AMPK signaling in dendritic cells: a metabolic sensor controlling the balance between immunity and tolerance

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AMPK

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Characterization of Dendritic Cell Metabolism by Flow Cytometry

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Abstract

In response to different stimuli dendritic cells (DCs) undergo metabolic reprogramming to support their function. Here we describe how fluorescent dyes and antibody-based approaches can be used to assess various metabolic parameters of DCs including glycolysis, lipid metabolism, mitochondrial activity and the activity of important sensors and regulators of cellular metabolism, mTOR and AMPK. These assays can be performed using standard flow cytometry and will allow for determination of metabolic properties of DC populations at single cell level and to characterize metabolic heterogeneity within them.

1 Introduction

Dendritic cells (DCs) play a key role in orchestration of pro-inflammatory as well as regulatory T cell responses. Classically, DCs undergo an activation program upon sensing of pathogen- or host-derived signals that endow them with the capacity to prime and polarize T cell responses. In recent years, it has become clear that DC activation is accompanied with, and supported by reprogramming of their cellular metabolism, and that their ability to drive different T cell responses is underpinned by distinct metabolic pathways (1, 2). Hence metabolic characterization of DCs provides valuable information about their biology and function in the context of different immune responses. Metabolomics and flux analysis are commonly used methods to assess metabolic properties of immune cells. These techniques generally require high cell numbers as input, which makes their use for metabolic profiling of scarce cell populations, such as DCs, problematic. Moreover, in such approaches information about metabolic heterogeneity within cell populations is lost. This protocol describes how metabolic properties, specifically those related to nutrient uptake, mitochondrial function and activity of key regulators and sensors of cellular metabolism, mTOR and AMPK, can be assessed at single cell level in primary and *in vitro*-cultured DCs using commercially available antibodies, fluorescent dyes and standard flow cytometry.

One of the central regulators of cellular metabolism is AMP-activated protein kinase (AMPK), which is activated in nutrient-poor conditions (1). AMPK controls activity of various metabolic pathways to inhibit anabolism and to promote catabolism. Another crucial metabolic kinase is mammalian target of rapamycin (mTOR), which opposes the functions of AMPK, as it is active in nutrient-rich conditions and drives metabolic processes associated with biosynthesis (1). A commonly used method to study activity of kinases involves assessing phosphorylation status of downstream targets. Here, we provide a flow cytometry protocol for the staining of phosphorylated proteins acetyl-CoA carboxylase (ACC) and S6, as markers for AMPK and mTOR activity, respectively.

Two major nutrient sources for cells are glucose and fatty acids. A shift towards glucose metabolism is commonly observed upon immunogenic DC activation (3), while rewiring of lipid metabolism is associated with tolerogenic DC function (4). The fluorescently labelled glucose-analog 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol4-yl)Amino)-2-Deoxyglucose (2-NBDG) can be used to measure glucose uptake in live cells, while BODIPY™ lipid probes allow for characterization of fatty acid uptake and intracellular lipid accumulation (5, 6). We describe a flow-cytometry based protocol that is applicable for, but not restricted to, staining with 2-NBDG, BODIPY4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Hexadecanoic Acid (BODIPY™ FL C_{16}) and 4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene (BODIPY™ 493/505) to characterize glucose uptake, fatty acid uptake and lipid accumulation, respectively, in live DCs.

Mitochondria serve as central metabolic hubs in cells, including DCs, through citric acid cycle activity, oxidative phosphorylation for ATP production and reactive oxygen species (ROS) production (2). Several fluorescent dyes allow for easy analysis of various mitochondrial parameters. Mitochondrial mass, a measure of overall mitochondrial content in a cell, can be determined through the use of various MitoTracker dyes, which are commercially available in different fluorescence spectra (7). Mitochondrial membrane potential ($\Delta\Psi_m$), the driving force of mitochondrial ATP production and as such an important measure of cellular metabolism, can be stained for with tetramethyl rhodamine methyl (TMRM) (8, 9). Mitochondrial ROS

(mtROS), which are by-products of oxidative phosphorylation, and total cellular ROS production, can be analyzed through the dye MitoSOX™ Red, which fluoresces proportional to mtROS production, and CM-H_2DCFDA, respectively (9-11).

Procedural steps for aforementioned stainings are described below to provide a framework for metabolic characterization of *in vitro*-cultured and primary human and murine DCs by standard flow cytometry as well as options to combine multiple dyes using advanced spectral flow cytometry.

2 Materials

2.1 Cellular systems

This protocol provides detailed information for measurements in primary murine splenic DCs, human blood-derived DCs and *in vitro* cultured DC populations, including murine bone-marrow derived DCs (BMDCs) and human monocyte-derived DCs (moDCs), but may also be applicable to other cell types.

