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Dear Scientific Editors,

Please, find enclosed our manuscript entitled, “Multicolor flow cytometry-based quantification of mitochondria and lysosomes in T cell”, to be considered for publication in *JoVE*.

T cells are an essential part of adaptive immunity, protecting us from pathogens and altered own cells. It is known that for T cells to be activated and perform their functions, they need to go through intensive metabolic reprogramming to meet their biosynthetic demands. Mitochondria and lysosomes are crucial participants in this cellular metabolic regulation, and the measurement of mitochondrial and/or lysosomal contents has been considered crucial indicators to evaluate the T cell metabolic and functional status. However, current available assays such as electron microscopy or immunofluorescent staining demands a fixed homogenous cell population with large cell numbers as starting material to obtain the best results. In this manuscript, we assembled a protocol that combines organelle-specific dyes such as LysoTracker/MitoTracker with surface markers staining to quantify the intracellular lysosomes and mitochondria in live immune cell populations.

Given the high interests of the vast and fast-moving field of immunometabolism and tumor immunology, we believe that the method presented in our manuscript will appeal to a great numbers of JoVE readers, which encompass biomedical, biochemical, and clinical science researchers. Moreover, the technique described here can be easily modified and applied to various cell types of interests.

This manuscript illstrates an original method developed in our lab and is not under consideration for publication by any other journal. All authors approved the manuscript and this submission. The authors have no conflicts of interest to declare. If you find our manuscript appropriate for *JoVE*, we would like to recommend the following researchers as potential scientific reviewers for this paper:

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Thank you for receiving our manuscript and considering it for review. We appreciate your time and look forward to your response.

Sincerely,

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TITLE:

Multicolor Flow Cytometry-based Quantification of Mitochondria and Lysosomes in T Cells

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KEYWORDS:

Flow cytometry, multicolor, T cell, lysosome, mitochondria, organelle-specific dyes

SUMMARY:

This article illustrates a powerful method to quantify mitochondria or lysosomes in living cells. The combination of lysosome- or mitochondria-specific dyes with fluorescently conjugated antibodies against surface markers allows the quantification of these organelles in mixed cell populations, like primary cells harvested from tissue samples, by using multicolor flow cytometry.

ABSTRACT:

T cells utilize different metabolic programs to match their functional needs during differentiation and proliferation. Mitochondria are crucial cellular components responsible for supplying cell energy; however, excess mitochondria also produce reactive oxygen species (ROS) that could cause cell death. Therefore, the number of mitochondria must constantly be adjusted to fit the needs of the cells. This dynamic regulation is achieved in part through the function of lysosomes that remove surplus/damaged organelles and macromolecules. Hence, cellular mitochondrial and lysosomal contents are key indicators to evaluate the metabolic adjustment of cells. With the development of probes for organelles, well-characterized lysosome or mitochondria-specific dyes have become available in various formats to label cellular lysosomes and mitochondria. Multicolor flow cytometry is a common tool to profile cell phenotypes, and has the capability to be integrated with other assays. Here, we present a detailed protocol of how to combine organelle-specific dyes with surface markers staining to measure the amount of lysosomes and mitochondria in different T cell populations on a flow cytometer.

INTRODUCTION:

The activation and proliferation of T cells are critical steps for mounting successful immune responses. Recent advances suggest that the cellular metabolism is tightly associated with both the development and functions of T cells. For example, naive T cells rely largely on oxidative phosphorylation (OXPHOS) to meet the energy demand during the recirculation among secondary lymphoid organs. Upon activation, naive T cells undergo drastic metabolic reprogramming, including the induction of aerobic glycolysis to increase ATP production and to fulfill the tremendous metabolic demands during cell differentiation and proliferation. The cells that fail to follow through the metabolic needs die by apoptosis[1,2]. During the metabolic reprogramming, mitochondria play important roles since they are the organelles largely responsible for the production of ATP to supply energy for the cell, and the cellular mitochondria content fluctuates during metabolic switches throughout T cell development and activation[3]. However, the accumulation of superfluous or damaged mitochondria can produce excess ROS that damage lipids, proteins, and DNA, and can eventually lead to cell death[4]

The excessive or damaged mitochondria resulting from metabolic alterations are removed by a specialized form of autophagy[5], known as mitophagy. Mitochondria are wrapped by autophagosomes and then fused with lysosomes for degradation. These close communications between mitochondria and lysosomes have generated great interest[6,7]. For example, oxidative stress stimulates mitochondria to form mitochondria-derived vesicles (MDVs) that are targeted to lysosomes for degradation in a phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) and parkin (an E3 ubiquitin ligase) dependent manner[8]. It was also found that mitophagy is essential for beige-to-white adipocyte transition[9,10]. More importantly, lysosomes are not merely a degradation compartment, but also a regulator of cellular signaling. Excessive substrate accumulation due to enzyme deficiencies results in lysosomal dysfunction by disrupting lysosomal membrane permeability and affects Ca^{2+} homeostasis[11]. The T cell functional defects in lysosomal acid lipase (LAL)[12] or lysosomal metabolite transporter knockout mouse model[13] further showed the importance of lysosomes in maintaining T cell homeostasis. Both mitochondria and lysosomes are inseparable parts of cellular metabolic regulation. Therefore, measurement of cellular mitochondrial content has been a crucial indicator to evaluate the T cell metabolic and functional status.

Assays commonly used to quantify cellular mitochondrial or lysosomal contents include immunoblot, electron microscopy, immunofluorescent (IF) staining, and PCR analysis of mitochondrial DNA copy numbers[14-16]. While immunoblot can quantitatively compare protein levels across different samples and electron or IF microscopy can visualize the morphological hallmarks of these organelles[17], these assays bear certain technical drawbacks. For example, it is time consuming to acquire sufficient number of cell images with high magnification and resolution or to compare the expression levels of a protein across dozens of samples, making these assays considered to be low throughput methods. Moreover, these assays can only be applied to homogenous cell population, such as cell lines, but not to tissue samples that are composed of mixed populations.

It is also difficult to apply these assays to rare populations, for which the requirement of minimal cell numbers from 10^6 to 10^8 is impossible to meet. Finally, cells are usually lysed or fixed during

the process, making them incompatible with other methods to extract further information. Compared with the traditional methods, fluorescent-based flow cytometry has a relatively high throughput – the information of all the cells within a sample composed of mixed cell populations can be analyzed and collected simultaneously. In addition, one can detect more than 10 parameters on the same cell and sort the cells based on desired phenotypes for further assays. Reactive fluorescent probes have been used to label lysosomes and mitochondria in live cells and can be detected by flow cytometry[18,19]. These organelle-specific probes are cell permeable and have physico-chemical characteristics that allow them to be concentrated in specific subcellular locations or organelles. Conveniently, these probes are available in various fluorescent formats, thus enabling their application for multicolor analysis.

This protocol describes in detail how to combine surface marker staining with lysosome- or mitochondria-specific dyes to label lysosomes or mitochondria in live cells. This is especially useful for samples generated from primary tissues and organs, which are often composed of heterogeneous cell populations. Researchers can identify cell populations of interest by their surface marker expression and specifically measure the lysosomal or mitochondrial contents through organelle-specific dyes in these cells. Here, we demonstrate the detailed procedure of flow cytometric analysis that evaluates the lysosomal or mitochondrial mass in the major splenic T cell subpopulations.

