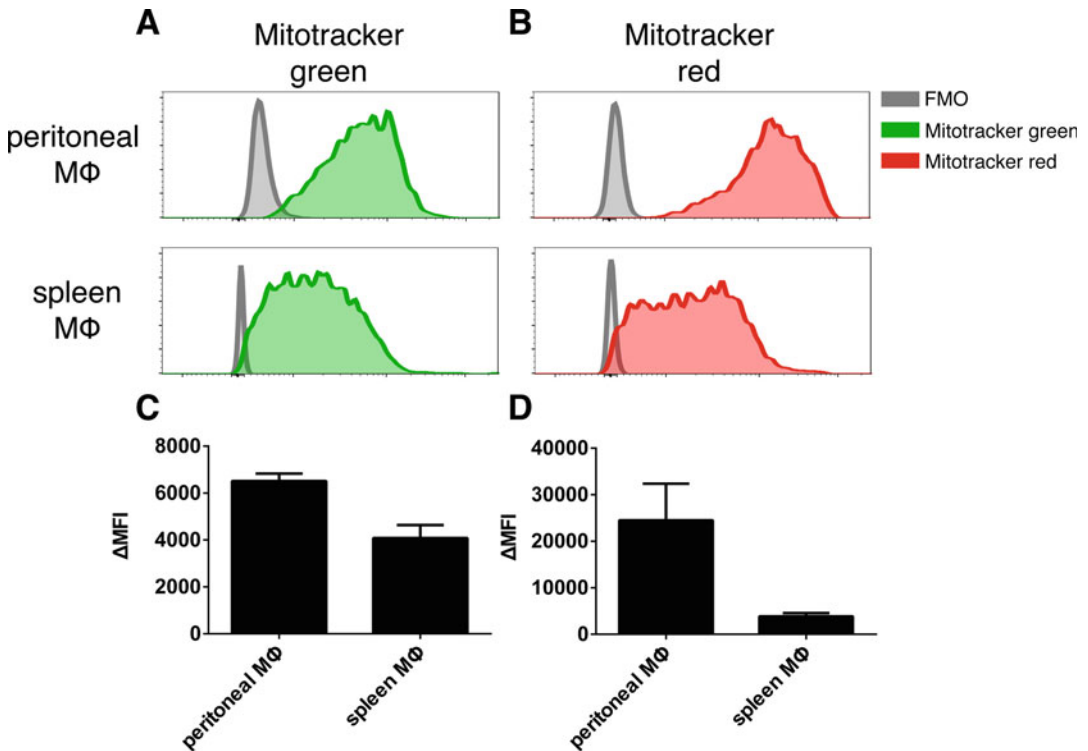


Fig. 2 Quantification of mitochondria mass and membrane potential in peritoneal and splenic macrophages. Cells were stained with mitochondria-specific dye for 15 min at 37 °C, followed by surface marker staining. The fluorescent signal of stained cells was acquired by the flow cytometer and analyzed. (a) Mitochondria mass of peritoneal and splenic macrophages was quantified by MitoTracker™ Green staining of peritoneal and splenic macrophages. (b) Mitochondria membrane potential of peritoneal and splenic macrophages was evaluated by MitoTracker™ Red staining. The green and red represent mitochondria-specific staining, and the gray line shows fluorescence minus one (FMO). The results are representative of one experiment with $n = 3$. The statistics of mitochondrial mass (c) and membrane potential (d) were performed by calculating the $\Delta\text{MFI} = \text{MFI (Mitochondria staining)} - \text{MFI (FMO)}$

2.2 Detection of Mitochondrial Mass, Activity, and ROS (See Notes 1 and 2)

1. MitoTracker Green FM, stock solution 1 mM in DMSO.
2. MitoTracker Red FM, stock solution 1 mM in DMSO.
3. CellROX™ Green (Thermo Fisher Scientific), stock solution 2.5 mM in DMSO.
4. MitoSOX™ (Thermo Fisher Scientific), stock solution 5 mM in DMSO.
5. Round-bottom FACS tube.

2.3 Surface Marker Staining

1. 2.4G2 hybridoma (ATCC® HB-197™) supernatant.
2. FACS buffer (1× PBS supplemented with 2% fetal bovine serum (FBS) and 1 mM EDTA).
3. PE-Cy7 Anti-mouse F4/80 (BioLegend, Cat#123114).
4. BV421 Anti-mouse F4/80 (BioLegend, Cat#123137).