2.2 Flow cytometry reagents and equipment

1. Phosphate buffered saline (PBS).
2. FACS buffer: 1% bovine serum albumin (BSA) in PBS.
3. Lineage-defining antibodies (Table 3-6).
4. αCD16/αCD32 (Fc-Block).
5. Viability dye (see **Note 1**).
6. 96-well V-bottom plate.
7. FACS tubes.
8. Flow cytometer equipped with lasers and emission filters suitable for the analysis of cells stained with the antibodies and dyes listed in the antibody panel (Table 3-7).

2.3 Intracellular staining for metabolic phosphorylated proteins

1. 16% formaldehyde, methanol free, Ultra Pure.
2. Formaldehyde, stabilized with methanol.
3. 100% methanol, stored at -20 °C.
4. 10x eBioscience™ Permeabilization Buffer (Perm buffer).
5. Conjugated antibody or primary and secondary antibodies (Table 2).
6. Complete RPMI: RPMI 1640 Medium GlutaMAX, 10 % fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol.
7. 24-well cell culture plate.
8. 96-well flat-bottom plate.
9. 1 mL syringe.
10. 5 ml FACS tubes.

2.4 Metabolic dyes

1. Metabolic dyes (Table 7).

3 Methods

Throughout the protocol, centrifugation is set at 300 x *g* for 4 minutes at 4°C, unless stated otherwise.

3.1 Intracellular staining of phosphorylated metabolic proteins

This protocol describes in detail the staining procedure of phosphorylated ACC and S6, as a direct reflection of AMPK and mTOR activity, respectively. This protocol can be applied to other intracellular and/or phosphorylated proteins as well (*see* **Note 2**).

In brief, the general procedure for intracellular staining for phosphorylated metabolic proteins consists of the following steps:

1. Fixation of cells
    - Phosphorylation is generally an unstable post-translational modification and therefore it is recommended to fix cells prior to any processing (*see* **Note 3**). Live and dead cells can be distinguished by strict gating in the FSC-A vs SSC-A plot (Figure 1A).
    - The fixation protocol is divided in 3 parts: (1) procedure for direct fixation of DCs in in tissues prior to their isolation; (2) fixation procedure of tissue- and blood-derived DCs after their isolation from tissues; (3) fixation procedure for *in vitro*-cultured DCs.
2. Permeabilization of cells.
3. Staining with primary antibody (see Note 4).
4. Staining with secondary antibody.
5. Cell acquisition on flow cytometer.

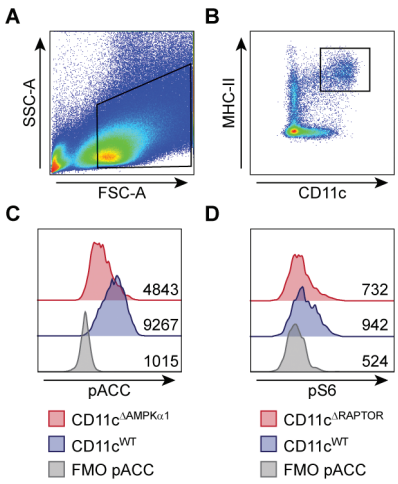

Figure 1: pACC and pS6 staining in murine tissue-derived DCs. (A) Strict gating of the FSC-A is required to remove death cells in a sample without viability staining. **(B)** pACC and pS6 data is shown from MHC-II^{+}CD11c^{+} splenic DCs. The signal of **(C)** pACC and **(D)** pS6 are lost in CD11c$^{\Delta AMPK\alpha1}$ and CD11c$^{\Delta raptor}$ mice, respectively.

3.1.1 Fixation

3.1.1.1 Fixation prior to tissue processing

1. Add 500 µL of 2% formaldehyde/PBS to a 24-wells plate, 1 well per tissue.
2. transfer tissue of interest to 2% formaldehyde/PBS (*see* **Note 5**).
3. Immediately smash the tissue with the back of a 1 mL syringe.
4. Fix the cells for 30 minutes at room temperature (RT).
5. Add 500 µL of RPMI (no additives) to the wells to lower the concentration of the fixative. Continue with the next step if all tissues have been in fixative for at least 30 minutes.
6. Centrifuge the 24-wells plate.
7. Transfer as much supernatant as possible, without transferring pieces of tissue, to a sterile 5 mL FACS tube.
8. Add 1 mL of RPMI (no additives) to the wells of the 24-wells plate and spin down the plate.
9. Repeat step 7-8.
10. After the second centrifugation of the plate, remove supernatant from the well as in step 7 and spin down the 5 ml FACS tubes. In the meantime, add 350 µL of RPMI (no additives) to the wells
11. After centrifugation, remove the supernatant from the tubes by decantation.
12. Resuspend the pellet (may not be visible) in the remaining +/- 150 µL of medium and transfer this solution back to the 24 well plate containing 350 µL of medium.
13. The wells containing the tissues should be now in a total of 500 µL of medium.
14. Process the tissues using your standard protocol to generate single cell suspensions (*see* **Note 6**).
15. After generation of single cell suspensions, store cells in 200 µL FACS buffer at 4 °C for a maximum of 1 week. Continue with step 3.1.2.