PROTOCOL

The mouse tissue harvest procedure described here has been approved by the Institutional Animal Care and Use Committee (IACUC) of National Yang-Ming University.

1. Preparation of Lymphocyte Suspension from Lymphoid Organs

1.1. Euthanize the mouse by an approved method such as CO_2 inhalation in a transparent acrylic chamber followed by cervical dislocation to ensure death.

Note: Keep the flow rate of CO_2 for a 20% displacement per minute of the chamber, and no higher than 5 psi (pound per square inch). It takes 2-3 min for a mouse to lose consciousness. Maintain the CO_2 flow at least for 1 min after the animal has stopped breathing (5 – 7 min overall).

1.2. Place the mouse on its back on a clean board (*e.g.* a polystyrene plastic board) and fix the limbs with tape. Rinse with 70% ethanol.

1.3. To harvest spleen, cut and open the abdominal cavity with 11-cm dissecting scissors (the spleen is below the stomach and above the left kidney). Remove the spleen with forceps and clean from connective tissues (use scissors if necessary).

1.4. To harvest the thymus, cut the diaphragm and the rib cage from both sides with dissecting scissors. Use forceps to lift up the sternum to reveal the entire thoracic cavity. Separate the thymus carefully from surrounding tissues with scissors, and remove any residual connective

tissue on a paper towel wetted with PBS.

1.5. Mechanically dissociate the tissues with a syringe plunger in a 6-cm dish containing 5 mL ice-cold FACS buffer (PBS supplemented with 2% fetal bovine serum (FBS) and 1 mM EDTA). Transfer the single-cell suspension to a 15-mL centrifuge tube; wash the 6-cm dish with additional 2 mL FACS buffer and combine the wash with cell suspension as well.

1.6. Centrifuge the cell suspension (300 x g, for 5 min at 4 °C) and discard the supernatant. Re-suspend the pellet with 2 mL of Ammonium-Chloride-Potassium (ACK) lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA) for 1 min at room temperature (RT) to lyse the red blood cells. Neutralize the ACK buffer by adding 10 mL FACS buffer to the cell suspension.

1.7. Centrifuge the cell suspension (300 x g, for 5 min at 4 °C) and discard the supernatant Re-suspend cells in 5 mL of complete RPMI medium (RPMI 1640 medium, supplemented with 44 mM $NaHCO_3$, 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 non-essential amino acids and 10% FBS).

1.8. Filter cell suspension through a cell strainer (pore size 70 µm) to remove cell clumps. Count cells with a hemocytometer or automated cell counter.

2. Quantification of Mitochondria and Lysosomes

Note: Perform the organelle-specific dye staining first because the optimal staining condition for these dyes is incubation at 37 °C. The surface marker staining can be significantly reduced because of antibody capping and internalization at that temperature.

2.1. Add 1 x 10^6 cells to round-bottom FACS tubes, centrifuge cells (300 x g, for 5 min at 4 °C) and discard supernatant.

2.2. Dilute the probe stock solutions to final working concentration (100 nM Mito Green or Lyso Green; henceforth mitochondria- or lysosome-specific dye, respectively) in pre-warmed 37 °C serum-free culture medium. This is the working solution for lysosome- or mitochondria-specific dye.

Note: Test multiple concentrations including the range of working concentrations suggested by manufacturer (20 – 200 nM for the mitochondria-specific dye and 50 – 75 nM for the lysosome-specific dye used here) in order to obtain the best staining efficiency. Choose a cell population that is known to have good expression of staining target (*e.g.* lysosomes) as positive control (*e.g.* human $CD14^+$ monocytes). Choose the working concentration based on good separation between negative and positive staining, as well as good cell viability.

2.3. Resuspend the cells in 100 µL of lysosome- or mitochondria-specific dye working solution and incubate in 5% CO_2 incubator at 37 °C for 15 min or 30 min, respectively.

2.4. Add 1 mL of ice-cold FACS buffer to stop the reaction. Pellet cells by centrifugation (300 x g, for 5 min at 4 °C) and discard supernatant. Cells are now ready for surface marker staining.

3. Surface Marker Staining

3.1. Block Fc receptors with 100 µL of pre-titrated 2.4G2 hybridoma supernatant on ice for 10 min. Wash cells with 1 mL of FACS buffer and centrifuge (300 x g, for 5 min at 4 °C). Discard supernatant.

3.2. Incubate the cells with 50 µL pre-titrated fluorescence-conjugated antibody combination in FACS buffer on ice for 20 min. Avoid light.

Note: Prepare single color-stained cells that include all the fluorescent antibodies present in the antibody combination and cells treated only with organelle-specific dye for setting voltages of each fluorescence detector and compensation adjustment on flow cytometer. Likewise, prepare the Fluorescence Minus One (FMO) as background controls by using cells stained with all the antibodies, but without staining with organelle-specific dyes.

3.3. Wash the cells by adding 1 mL of FACS buffer and pellet by centrifugation (300 x g, for 5 min at 4 °C). Discard supernatant.

3.4. Resuspend the cells in 300 µL of FACS buffer containing 1 µg/mL propidium iodide (PI) and immediately analyze the samples on flow cytometer.

4. Sample Collection on Flow Cytometer

4.1. Turn on the computer, flow cytometer, FACS acquisition software and any other accessory devices depending on the machine platform. Run PBS for around 1 min to ensure the flow line is filled with PBS and sheath buffer. Make sure the flow stream runs smoothly.

4.2. Open new experiment and select cytometer parameters (fluorescent detectors) according to manufacturer's instruction. Filter cells through 70-µm cell strainer and vortex prior to the acquisition of each sample to avoid clumps and doublet formation.

4.3. Make dot plots of forward scatter (FSC) and side scatter (SSC), which represent cell size and granularity, respectively. Optimize the voltages of FSC and SSC to make sure the cells of interest appear in the plots. Make dot plots of FSC-A *vs.* FSC-W (or FSC-H) and draw a gate for the single cells.

4.4. Make dot plots of each fluorescent parameter. Use unstained cells to determine background signal and use single color-stained controls to adjust voltages of each fluorescent parameter, so that positive and negative cell populations are clearly distinguishable and within the dynamic range of acquisition.

4.5. After all the voltages are set, use auto-compensation if available, or manually adjust the compensation with unstained and single color-stained controls to correct the spectral spillover. Apply the compensation to samples.

4.6. Make a dot plot of each parameter and draw gates according to your experimental set up. For example, FSC-A *vs.* FSC-H (or FSC-W) to exclude doublets, followed by FSC-A *vs.* SSC-A for lymphocyte gating; SSC-A *vs.* PI (live cells) and CD4 *vs.* CD8 (**Figure 1**).

4.7. Collect enough events (total cell number) for meaningful comparison between populations. Typically, at least 1,000 events in the population of interest need to be obtained[20].

4.8. After all the samples are acquired, export flow data to be analyzed by flow cytometry analysis software. Save the experiment as template to preserve the cytometer setting and parameters for applying to similar experiments in the future.