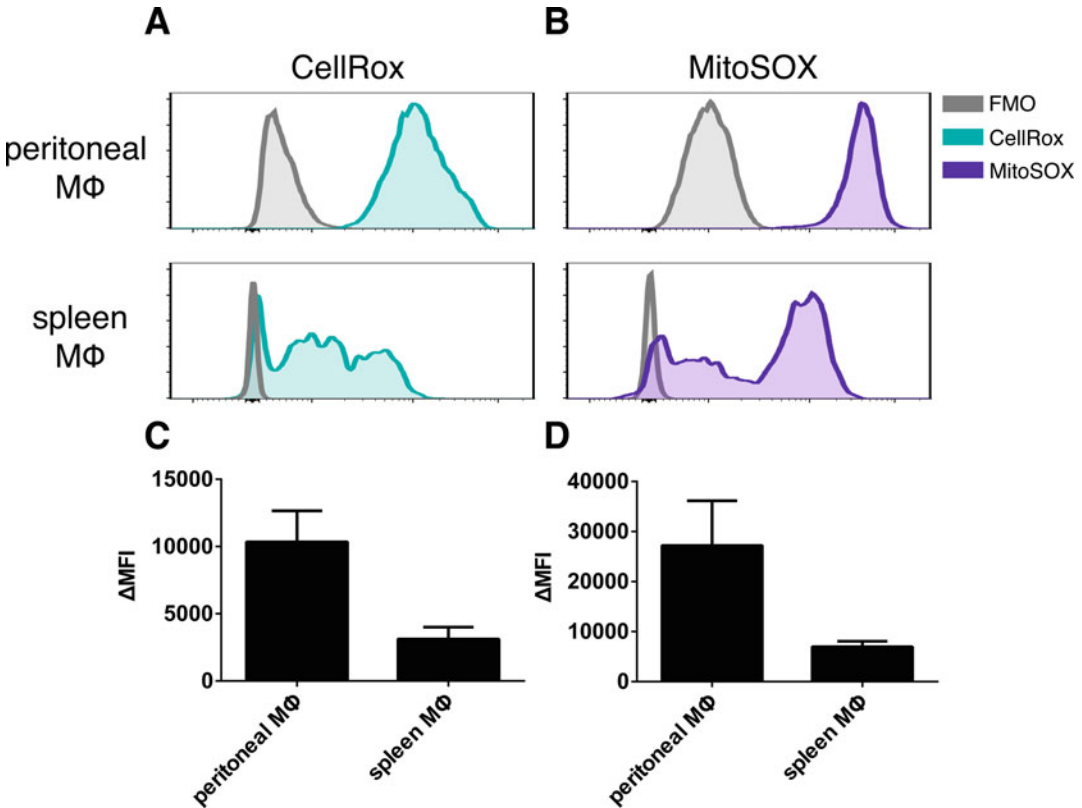


Fig. 3 Measurement of cellular and mitochondrial ROS in peritoneal and splenic macrophages. Cells were stained with surface marker staining, followed by reactive oxygen species-specific dye for 15 min at 37 °C. The fluorescent signal of stained cells was acquired by the flow cytometer and analyzed. **(a)** The level of total cellular ROS in peritoneal and splenic macrophage was evaluated by CellROX staining. **(b)** MitoSOX™ staining of peritoneal and splenic macrophages measures the mitochondrial ROS level. The blue and purple represent mitochondria staining, and the gray one shows FMO. The results are representative of one experiment with $n = 3$. The statistical analysis was done by calculating the $\Delta\text{MFI} = \text{MFI (reactive oxygen species staining)} - \text{MFI (FMO)}$ for cellular **(c)** and mitochondrial **(d)** ROS

5. APC Anti-mouse CD11b (BioLegend, Cat#101212).
6. PE-Cy7 Anti-mouse CD11b (BioLegend, Cat#101215).
7. Propidium iodide solution.
8. DAPI.

3 Methods

3.1 Tissue Harvest and Generation of the Single-Cell Suspension

1. Euthanize the mouse by an approved method such as CO₂ asphyxiation in a transparent acrylic chamber. Rinse the target area with 75% ethanol.
2. To harvest the peritoneal cells, intraperitoneally inject 5 mL serum-free DMEM medium with a 24G needle. Gently

massage the peritoneal cavity, and harvest the peritoneal cell-containing lavage as much as possible with the syringe. Transfer the lavage to a 15 mL centrifuge tube and leave on ice until assay. This is the peritoneal single-cell suspension.

3. Open the peritoneal cavity and locate the spleen. The spleen is at the left upper quadrant of the abdomen, carefully remove the surrounding connective tissue and harvest the spleen.
4. Dissociate the spleen by pressing gently with a syringe plunger in a 6 cm dish containing 5 mL ice-cold serum-free DMEM. Transfer the single-cell suspension to a 15 mL centrifuge tube, wash the 6 cm dish with an additional 2 mL serum-free DMEM, and pool the cell suspension together. This is the splenocyte single-cell suspension.
5. Centrifuge the cell suspension for 5 min at $450 \times g$, 4°C and discard the supernatant. Resuspend the cell pellet with 2 mL ACK lysing buffer for 2 min at room temperature (RT) to lyse the red blood cells. At the end of the reaction, add 13 mL of PBS to the cell to neutralize the ACK lysing buffer.
6. Pellet the cells again by centrifuging for 5 min at $450 \times g$, 4°C and resuspend peritoneal cells in 1 mL, splenocytes in 3 mL serum-free DMEM.
7. Filter cell suspension through nylon mesh to remove any clumps to obtain the single-cell suspension. Enumerate the cell number.