3.1.1.2 Fixation of single cell suspensions after tissue or blood processing

1. Process tissues or blood according to your standard protocol to obtain single cell suspensions or PBMCs, respectively.
2. Plate the desired number of isolated cells (Table 2) in a 96-wells V-bottom plate.
3. Fill the wells to 200 µL with PBS.
4. Centrifuge the plate and remove supernatant by flicking.
5. Repeat step 3-4.
6. Stain cells with a viability stain of your choice (*see* **Note 7**).
7. Repeat step 3-4.
8. Add 200 µL of complete medium.
9. Incubate the cells for 1-2 hours at 37 °C (*see* **Note 8**).
10. Take the plate out of the incubator and immediately add 65 µL of 16% ultra-pure methanol free formaldehyde, to reach a final concentration of 4% (*see* **Note 9** and **Note 10**).
11. Fix cells for 15 minutes at RT (*see* **Note 11**).
12. Repeat step 3-4 twice, but use FACS buffer instead of PBS.
13. Store cells in 200 µL FACS buffer at 4 °C for a maximum of 1 week. Continue with step 3.1.2.

3.1.1.3 Fixation of in vitro-cultured DCs

1. Plate the desired number of cells (Table 2) in complete RPMI in a 96-well flat bottom plate. Include wells for the controls: a staining with the secondary antibody only and an unstained sample.
2. Fill the wells to 200 µL with complete RPMI and stimulate cells with your compound of interest.
3. Take the plate out of the incubator and immediately add 65 µL of 16% ultra-pure methanol free formaldehyde, to reach a final concentration of 4% and resuspend.
4. Fix cells for 15 minutes at RT.
5. Put the plate on ice and carefully loosen the adherent cells by scraping with 200 µL pipet tips.
6. Transfer cells to a V-bottom plate.
7. Centrifuge the plate.
8. Discard supernatant by flicking the plate.
9. Add 200 µL FACS buffer.
10. Repeat steps 7-9 twice.
11. Store plate at 4 °C for a maximum of 1 week. Continue with step 3.1.2.

3.1.2 Staining procedure

1. Centrifuge the plate and remove supernatant by flicking.
2. Add 200 µL of 1x eBioscience permeabilization buffer to each well.
3. Centrifuge the plate and remove supernatant by flicking.
4. Repeat steps 2-3.
5. Resuspend cells in 100 µL of 100% methanol for further permeabilization (*see* **Note 12**).
6. Incubate for 20 minutes at -20 °C.
7. Repeat steps 2-3 twice.
8. Resuspend cells in 20 µL of 1x eBioscience permeabilization buffer containing the primary antibody and Fc-Block (αCD16/αCD32) (*see* Table 1).
9. Incubate for 30 minutes at 4 °C.
10. Repeat steps 2-3.
11. Resuspend cells in 30 µL of 1x eBioscience permeabilization buffer containing the secondary antibody and the desired antibody cocktail mix (*see* **Note 13** and Table 3-6).
12. Incubate for 30 minutes at 4 °C.
13. Fill wells up to 200 µL with FACS buffer.
14. Centrifuge the plate and remove supernatant by flicking.
15. Resuspend cells in the appropriate volume of FACS buffer to be acquired in a flow cytometer. A representative example of pACC and pS6 staining is shown in Figure 1.

3.2 Glucose and Lipid Metabolism

Various fluorescent dyes are available to evaluate nutrient uptake and accumulation using flow cytometry. Here, we provide a protocol for assessing glucose uptake (2-NBDG), cellular neutral lipid droplet content (BODIPY™ 493/505) and uptake of long-chain fatty acids (BODIPY™ FL C_{16}).

Table 1: Antibodies used for staining of intracellular (phosphorylated) proteins.