5. Data Analysis

Note: There are many tools to analyze flow cytometric data, both commercial and free software. The acquisition software of the flow cytometers can also work for data analysis. Here we used FlowJo as an example.

5.1. Start the software and drag the FCS files into the workspace. Click on a sample to open a graphic window. Select parameters to be presented on X and Y axis by clicking on the X and Y axes. Use the toolbar to draw gates according to differentially expressed markers or defined phenotypes of cell populations.

5.2. Add gates of each parameter as shown in **Figure 1**. Drag gates to all samples in the workspace, so that they get identical gating.

5.3. Drag the plots to layout editor to create graphical reports.

5.4. Display the mean fluorescence intensity (MFI) for each population of interest by clicking the statistics button (Σ) in a graphic window and choosing “Mean” and the appropriate parameter, for example, the channel used to detect organelle-specific dye. Then click “Add”.

5.5. Click the table editor icon and drag MFI to table editor to create statistical reports. Save as Excel, Text or CSV file to calculate delta MFI (ΔMFI) as MFI (organelle-specific dyes) - MFI (FMO).

Note: Do not calculate MFI between samples acquired with different cytometer settings (voltages, fluorescent channels or compensation). Comparisons of values derived from experiments with different settings are meaningless and biased.

5.6. Export all the values from the workspace and plots from layout editor for making tables and

figures.

REPRESENTATIVE RESULTS

Identification of major T cell subsets in spleen and thymus

In brief, single cell suspensions from spleen and thymus were lysed of red blood cells, incubated with 2.4G2 supernatant, followed by organelle-specific dye and surface marker staining with fluorescence-conjugated antibodies (**Table 1**). The developmental progression of thymocytes can be described by using antibodies to the co-receptors CD8 and CD4. The most immature thymocytes do not express CD4 or CD8 and are called double negative (DN). Then, they up-regulate both CD4 and CD8 and become double positive (DP). Finally, they down-regulate CD4 or CD8 and become mature single positive (SP) cells ready to leave the organ. The earliest stages of T cell development, when the cells do not express the co-receptors CD4 and CD8, can be further classified on the basis of CD44 and CD25 expression. The earliest progenitors are $CD44^+$ $CD25^-$ (DN1), and then they start expressing CD25 and become $CD44^+CD25^+$ (DN2) cells. After that, the cells down-regulate CD44 and become $CD44^-CD25^+$ (DN3) cells and, finally, they down-regulate CD25 as well to become $CD44^-CD25^-$ (DN4) cells that are on the way to expressing CD4 and CD8 and becoming double-positive (DP) cells.

In the spleen, the T cells can be divided into cytotoxic $CD8^+$ and helper $CD4^+$ T cells. Both of these populations can be further divided into naive, effector and memory subsets based on CD62L and CD44 staining. The detailed gating strategy is shown in **Figure 1**.

Quantification of mitochondria mass in different T cell subsets

Mitochondria-specific dyes were used to measure the amounts of mitochondria in T cell populations. We found that the mitochondrial contents were the lowest in DN1, peaked at DN2-DN3, and then slightly decreased in DN4 and DP thymocytes and were even lower in CD4 and CD8 SP thymocytes (**Figure 2A**). However, CD4 or CD8 SP thymocytes had higher mitochondria staining MFI than splenic CD4 or CD8 T cells (**Figure 2B**). These observations suggest that mitochondrial content fluctuates during T cell development with immature thymocytes containing more mitochondria than their mature counterparts do, and the naive T cells that recirculate in oxygen-rich blood have even lower mitochondrial mass. Interestingly, this reduction of mitochondrial contents was more prominent in the CD8 than the CD4 T lineage (**Figure 2B**), and T cell activation further decreased it. Among activated T cells, $CD4^+$ memory T cells had slightly more mitochondria compared to effectors; however, the opposite was the case for $CD8^+$ T cells: $CD8^+$ memory T cells had the lowest mitochondrial mass among all T cell subsets (**Figure 2B**).

Lysosomal content measurement in various subpopulations of T cells

Using lysosome-specific dye, we observed relatively low, but detectable, lysosomal contents in all T cell populations, with more prominent presence of lysosomes in DN1 thymocytes (**Figure**

3A). No significant difference was found among the thymocyte subsets from DN1 onward. In the periphery, a relatively high number of lysosomes was found in memory and effector $CD8^+$ T cells. This phenomenon is in line with previous studies showing that activated $CD8^+$ T cells increased their expression of lysosomal-associated membrane protein 1 (LAMP-1)[21,22], reflecting their possession of lysosomal-cytotoxic granules (**Figure 3B**)

FIGURE AND TABLE LEGENDS

Figure 1: Gating strategy of splenic lymphocytes and thymocytes. Cells were pre-gated on FSC/SSC and PI^- to obtain live singlets. **(A)** Total thymocytes from mice were stained with CD4, CD8, CD44, and CD25. CD4 and CD8 expressions were used to distinguish $CD4^+CD8^-$ SP, $CD4^-CD8^+$ SP, $CD4^+CD8^+$ DP, and $CD4^-CD8^-$ DN subsets. The $CD4^-CD8^-$ DN subset was further divided into $CD44^+CD25^-$ DN1, $CD44^+CD25^+$ DN2, $CD44^-CD25^+$ DN3, and $CD44^-CD25^-$ DN4 subpopulations. **(B)** Total splenocytes from mice were stained with TCRβ, CD4, CD8, CD44, and CD62L. The $TCR\beta^+$ cells were separated into $CD4^+$ and $CD8^+$ T cells, which were further divided into naive ($CD44^-CD62L^+$), memory ($CD44^+CD62L^+$), and effector ($CD44^+CD62L^-$) populations. The results are representative of three independent experiments with *n* = 3-4.

Figure 2: Representative histograms showing mitochondria staining in each cell population. Cells were stained with mitochondria-specific dye for 15 min at 37 ˚C, followed by surface marker staining. Fluorescent signal of stained cells was acquired by flow cytometer and analyzed on. **(A)** Mitochondria staining of DN and DP thymocytes. **(B)** Mitochondria staining of CD4SP and SD8SP thymocytes and splenic T cell populations. ΔMFI = MFI (Mitochondria staining) – MFI (FMO). The solid red line represents mitochondria staining, the solid blue line shows FMO control, and the dashed red line represents mean fluorescence intensity of mitochondria staining in each population. The results are representative of three independent experiments with *n* = 3-4.

Figure 3: Representative histograms showing lysosome staining in splenic lymphocytes and thymocytes. Cells were stained with lysosome-specific dye for 30 min at 37 ˚C, followed by surface marker staining. Fluorescent signal of stained cells was acquired by flow cytometer and analyzed. **(A)** Lysosome staining of DN and DP thymocytes. **(B)** Lysosome staining of CD4SP and SD8SP thymocytes and splenic T cell populations. ΔMFI = MFI (Lysosome staining) - MFI (FMO). The solid green line represents lysosome staining, the solid blue line shows FMO, and the dashed red line represents mean fluorescence intensity of lysosome staining in each population. The results are representative of one experiment with *n* = 5.