3.2 Quantification of Mitochondria Mass and Membrane Potential

1. Freshly prepare the mitochondria-specific dye working solution by mixing 0.1 μL MitoTracker stock solution to 1 mL ice-cold serum-free DMEM (*see Note 3*).
2. Add 1×10^6 peritoneal cells or 2×10^6 splenocytes to round-bottom FACS tubes, and pellet the cells by centrifuging for 5 min at $450 \times g$, 4°C (*see Note 4*). Discard the supernatant.
3. Resuspend the cells in 100 μL of mitochondria-specific dye working solution and incubate for 15 min in 5% CO_2 incubator at 37°C (*see Note 5*).
4. At the end of the incubation time, add 1 mL ice-cold FACS buffer to stop the reaction, and centrifuge for 5 min at $450 \times g$, 4°C . Discard the supernatant. Cells are now ready to proceed with surface marker staining.

3.3 Surface Marker Staining

1. Resuspend the cells in 100 μL of 2.4G2 hybridoma supernatant and incubate on ice for 10 min to block Fc receptors. Wash the cells by adding 1 mL of ice-cold FACS buffer, and centrifuge for 5 min at $450 \times g$, 4°C . Discard the supernatant.

2. Resuspend the cells with 100 μL of FACS buffer containing the pre-titrated fluorescence-conjugated antibodies and incubate the mixture on ice for 20 min. Avoid light exposure during the incubation period.
3. Wash the cells by adding 1 mL of ice-cold FACS buffer, and centrifuge for 5 min at $450 \times g$. Discard the supernatant.
4. Resuspend the pellet in 350 μL of FACS buffer containing 1 $\mu\text{g}/\text{mL}$ propidium iodide (PI) and immediately analyze the samples on the flow cytometer.

3.4 Reactive Oxygen Species Detection

1. To measure cellular ROS, the samples should be stained with surface markers first (Subheading 3.3, steps 1–3), followed by reactive oxygen species detection procedures (*see* Note 6).
2. To make the CellROX working solution, add 0.4 μL CellROX stock to 200 μL of warm serum-free DMEM. MitoSOX working solution is made of 0.2 μL MitoSOX stock in 200 μL warm serum-free DMEM. Both working solutions should be freshly prepared. Resuspend the cell pellet from Subheading 3.3 in working solutions and incubate at 37°C for 30 min. For the control (fluorescence minus one, FMO), add 200 μL warm serum-free DMEM only. Avoid light exposure during the incubation period.
3. Add 1 mL ice-cold FACS buffer to each sample and centrifuge for 5 min at $450 \times g$ 4°C . Discard the supernatant.
4. Resuspend the pellet in 100 μL ice-cold FACS buffer containing 0.1 μL DAPI stock solution and immediately analyze the samples on the flow cytometer and perform data analysis (*see* Note 7).

3.5 Data Analysis

1. Once the data are collected, use the analytical software of choice to create a dot plot and gate on the populations of interest. FSC-A vs. SSC-A is used to identify cell populations, followed by FSC-A vs. FSC-H for singlet gating, FSC-A vs. PI to gate on live cells. Cell surface markers are used to mark the population of interests. Here, for example, we used F4/80 vs. CD11b for macrophages gating (Fig. 1).
2. To visualize the fluorescence of mitochondria-specific dye in the population of interests, choose the “Histogram” function (Figs. 2a, b and 3a, b). Calculate the mean fluorescence intensity (MFI) for each population of interest.
3. To perform statistical analysis, generate delta MFI (ΔMFI) for each cell population by calculating MFI (organelle-specific dyes)—MFI (FMO) (Figs. 2c, d, and 3c, d).

4 Note

1. These organelle-specific dyes are very sensitive to freeze–thaw cycles as well as light and air exposure. Aliquot the stock solution in a small volume to avoid repeated freeze–thaw and light exposure and to minimize the dye from constant air exposure.
2. All of these mitochondria-specific dyes have cytotoxicity at high concentrations, a higher staining concentration than that used in the current protocol is not recommended.
3. To maintain good cell viability throughout the procedures, it is recommended to use a serum-free culture medium during the organelle-specific dyes staining step.
4. We recommend careful titration and optimization of the staining procedures for the cell population of interests. Once the conditions are set, fix the cell-to-dye ratio to maintain the consistent staining intensity.
5. To efficiently label the mitochondria-specific dyes, the reaction has to been performed at 37 °C. We recommend proceeding with surface marker staining at 4 °C upon the completion of the mitochondrial-dye staining to achieve the best staining results.
6. Due to the sensitivity of ROS-specific dyes and the versatile nature of ROS, perform the surface marker staining first, followed by the ROS-specific dye staining. Upon the completion of the ROS-specific dye staining procedure, analyze the sample immediately.
7. Before harvesting the sample, filter cell suspension through a cell strainer to avoid cell clumps.

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