Target	Source	Identifier	Control	Reference for control
Phospho-Acetyl-CoA Carboxylase (Ser79)	Cell Signaling	11818	Metformin	(19)
Phospho-S6 (Ser240) - PE	BD Biosciences	560430	LPS	(20)
Goat anti-Rabbit AF647	ThermoFisher	A-21244	-	-

3.2.1 Staining procedure

1. Plate the desired number of live cells (Table 2) in complete RPMI in 96-wells V bottom plate. Include wells for the controls treated with 2-DG (in case of 2-NBDG, *see* **Note 14**) or incubated in PBS containing 10% FCS (in case of BODIPY™ FL C_{16} and BODIPY™ 493/505, *see* **Note15**)
2. Fill wells with PBS to up to 200 µL.
3. Spin down and remove supernatant.
4. Repeat steps 2-3.
5. Stain cells with the viability dye of your choice and Fc-Block (αCD16/αCD32) in PBS.
6. Incubate cells for 15 minutes at RT.
7. Repeat steps 2-3.
8. Incubate cells with either 50 µM of 2-NBDG, 5 µM of BODIPY™ 493/505 dye or 10 µM BODIPY™ FL C_{16} (diluted in PBS) and incubate cells for 15 minutes at 37 °C in CO_2 incubator. (*see* **Note 16, Note 17, Note 18** and **Note 19**)
9. Add FACS buffer to wells to reach 200 µL.
10. Centrifuge the plate and remove supernatant.
11. Resuspend cells in 30 µL of FACS buffer containing antibody cocktail mix (*see* Table 3-6).
12. Incubate for 30 minutes at 4 °C.
13. Repeat steps 9-10.
14. Resuspend cells in the appropriate volume of FACS buffer to be acquired in a flow cytometer. See Table 7 for information on excitation and emission wavelengths for each dye. A representative example of 2-NBDG and BODIPY™ FL C_{16} staining in DCs is shown in Figure 2.

3.3 Mitochondrial Metabolism

Mitotracker and TMRM are dyes commonly used to measure mitochondrial mass and mitochondrial membrane potential, respectively (*see* **Note 20)**. Due to the positive charge of these probes, they can enter live, polarized mitochondria, but not depolarized mitochondria. While TMRM is a dye available only with a specific emission wavelength, different Mitotrackers are commercially available with fluorescence in different wavelengths (i.e. Mitotracker Green FM and Mitotracker Deep Red FM). The following protocol is optimized for Mitotracker Green FM, but can also be applied to the other available Mitotracker dyes (*see* **Note 21**).

3.3.1 Staining procedure

1. Plate the desired number of cells (Table 2) in complete RPMI in a 96-wells V bottom plate. Include one well for FCCP-treated cells as control (*see* **Note 22**).

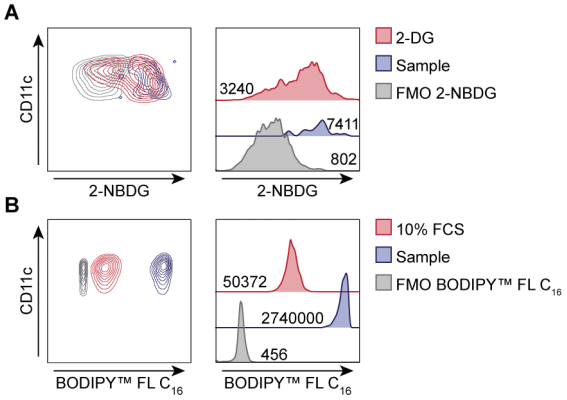

Figure 2: 2-NBDG and BODIPY™ FL C_{16} staining in murine tissue-derived MHCII$^+$CD11c$^+$ DCs. (A) 2-DG functions as a negative control for 2-NBDG staining. **(B)** 10% FCS can be used as a negative control for BODIPY™ FL C_{16} staining.

2. Fill wells up to 200 µL with complete RPMI.
3. Centrifuge the plate and remove supernatant.
4. Repeat steps 2-3.
5. Stain cells with the viability dye of your choice and Fc-Block (αCD16/αCD32) in PBS.
6. Incubate cells for 15 minutes at RT.
7. Repeat steps 2-3.
8. Incubate cells with 10 nM of TMRM and 20 nM of Mitrotracker Green FM for 15 minutes at 37 °C in complete RPMI.
9. Repeat steps 2-3.
10. Incubate control well with 50 µM of FCCP for 15 minutes at 37 °C in complete RPMI.
11. Centrifuge the plate and remove supernatant.
12. Fill wells up to 200 µL with FACS buffer.
13. Centrifuge the plate and remove supernatant.
14. Resuspend cells in 30 µL of FACS buffer containing antibody cocktail mix (*see* Table 3-6).
15. Incubate for 30 minutes at 4 °C.
16. Repeat steps 11-12.
17. Resuspend cells in the appropriate volume of FACS buffer to be acquired in a flow cytometer. See Table 7 for information on excitation and emission wavelengths for each dye. A representative example of Mitotracker Green FM and TMRM staining in DCs is shown in Figure 3.