Table 1: Multicolor flow cytometry panel designs. The fluorochrome and concentration of antibodies/organelle dyes in the staining mixtures are listed here. It is strongly recommended to titrate and test each antibody when setting up new staining panels.

DISCUSSION

This protocol combines organelle-specific dyes and surface marker staining to quantify the amount of mitochondria or lysosomes in different T cell populations. This method was developed

to overcome the limitation of cell number and homogeneity requirements for traditional methods, such as electron microscopy and immunoblot analysis. It is especially useful in analyzing rare cell populations and simultaneously examining multiple cell types at the same time.

The duration of the procedure and the temperature of incubation are the most critical factors in this protocol. Proper organelle labeling requires accurate titration of organelle-specific dyes. Cells should also be strictly kept on ice to enhance the viability and avoid antibody capping and internalization. In addition, the fluorescence of these organelle-specific dyes is vulnerable to light exposure and temperature changes. Thus, immediate analysis of samples once the staining procedure is completed is very important. We have consistently been able to complete the whole experiment within 2 hours.

Since this protocol relies on the capability of detecting multicolor fluorescence by flow cytometer to evaluate intracellular components, the acquisition settings (*e.g.* PMT voltages) and the compensation of spectra overlap (the spillover of fluorescence into neighboring channels) can heavily influence the intensity of the signal. This is especially relevant when the surface markers needed to identify the relevant cell types increase above six. In this situation, experience in designing multicolor flow cytometry experiments becomes very important for high quality data acquisition.

The immune system protects the body from pathogen insults and infections, and T cell activation has pivotal role in providing essential signals to other immune cells. Recent studies suggest that different T cell populations have unique metabolic programs, and mitochondrial and lysosomal contents are crucial indicators of metabolic changes. However, this phenomenon is not unique to T cells. The differentiation and activation of innate immune cells, such as macrophage and dendritic cells, are also closely linked to their metabolic status[23,24]. The advantage of this protocol is that it can be adapted to examine any cell populations of interest by using antibodies against lineage-specific markers. Moreover, in combination with other organelle-specific dyes, such as ER- or Cyto-ID, it has the potential to evaluate different organelles or cellular compartments in various types of cells.

The establishment of this mitochondria or lysosome quantification method is not only instrumental to study the metabolism in T cells, but also has great potential to benefit research that focuses on metabolic changes in disease settings, such as cancer or autoimmune diseases[25,26].

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DISCLOSURES:

The authors declare no conflicts of interests.

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Figure 1

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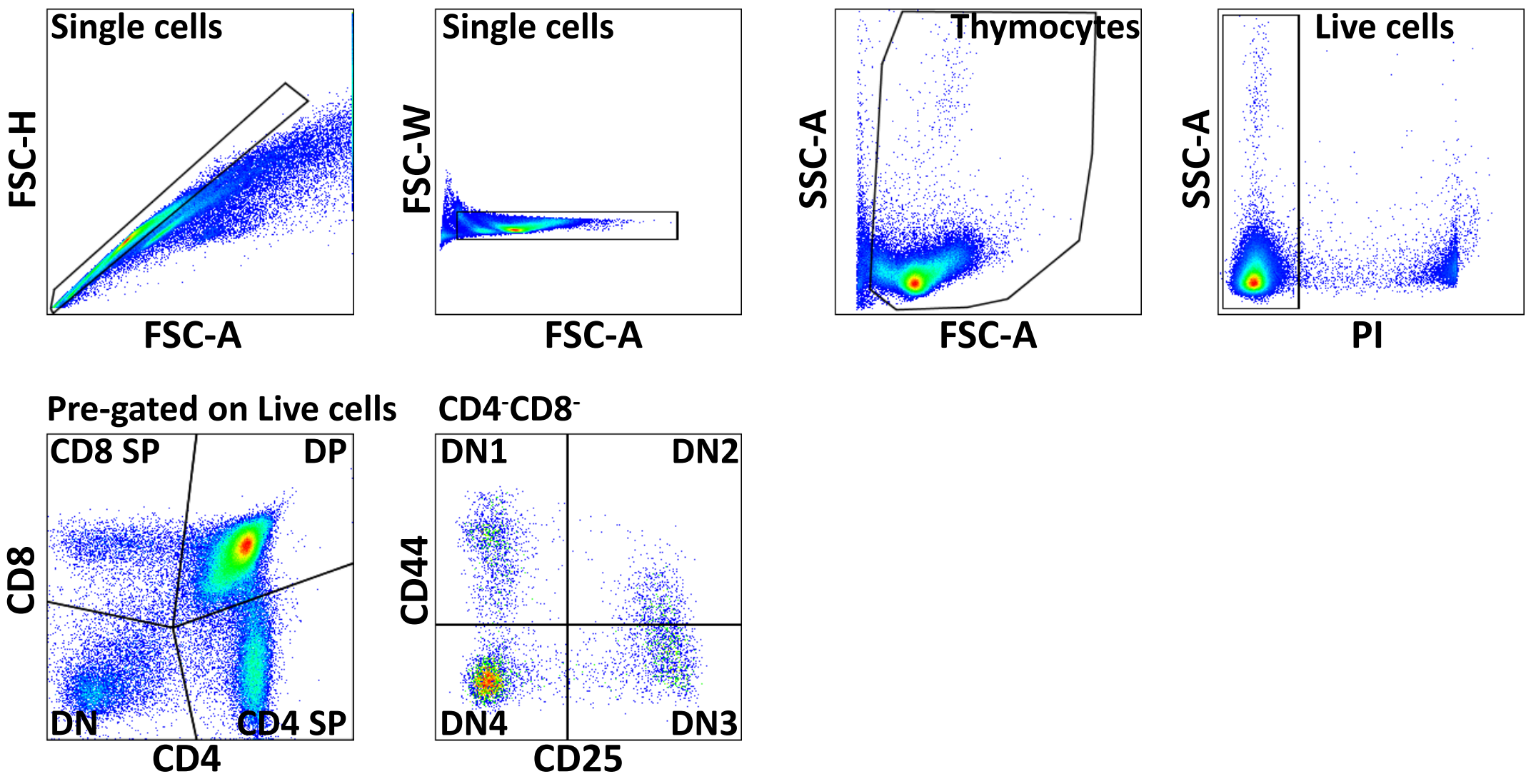

Figure 2

Click here to access/download;Figure;20180904 Fig2.pdf

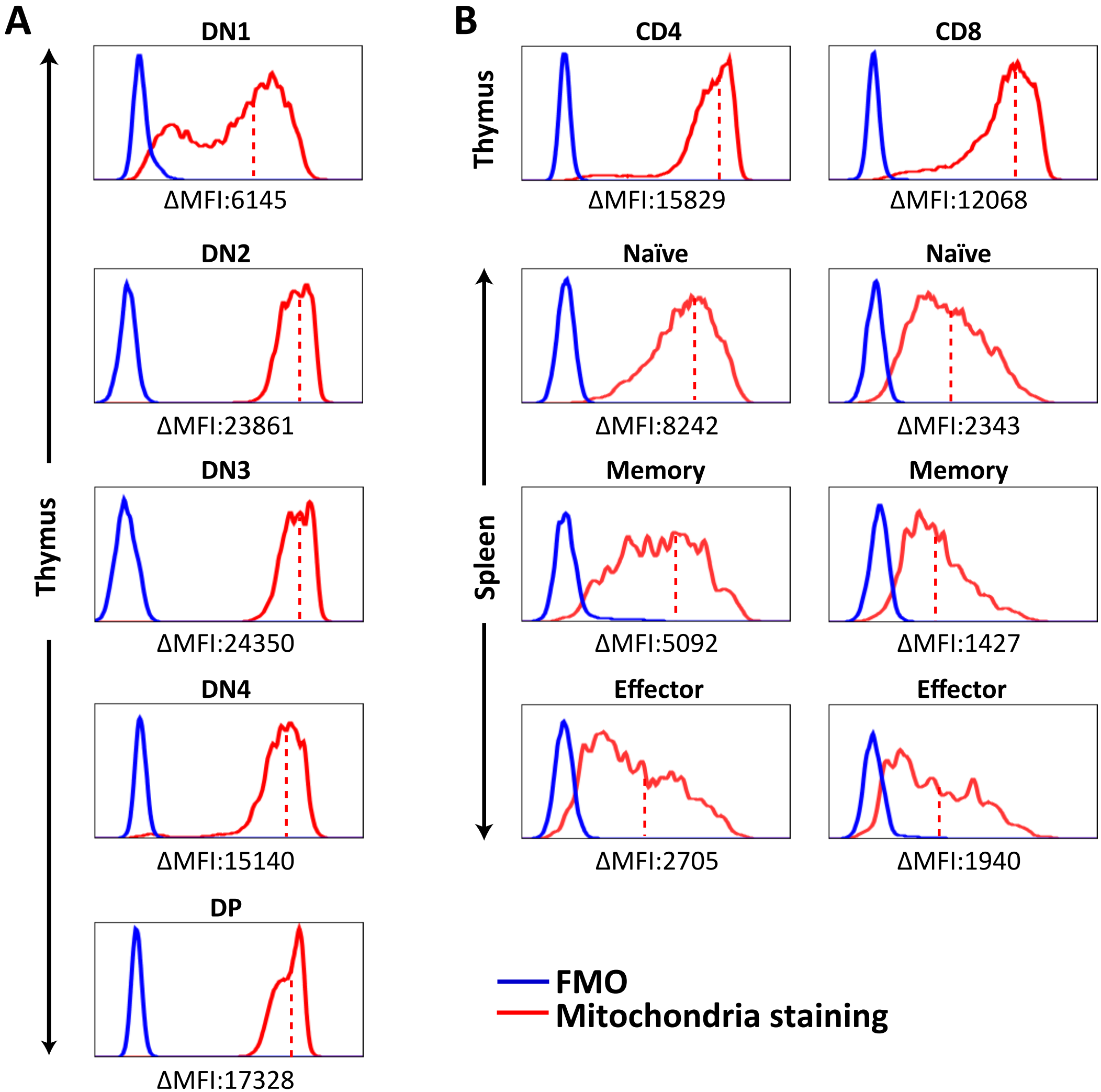

Figure 3

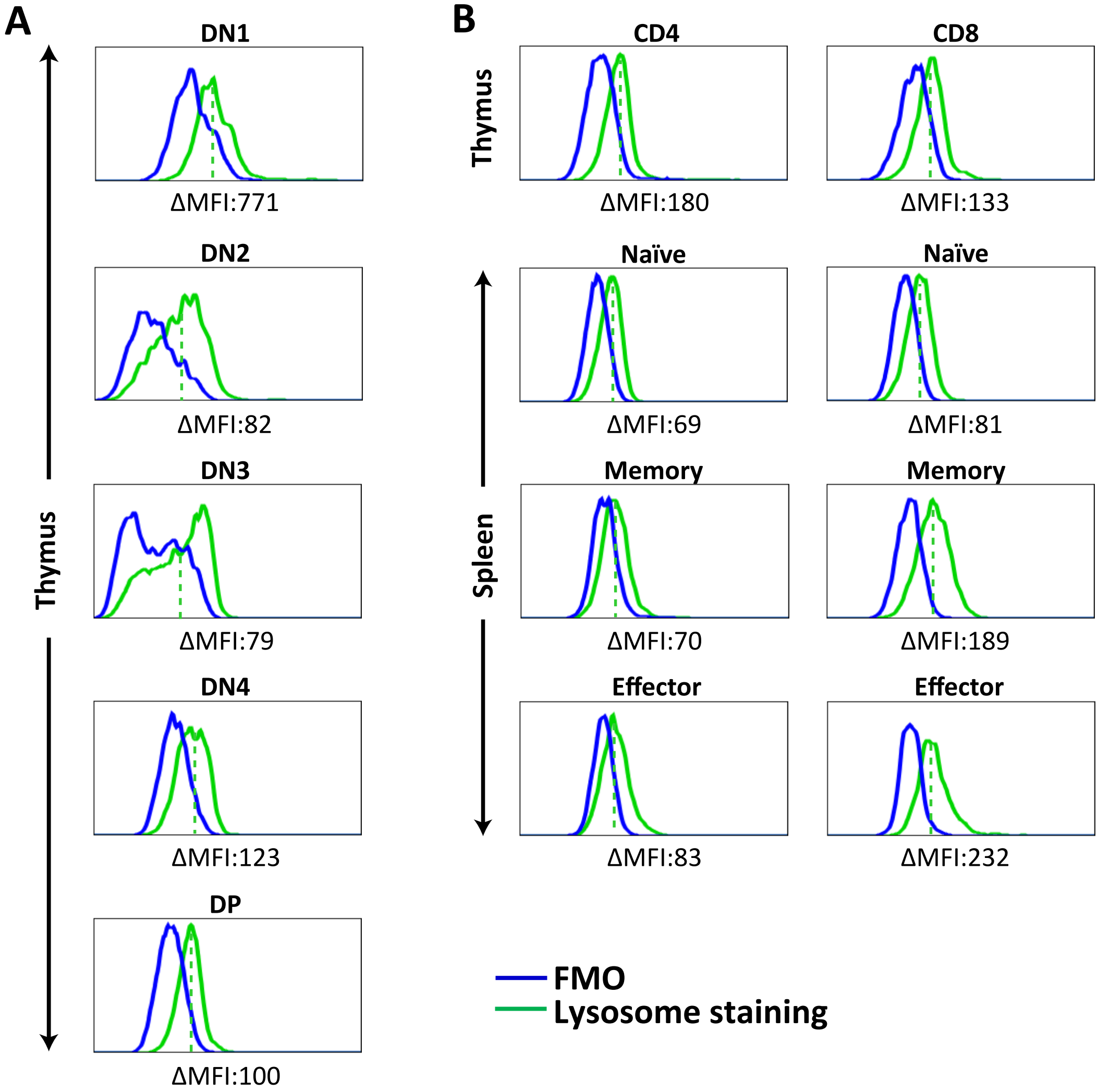

Table 1 Click here to access/download;Table;Jove Table 1.xlsx

	Fluorescence	Antibody	100 µL/10^6
Thymus	FITC	MitoTracker/LysoTracker Green	0.1 µM
	PE-Texa Red	propidium iodide	0.1 µg
	PE	CD25	50 ng
	PE/Cy7	CD44	25 ng
	APC/Cy7	CD8	50 ng
	BV785	CD4	25 ng
Spleen	FITC	MitoTracker/LysoTracker Green	0.1 µM
	PE-Texa Red	propidium iodide	0.1 µg
	PE	CD62L	12.5 ng
	PE/Cy7	CD44	25 ng
	APC/Cy7	CD8	50 ng
	BV510	TCRβ	50 ng
	BV785	CD4	25 ng