3.4 ROS Staining

A variety of commercial kits and dyes are available to detect intracellular production of ROS via flow cytometry. Here we describe a staining procedure for CM-H_2DCFDA and MitoSOX™ Red for DCs, that detect total intracellular ROS and mitochondria-derived superoxide, respectively (*see* **Note 23**).

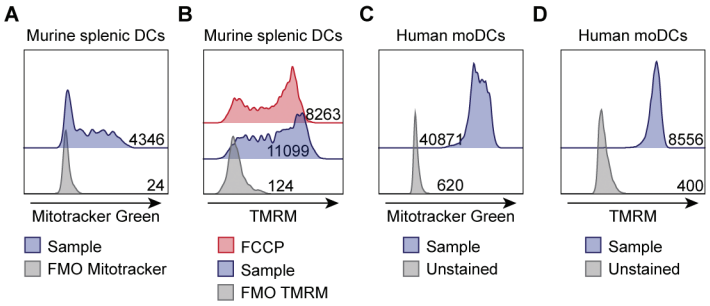

Figure 3: Mitotracker Green FM and TMRM staining. (A+B) Mitotracker Green FM and TMRM staining in murine tissue-derived DCs gives a heterogenous staining. Signal can be quantified using MFI or the frequency of positive cells. **(C+D)** Staining of moDCs, a homogenous population, with the same dyes, gives a single population. In these cells signal can be quantified using MFI.

3.4.1 Staining procedure

1. Plate the desired number of cells (Table 2) in complete RPMI in 96-wells V bottom plate. If desired plate 2 additional wells for positive (H_2O_2) and negative (N-acetyl cysteine (NAC)) control.
2. Fill wells up to 200 µL with complete RPMI.
3. Spin down and remove supernatant.
4. Repeat steps 2-3.
5. Incubate controls well with 0.03 % H_2O_2 or 1 µM of NAC for 1 hour at 37 °C in complete RPMI (*see* **Note 24**).
6. Repeat steps 2-3.
7. Stain cells with the viability dye of your choice and Fc-Block (αCD16/αCD32) in PBS.
8. Incubate cells for 15 minutes at RT.
9. Repeat steps 2-3.
10. Incubate cells with 5 µM of MitoSOX™ Red or 1 µM of CM-H_2DCFDA for 15 minutes at 37 °C in complete RPMI.
11. Fill wells up to 200 µL with FACS buffer.
12. Centrifuge the plate and remove supernatant.
13. Resuspend cells in 30 µL of FACS buffer containing antibody cocktail mix (*see* Table 3-6).
14. Incubate the cells for 30 minutes at 4 °C.
15. Repeat steps 11-12.
16. Resuspend the cells in the appropriate volume of FACS buffer to be acquired in a flow cytometer. See Table 7 for information on excitation and emission wavelengths for each dye. A representative example of MitoSOX™ Red and CM-H_2DCFDA staining in DCs is shown in Figure 4.

3.5 Metabolic panel tested on Cytek Spectral flow cytometer

Metabolic dyes can be measured with standard flow cytometers, but spectral overlap between a number of these dyes makes it difficult to design a panel in which multiple dyes are combined. However, with the Five-laser Cytek Aurora, a spectral flow cytometer, one can combine up to 4 dyes in one panel, namely 2-NBDG, BODIPY™ FL C_{16}, TMRM and Mitotracker Green FM.

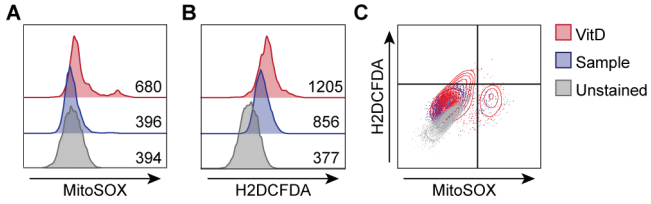

Figure 4: MitoSOX and H2DCFDA staining in *in vitro*-cultured human moDCs. Vitamin D (VitD) was used as positive control (18). **(A)** MtROS and **(B)** total ROS production are increased in VitD-treated DCs. **(C)** MtROS and total ROS can stained together and distinguished from each other. In these cells signal can be quantified using MFI.

Therefore, spectral flow cytometry allows for metabolic profiling using multiple metabolic dyes in parallel when the number of cells is limited, for example in the case of murine tissue-derived DCs (Figure 5).