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10 cm Graefe forceps (straight and serrated)	Dimeda		
11 cm Iris scissors (straight with sharp/sharp hea	Dimeda		
15 mL centrifuge tube	Thermo Fisher Scientific	339650	
5 mL syringe	TERUMO		
6 cm Petri dish	α-plus		
70 µm nylon cell strainer	SPL Lifesciences	93070	
Ammonium chloride (NH_4Cl)	Sigma	A9434	
Anti-mouse CD25	Biolegend	102007	Clone:PC61
Anti-mouse CD4	Biolegend	100453	Clone:GK1.5
Anti-mouse CD44	Biolegend	103029	Clone:IM7
Anti-mouse CD62L	Biolegend	104407	Clone:MEL-14
Anti-mouse CD8	Biolegend	100713	Clone:53-6.7
Anti-mouse TCRβ	Biolegend	109233	Clone:H57-579
BD LSRFortessa	BD Biosciences		
Ethylenediaminetetraacetic acid (EDTA)	Bio basic	6381-92-6	
FALCON 5ml Polystyrene Round-Bottom Tube (FACS tube)	BD Biosciences	352052	
FcRgamma II (CD32) Hybridoma (2.4G2)	ATCC	HB-197	
Fetal Bovine Serum (FBS)	Hyclone	ATD161145	
Flowjo, LLC	BD Biosciences		
Hydroxyethyl piperazineethanesulfonic acid (HEP	Sigma	H4034	
L-glutamine (200 mM)	Gibco	A2916801	
LysoTracker Green DND26	Thermo Fisher Scientific	L7526	
MEM Non-essential amino acids (100X)	Gibco	11140050	
MitoTracker Green FM	Thermo Fisher Scientific	M7514	
Penicillin-Streptomycin (10,000 U/mL)	Gibco	15140122	
Potassium bicarbonate (KHCO3)	J.T.baker	298-14-6	
Propidium iodide solution	Sigma	25535-16-4	
RPMI 1640 medium (powder)	Gibco	31800089	
Sodium bicarbonate (NaHCO3)	Sigma	S5761	

Sodium pyruvate (100 mM)	Gibco	11360070	

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Title of Article: Multicolor flow cytometry-based quantification of mitochondria and lysosomes in T cell

Author(s): C-W. Wei, T-A. Zhou, I. Dzhagalov, C-L. Hsu

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Editorial Team
JoVE
September 4th, 2018

Dear Scientific Editors,

Thank you for the opportunity to revise our manuscript. The constructive advice of the editor and reviewers have substantially improved our paper. Based on the suggestions, we added in two additional sessions to describe sample collection on flow cytometer and data analysis, and carefully reviewed the manuscript for any typos or errors. Attached is our detailed response to their comments. Please note that all changes in the manuscript have been highlighted in yellow color. We believe that we have addressed all of the comments and that our manuscript has been significantly strengthened by these modifications.

We appreciate your time and look forward to your response.

Sincerely,

Responses to the editors:

Thank you for your thorough review of our manuscript. Each comment or concern has been carefully revised and responded. Changes are highlighted in the revised manuscript.

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

We are grateful for this opportunity to proofread the manuscript and have corrected spelling or grammar issues.

2. Please spell out each abbreviation the first time it is used.

Each abbreviation has been spelled out on the first time of usage:
line 80: immunofluorescent (IF),
line 139: fetal bovine serum (FBS)
line 144: ammonium-chloride potassium (ACK) lysing buffer,
line 135: room temperature (RT),
line 195: fluorescence minus one (FMO),
line 201: propidium iodide (PI),
line 214: forward scatter (FSC), side scatter (SSC),
line 278: double negative (DN), double positive (DP), single positive (SP),
line 315: lysosomal-associated membrane protein 1 (LAMP-1).

3. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "MitoTracker" /"LysoTracker" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

We appreciate this reminder and have replaced most of the terms with "Lysosome/ mitochondria-specific dyes" or "organelle-specific probes" and significantly reduced the number of using "MitoTracker/LysoTracker" in the paragraphs.

4. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

The ethics statement has been moved to line 115, in front of the beginning of protocol.

5. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

We have revised the protocol steps accordingly.

6. Please specify all surgical instruments used in each step.

We have added the specifications of surgical instruments in each step and materials.

7. 2.2: What happens after centrifugation, discard the supernatant?

To clarify the step, we have revised the sentence to “Wash the cells with 1 mL FACS buffer and centrifuge (300 x g, for 5 min at 4 °C) and discard the supernatant.”

8. 3.2: Please describe how to analyze the samples on a flow cytometer.

We added additional section 4 “Sample collection on flow cytometry” and section 5 “Data Analysis” in the revised manuscript.

9. Please include single-line spaces between all paragraphs, headings, steps, etc.

Single-line spaces are inserted between all paragraphs, headings, and steps.

10. Figures 2 and 3: Please line up the left and right panels better.

Figure 2 and 3 have been revised to have better presentation.

11. Table 1: Please change “µl” to “µL”, and include a space between all numbers and their units (i.e., 100 µL, 0.1 µM, 0.1 µg, 25 ng, etc.).

All units used in the manuscript has been carefully examined and corrected to µL with a space between numbers and their units.

12. References: Please do not abbreviate journal titles.

We have examined all the references and updated the style not to abbreviate journal titles.

Response to Reviewers' comments:

Reviewer #1:

Major Concerns:

The manuscript is well written with a clear description of the necessity of this protocol. However the expectative indicated in the summary and abstract are not fulfilled in the protocol. The authors introduced the analysis of rare cells or the possibility to quantify mitophagy but none of these challenges were take into consideration in the methodology or in the representative results. Without this, current protocol reduced enthusiasm for the manuscript. Here, the list of the issues:

1-The protocol could not be used to combine both mitochondrial and lysosomal staining. First, because they use the same fluorochrome, the green one, and secondly time of incubation for the mitotracker probe is half of the time that is required for the lysotracker probe. In the introduction it is well mentioned the interesting process of mitophagy. So how can be distinguished or computed those cells with defective or damaged mitochondria content? Current protocol makes impossible its application to measure mitochondrial autophagy for instance.