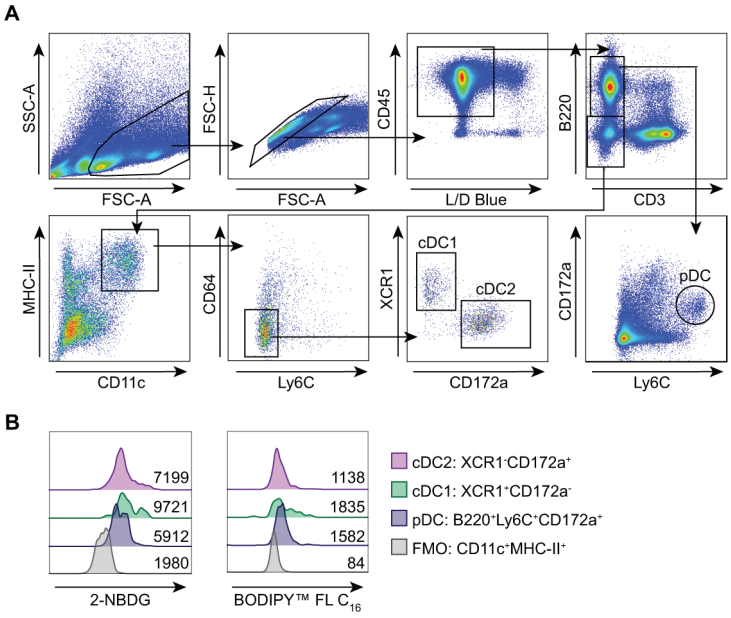

Figure 5: Mitochondrial dye staining using spectral flow cytometry. **(A)** Gating strategy to identify DC subsets in murine splenocytes. **(B)** cDC1s and cDC2s show differences in uptake of 2-NBDG and BODIPY™ FL C_{16}.

Table 2: Recommended number of cells to ensure a proper staining for identification of phosphorylated proteins and metabolic dyes in DCs.

Source of cells	Cells concentration	Minimum number of cells
Pure DC population (from *in vitro* DC cultures)	$1x10^6$/mL	50000
Mixed population (from tissues/PBMCs)	$10x10^6$/mL	1000000

Table 3: Lineage-defining antibodies required for identification of primary murine DC subsets.

Marker	Target population	Clone
CD3	T cells	17A2
B220	B cells/pDCs	RA3-6B2
Ly6C	Monocytes/pDCs	HK1.4
CD64	Macrophages	X54-5/7.1
CD11c	Monocytes/DCs	N418
MHC-II	Monocytes/DCs	M5/114.15.2
XCR1	cDC1	ZET
CD11b	cDC2	M1/70
CD172a	cDC2/pDCs	P84
Siglec-H	pDCs	551
CD16/32	FC-receptors	93

Table 4: Lineage-defining antibodies required for identification of primary human DC subsets.

Marker	Target population	Clone
CD3	T Cells	UCHT1
CD19	B Cells	HIB19
CD56	NK Cells	NCAM16.2
CD11c	Monocytes/DCs	Bu15
HLA-DR	Monocytes/DCs	L243
CD16	Monocytes	3G8
CD14	Monocytes	MΦP9
CD1c	cDC2	L161
CD141	cDC1	AD5-14H12
CD123	pDC	9F5
CD16/32	FC-receptors	polyclonal

Table 5: Lineage-defining antibodies for identification of human *in vitro*-cultured DC populations.

Marker	Clone
CD11c	h: Bu15
HLA-DR	h: L243/
CD14	MφP9
CD1a	HI149

Table 6: Lineage-defining antibodies for identification of mouse *in vitro*-cultured DC populations.

Marker	Clone	Differentiation Protocol
CD11c	N418	GM-CSF/FLT3L
MHC II	M5/114.15.2	GM-CSF/FTL3L
CD11b	M1/70	GM-CSF/FLT3L
CD115	AFS98	GMCSF
CD135	A2F10	GMCSF
CD24	M1/69	FLT3L
CD172a	P84	FTL3L
Siglec-H	551	FTL3L

Table 7: Metabolic dyes.

Dye	Source	Identifier	Concentration	Excitation	Emission
MitoTracker™ Deep Red FM	ThermoFisher	M22426	10 nM	540	595
MitoTracker™ Green FM	ThermoFisher	M7514	20 nM	490	516
TMRM	ThermoFisher	T668	20 nM	548	574
2-NBDG	ThermoFisher	N13195	50 µM	465	540
BODIPYTM FL C16	ThermoFisher	D3821	200 nM	488	508
BODIPYTM 493/503	ThermoFisher	D3922	5 µM	493	503
HCS LipidTOX™ Deep Red	ThermoFisher	H34477	1:200	644	665
MitoSOX™ Red	ThermoFisher	M36008	5 µM	510	580
H2DCFDA (H2-DCF, DCF)	ThermoFisher	D399	1 µM	492-495	517-527

4 Notes

1. Cells can be stained with the viability dye of your choice. We successfully tested LIVE/DEAD™ Fixable Aqua (1:400 dilution when performing experiments on a BD FACSCanto II, BD FACS LSR or BD FACS Fortessa X20 flow cytometer and 1:1000 dilution when performing experiments on a Cytek Aurora Spectral flow cytometer) and LIVE/DEAD™ Fixable Blue and Zombie NIR™ Fixable (both 1:1000 on a Cytek Aurora Spectral flow cytometer).
2. Not all unconjugated antibodies are suitable for flow cytometry. Make sure to have a proper positive and negative control to test if the staining is reliable.
3. To maintain the *in situ* phosphorylation status of the protein of interest it is recommended to fix tissues directly after harvesting. In our hands, this protocol works well for murine spleen, lymph nodes and tumors, but not for lamina propria of the murine small intestine. Furthermore, if a tissue is also used for experiments that require live cells, it is recommended to fix only part of the tissue.
4. If the antibody of interest is already conjugated to a fluorochrome (e.g. pS6-PE (Table 1)), this step can be skipped and the antibody can be included in the antibody cocktail together with lineage-defining antibodies.
5. This protocol is optimized for murine lymphoid tissues such as spleen and lymph nodes. In case of tissues other than spleen or lymph nodes, one can use the standard protocol implemented in your laboratory, replacing the solution normally used to collect the tissue of interest with 2% formaldehyde/PBS. Wash the fixative away with medium or PBS before digesting the tissue.
6. Fixing the samples prior to tissue processing might result in less efficient digestion of the tissues and, as a consequence, reduced number of cells. Digestion of spleen and lymph nodes using DNase I and Collagenase D is still effective when performed on fixed samples.
7. When processing tissue samples before fixing the cells, we recommend to stain the cells with a viability dye before resting the cells in the incubator. Not only the mechanic procedure to obtain the cells can lead to cell death, but the heterogeneity of cells makes it difficult to distinguish live and dead cells by size and complexity. On the other hand, *in vitro*-cultured DCs comprise a more homogenous population when analyzing the samples by size and complexity and the removal of dead cells based on these parameters can be performed easier. Additionally, the viability of *in vitro*-cultured DCs is normally high. However, if working with additional treatments that affect viability DCs, we recommend to include a viability dye staining.
8. The processing procedure to obtain a single cell suspension from tissues can interfere with the *in situ* phosphorylation status of the protein of interest. Therefore, we recommend to leave cells following their isolation for 1-2 hours in an CO_2 incubator. This resting of the cells will help restore phosphorylation status more comparable to their original *in situ* profile.
9. In our experience, adding 22 µL of the standard 37% formaldehyde, which contains methanol as a stabilizer, is also an option. However, this option provides a higher background signal and our advice is to use 16% ultra-pure formaldehyde.
10. 4% ultra-pure formaldehyde can damage epitopes in tissues. Test your panel to see whether binding of your antibodies of interest are affected and perform a titration from

1-4% of ultra-pure formaldehyde if needed to improve staining signal. In our experience XCR1 binding is highly affected by fixation. Staining cells with XCR1 after the fixation might lead to the appearance of false CD172a^{+}XCR1^{+} DC population. To prevent this, stain cells with XCR1 before fixation, for example, together with the viability dye.