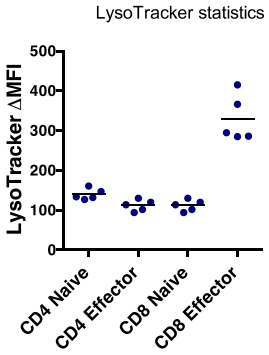
We thank Reviewer 1's for the comments and would like to clarify that we did not and do not recommend to combine mitochondrial and lysosomal staining in the same sample. We will revise the wording in our manuscript to avoid this misunderstanding. Secondly, the proposed protocol here does not intend and cannot be used to distinguish normal to damaged mitochondria. It is simply a method to quantify cellular mitochondrial contents. Further biochemical analyses are required to study mitophagy process or identify damaged mitochondria, but these go beyond the scope of the current protocol.

2- The authors mention that this protocol is useful for mitochondria/lysosome quantification in rare cells. However, they use tissues where lymphocytes are the most abundant cells. How accurate is their protocol in non-lymphoid tissues where lymphocytes are less abundant? What is the minimum number of target cells required to obtain a significant ΔMFI for both mitotracker and lysotracker?

In this manuscript we used lymphoid tissues as an example to show that one can quantify mitochondrial/lysosomal contents in different cell populations of interest. We showed that this method could successfully quantify mitochondrial/lysosomal contents in immune cell sub-populations such as the rare double-negative 1 (0.75% of all cells in the thymus) or the abundant double-positive thymocytes (85%). One can definitely apply this method in non-lymphoid tissues (eg. tumor or tissue biopsy). In principle, as long as the cell population of interest has distinct surface marker expression and is at least 1,000 in number, one should be able to perform the analysis with confidence (line 232-233).

3- How repetitive and significant are the data? How ΔMFI varies between staining? When comparing surface staining between figure 2A and 3A for DN3 populations a noticeable difference is observed that affects the MFI.

We have optimized our protocol so that the results are with good consistency. We showed only representative results in the figures for simplification, but we do have at least 5 technical repeats for each experiment as shown here.



It is not surprising to find MFI differences between Fig. 2A (mitochondrial staining) and 3A (lysosomal staining) since different dyes were applied.

4- Mitochondria are very dynamic organelles that suffer processes such as fusion or fission. It is not clear in the protocol whether authors are measuring the amount of mitochondria as a mass or as a number. Visualization of mitochondrial content by IF or by imaging flow cytometry and comparing it with multicolor flow cytometry will render data more useful.

The protocol provides a method to measure the amount of mitochondria, not number. To quantify the absolute number of mitochondria, one needs to apply methods such as measuring citrate synthase, cardiolipin content, or mitochondrial DNA copy. The MitoTracker signals detected by flow cytometry and the mitochondria contents by microscopy do have a strong correlation as shown in *Buck et al., Cell 2016*.

Minor Concerns:

5- How the metabolism of T cell is affected by euthanizing mice with CO_2 for 5-7 minutes?

It is unclear if euthanizing mice with CO_2 affects the T cell metabolism nor not. Nevertheless, it is a well-recognized IACUC protocol for humane euthanasia and is applied for the majority of the studies using mouse models, including T cell metabolism studies.

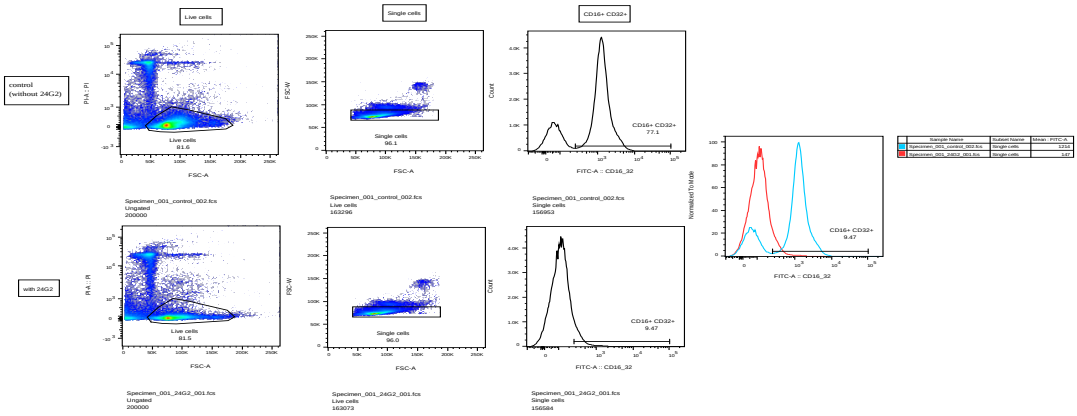
6- The protocol should also include the time lasting from sacrifice to analysis since when performing experiments with several mice this would be a critical step.

The whole procedure takes ~2 hour from tissue harvest to analysis and we added this information to the text (line 367-368). We have not performed detailed analysis to compare the effects of sample preparation time on the readout. It is critical to acquire the samples on the flow cytometer as soon as the staining is completed (noted in protocol step 3.4). Nevertheless, we do recommend to streamline the experiment with sufficient support if large number of mice are involved.

7- Fc receptors are blocked by using 2.4G2 hybridoma instead of purified IgG. Should be hybridoma titrated and used according to its titration?

Yes, the hybridoma supernatant should be titrated before application. We have added this to the text (line 185). We performed the titration and tested its effectiveness by CD16/32 surface staining after 2.4G2 supernatant incubation. The results are shown below. However, the details of how antibody titration are done goes beyond the scope of the current manuscript.

8-



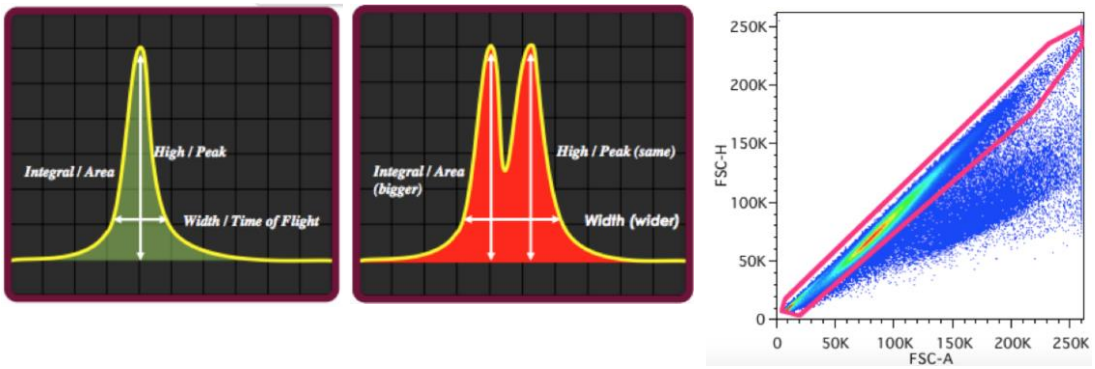
Controls for compensation. In the protocol, single staining for spillover assessments is indicated after mitotracker/lysotracker staining. Single staining must be performed for all dyes, including mitotracker and lysotracker as well as for all the cell surface fluorochrome-conjugated antibodies.

All the experiments performed were with proper compensation setups. We included this information in the revised manuscript as suggested (line 192-196).

9- In Figure 1, a relevant number of cells are discarded because they are not considered as single cells. When working with a cell lineage, FSC-A vs FSC-H linearity could be considered as a parameter for doblet exclusion. But what happens when working with homogenate tissues where many type of cells with different size (FSC parameter) are present? Can you consider only one type of FSC-A vs FSC-H linearity?

The FSC-A vs. FSC-H gating is not assuming a single linearity; it is used to determine the accuracy of the electric pulse. The principle is explained below:

Since the flow cytometer analyses the sample at single cell basis, one can create a gate that eliminates cells that show an increase in pulse area without an increase in pulse height.



10- It is well explained the rationale for CD62L vs CD44 staining to distinguish naïve, memory and effector T cells. What about the rationale for CD44 vs CD25?

The usage of CD44 (Pgp-1) and CD25 (IL-2R) to distinguish different stages of CD4-CD8- (double-negative or DN) T cells is a well-validated system (*Godfrey et al., J.Immunol, 1993*). The earliest stages of T cell development, when the cells do not express the co-receptors CD4 and CD8, can be classified on the basis of CD44 and CD25 expression. The earliest progenitors are CD44+ CD25- (DN1), then they start expressing CD25 and become CD44+CD25+ (DN2) cells. After that the cells down-regulate CD44 and become CD44-CD25+ (DN3) cells and, finally, they down-regulate CD25 as well to become CD44-CD25- (DN4) cells that are on the way of expressing CD4 and CD8 and become double-positive (DP) cells. We added this information to the text (lines 276-286).

11- The assertion that "naïve T cells have the lowest mitochondria mass" (pag 6 line 165) is not accurate, because when compared with memory and effector T cells these two later show less mitochondria content.

In this sentence, we were referring to thymocyte populations. We re-phrased it, so that it is clear that the mitochondrial mass of naïve T cells is lower than that of thymocyte populations (lines 298-301).

12- In Figure 3. How specific and significant is the staining for lysotracker? Did the authors perform a statistical test to conclude differences in the staining? Some of these probes induce shift in the background fluorescent that have not to be taken as a positive staining.

LysoTracker has been considered and tested as a specific marker for lysosomes (*Chen et al., Sci Rep 2015*). For each experiment, we do have at least 3 technical repeats to ensure the validity of results. It is also noteworthy that we presented our results as ΔMFI = mean fluorescence intensity (LysoTracker) - mean fluorescence intensity (surface marker only), therefore we are confident that the shifts in the

staining as low but positive signals. But, perhaps, the best argument for the validity of the staining is that cells with the highest LysoTracker staining are the ones known to have large lysosomal compartments (effector and memory $CD8^+$ T cells).

13- About the lysosomal content in memory and effector CD8 T cells, did the authors perform intracellular staining for other lysosomal markers to corroborate their conclusions on lysotracker staining?

We did not perform such comparisons, since LysoTracker is only applicable to intact cells and cannot be combined with intracellular staining. However, *Shen et al., J Leu Biol, 2006* also detected increase of lysosomal content in memory and effector CD8 T cells using different assays, further corroborates our findings with LysoTracker.

14- The list of dyes in table 1 does not mention the lysotracker.

We have included this information in the table.

15- In the materials list, flow cytometer is a BD LSRfortessa instead of BD LSRfortrees.

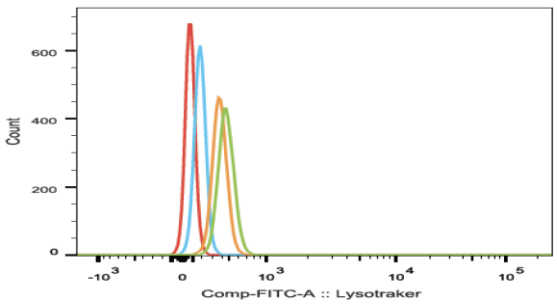
We have corrected this typo.

Reviewer #2:

Minor Concerns:

I would like to suggest careful editing of the text to the authors because I found some typos. It might be helpful if the authors can provide the method to optimize concentration of MitoTracker and LysoTracker, although this is not mandatory.

We appreciate the reviewer's comment and have carefully edited the text. Below we showed the example of LysoTracker titration results:



	Sample Name	Subset Name	Count	Median : Comp-FITC-A
	Specimen_001_S7_ 100 nM_003_008.fcs	CD14	4554	502
	Specimen_001_S7_ 75 nM_002_007.fcs	CD14	4529	423
	Specimen_001_S7_ 50 nM_001_006.fcs	CD14	4785	196
	Specimen_001_S7_ 0 nM_005.fcs	CD14	4643	84.8

We performed the LysoTracker titration using human monocytes ($CD14^+$). It is recommended to choose a cell population that is known to have good expression of staining target (eg. Lysosomes). 100nM was chosen based on two factors: 1) good separation between negative to positive staining; 2) good cell viability. We reflected this in the text (line 170-175).

Reviewer #3:

Minor Concerns:

1. There are several typos that require to be corrected. Line 78 "uncertainly" changes to "uncertainty", Line 79 "immune-blot" changes to "immunoblot" and Line 217 "It's" changes to "It is".

We thank the reviewer's reminder and have corrected these typos.

2. Since LysoTracker and MitoTracker dyes are two key reagents used in this manuscript. It is suggested that the authors need to write a paragraph to introduce how these two dyes are working.

We agree that this information should be included in the manuscript and have added it (line 97-100).

3. In Protocol, the authors may need to explain why MitoTracker or LysoTracker staining should be performed before surface marker staining. What will happen if surface marker staining is performed first?

The MitoTracker or LysoTracker staining was performed first because the optimal staining condition for these organelle-specific dyes is incubation at 37˚C. If the surface staining is performed first and then cells are incubated at 37˚C, the surface marker staining can be significantly reduced because of antibody capping and endocytosis of antibody-bound surface markers. We have added text to indicate this (line 159-161).

4. In Line 108, the authors described the location of spleen (on the left side…). However, It is unclear the position of the spleen is from ventral view or dorsal view. It is suggested that the authors need to specify.

We have edited this description and clarified the position of the spleen (line 126-131).

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Editorial Team
JoVE
September 17th, 2018

Dear Scientific Editors,

Thank you for the comments. We have revised the manuscript to address each comment accordingly. Changes are marked in the revised manuscript via the track function.
We appreciate your time and look forward to your response.

Sincerely,

Responses to the editors (20180913 edits):

Thank you for the comments. We have revised the manuscript to address each comment accordingly. Changes are marked in the revised manuscript via the track function.

1. Please reduce your use of "MitoTracker", "LysoTracker", and "FlowJo" further; generally, we recommend to introduce generic terms for commercial products on first use and use them for the remainder of the manuscript.

We now have removed majority of the terms "MitoTracker", "LysoTracker", "FlowJo" and replaced with generic terms such as "mitochondria-specific dye" or "lysosome-specific dye". (please see line 165, 174, 245, 299, 335-341, 344-350). The few remaining ones are to specify the dyes and concentrations used in the protocol (line 163, 168).

2. You say that your euthanasia method has been approved by your IACUC; has your protocol as a whole been approved as well? Please specify this.

Yes, the whole procedure is approved by IACUC. We have now specified this in the manuscript (line 113).

3. The protocol is currently 3.5 pages long; however, we only have a 2.75 page limit for filming. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted the essential steps of the protocol for the video (line 155-239), which is about 2 pages long.