11. Fixation can also be performed at 37 °C without affecting the phosphorylation signal. However, in our experience, the recovery of cells is higher when fixation was performed at RT.
12. 100% methanol should be stored at -20 °C and taken out of the freezer right before addition to the cells. Be aware that methanol can damage some epitopes, thereby interfering with cell staining. We observed that CD11c is sensitive to methanol permeabilization, leading to signal loss and making it challenging to identify DCs in tissues. To prevent that, we recommend to stain CD11c prior to methanol permeabilization, for example together with the viability dye. Carefully select your CD11c fluorochrome because some of them are methanol sensitive and cannot be used prior to methanol permeabilization. In our experience, CD11c-BV421 signal is still retained after methanol permeabilization.
13. For staining of *in vitro*-cultured human moDCs, compounds like Vitamin D, retinoic acid or dexamethasone can markedly decrease CD1a expression. In that case, we recommend to stain for only CD11c and HLA-DR. In case of bone marrow-derived DCs differentiated in the presence of GM-CSF, be aware that this is a mixed population of DCs and macrophage-like cells (12) and additional markers might be required depending on your research question.
14. Even though 2-NBDG has been commonly used as a readout for glucose uptake, its use for this purpose was recently questioned (13). Interpret your results carefully and make sure to always include appropriate controls. The following molecules can be used as controls for 2-NBDG staining: (1) 2-DG acts as a substrate for hexokinase to block its activity and directly competes with 2-NBDG; (2) 4,6-O-ethylidene-α-D-glucose (4,6-O) is a glucose analogue that binds to the external site of glucose transporters (GLUTs) but is not transported into cells; (3) cytochalasin B (CytB) diffuses through the cell membrane and binds to the internal site of GLUTs, also preventing entry of glucose into cells (14).
15. Staining cells in PBS 10% FCS serves as a negative control for the uptake of BODIPY™ FL C_{16} and BODIPY™ 493/505. The lipids present in FCS are targets for BODIPY™ 493/505, reducing the availability of the dye for binding to cellular lipid structures. Additionally, lipids present in FCS will directly compete with BODIPY™ FL C_{16} for uptake by cells, thereby reducing BODIPY™ FL C_{16} uptake.
16. As an alternative to BODIPY™ 493/503, HSC LipidTOX is a commercially available dye that stains neutral lipids. This neutral lipid staining can be performed on fixed and permeabilized as well as unpermeabilized cells, which makes this dye an interesting option if cells need to be fixed. According to the manufacturer's instruction, HSC LipidTOX should be diluted 200x and stained in PBS.
17. ThermoFisher provides BODIPY™ lipid probes for multiple purposes, including the staining of membrane lipids, uptake of fatty acids of various lengths and nonpolar probes for neutral lipid staining with different fluorescence. This protocol may be applicable to other BODIPY™ lipid probes as well, but carefully read the manufacturer's instructions.
18. BODIPY™ 493/505 and BODIPY™ FL C_{16} are hydrophobic dyes that do not disperse

readily into aqueous solutions. To ensure proper dissolving of the dye, vigorously vortex them before preparing the staining mix.

19. The metabolic dyes can be stained together with the viability dye. In that case, make sure to stain your cells at 37 °C instead of RT. The viability dyes can resist higher temperatures while most of the metabolic dyes allow less flexibility in this respect and require a specific temperature for optimal staining.
20. Mitotrackers and TMRM are both lipophilic cationic dyes that stain live, polarized mitochondria. However, differently from TMRM, Mitotrackers bind to intramitochondrial protein thiols and remain bound, even after depolarization. Because of these differences, TMRM and Mitotracker are commonly used to measure mitochondrial membrane potential and mitochondrial mass, respectively.
21. Besides Mitotracker Green FM and Mitotracker Deep Red FM, there are also other Mitotrackers options, including Mitotracker Red FM and Mitotracker Red CMXRos. However, these Mitotrackers, in contrast to Mitotracker Green FM and Mitotracker Deep Red FM, accumulate in mitochondria in a membrane potential-dependent manner (in a similar way as TMRM). If one is interested in mitochondrial mass, the preferred choice would be Mitotracker Green FM or Mitotracker Deep Red FM. One important difference between these two dyes is the fact that Mitotracker Deep Red FM is a dye that is well-retained in the mitochondria after fixation of the cells, while Mitotracker Green FM is not.
22. Even though Mitotracker Green FM and Mitotracker Deep Red FM are considered mitochondrial mass dyes, both of their signals seem to be at least partially affected by mitochondrial membrane potential (15). Mitotracker Deep Red FM seems to be more affected than Mitotracker Green FM and the combination of both dyes has been used to identify dysfunctional mitochondria (16, 17). It is important to have an appropriate negative mitochondrial membrane potential control, such as ionophore FCCP, that depolarizes the mitochondrial membrane, to ensure that your Mitotracker staining is not being affected by membrane potential and, also, to properly demonstrate that the TMRM staining is affected by the membrane depolarization.
23. CM-H_2DCFDA (and its derivatives) are non-specific ROS dyes that report general ROS production but cannot be used with fixed cells. If one needs to study ROS production in fixed or permeabilized cells, CellROX™ Green can be used. For more specific ROS detection, one can use dihydroethidium (DHE) (which detects intracellular levels of superoxide) or DAF-FM Diacetate (which detects intracellular levels of NO).
24. Incubation time for the positive control well can be reduced by increasing the percentage of H_2O_2. Be aware that H_2O_2 can be toxic if cells are exposed to high concentrations for a longer period of time.

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