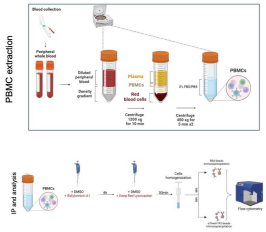
May 10, 2024

Flowcytometry analysis of lysosomal pulldown with anti-TMEM192 magnetic beads from PBMCs



DOI
dx.doi.org/10.17504/protocols.io.n2bvj378xlk5/v1

Enrico Bagnoli[1], Karolina Zeneviciute[1], Esther Sammler[1], Dario R Alessi[1]

[1]Medical Research Council Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK

ASAP Collaborative Rese… | Enrico Protocols

Enrico Bagnoli
MRC PPU

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.n2bvj378xlk5/v1

Protocol Citation: Enrico Bagnoli, Karolina Zeneviciute, Esther Sammler, Dario R Alessi 2024. Flowcytometry analysis of lysosomal pulldown with anti-TMEM192 magnetic beads from PBMCs. **protocols.io https://dx.doi.org/10.17504/protocols.io.n2bvj378xlk5/v1**

Protocol status: Working
We use this protocol and it's working

Created: February 13, 2024

Last Modified: May 10, 2024

Protocol Integer ID: 95163

Keywords: Organelle-IP, Lyso-IP, Flowcytometry, PBMCs, TMEM192, Immunoprecipitation

Funders Acknowledgement:
Aligning Science Across Parkinson's
Grant ID: ASAP-000463

Abstract

We describe a method that allows the staining of intact lysosomes using lysotracker for flow cytometry analysis. The analysis can provide a quantitative indication of the level of enrichment of the organelle immunoprecipitation (% of lysososomal+ beads) and can be used as a quality control check for the purity of the immunoprecipitated lysoIPs. Since only a small fraction of the immunoprecipitated material is needed for flow cytometry, this technique can be coupled with other assays, like western blotting or proteomics. While this protocol is focused on the staining and detection of lysosomes, it can be easily modified to analyse different organelles by changing the staining procedure and adjusting the parameters of the cytometer.

Intro

1 The following protocol allows the detection and analysis of the lysosomes isolated by immunoprecipitation with anti-TMEM192 magnetic beads from peripheral blood mononuclear cells (PBMCs). Cells were incubated with or without the lysotracker prior to the lysosomal immunoprecipitation. Additionally, PBMCs were treated with the V-ATPase inhibitor Bafilomycin A1, that blocks the acidification of the lysosomes and drastically reduces the signal. We included this step as a further proof of the functionality of the isolated lysosomes, but it can be avoided if unnecessary.
This protocol can be adapted to any other cell type.

Note

We noticed that TMEM192 beads have a strong autofluorescence signal, particularly in the FITC and mCherry channels. Using a higher excitation wavelength solved this issue. Specifically, we used the Deep Red Lysotracker (max ex 647, max em 668) and used the APC channel for flow cytometry analysis.

Materials and reagents

2

- PBMCs (or other cells)
- Bafilomycin A1 (Enzo LifeScience, BML-CM110-0100)
- Deep Red Lysotracker (Thermo, L12492)
- DMSO
- anti-TMEM192 magnetic beads
- BSA magnetic beads
- KPBS
- Flow cytometry tubes
- Flow cytometer with APC lasers and filters
- Water bath

PBMCs extraction

3

- Extract PBMCs as outlined in the protocol https://www.protocols.io/view/purification-of-tag-less-lysosomes-from-cells-isol-b5ziq74e (steps1-11)

- Divide PBMCs into different tubes, according to the experimental design.

Note

We divided cells from each donor into 4 tubes.
Tube 1: - Baf, - Tracker
Tube 2: - Baf, + Tracker
Tube 3: + Baf, - Tracker
Tube 4: + Baf, + Tracker

- Leave PBMCs in water bath at 37 °C

Bafilomycin A1 treatment

4

4h

- Prepare a [M] 100 micromolar (µM) working stock of Bafilomycin A1 in DMSO.
- Add Bafilomycin to tube 3 and 4 to a final concentration of [M] 200 nanomolar (nM)
- Add the same volume of DMSO to tube 1 and 2
- Incubate at 37 °C for 04:00:00 in the waterbath, gently mixing cells every 20min to avoid cell attachment

Note

Duration and concentration of the Bafilomycin A1 treatment can be tailored according to the specific requirements

Lysotracker staining

5

30m

- Prepare [M] 1 millimolar (mM) stock of Lysotracker in DMSO, if not already prepared
- Make 2 µM solution (2 µL l of Stock in 1000 µL of buffer)
- Add dye to a final concentration of [M] 50 nanomolar (nM) (1:40 dilution of 2 µM working solution), into Tube 2 and Tube 4, mix gently and incubate 37 °C 00:30:00
- Add the corresponding volume of DMSO into Tube 1 and 3.

Immunoprecipitation

6 - Perform endogenous organelle IP as in **dx.doi.org/10.17504/protocols.io.x54v9yp51g3e/v1** (steps 12-33)

Note

The IP is normally performed using 50 µl of beads/sample. However, only a small fraction of the beads is analysed (approx 1/200 of initial beads), the rest of the IP can be used for WB or other techniques. The initial amount of beads can also be scaled down.

- At the end of the IPs, for each of the initial tubes, there should be 2 samples, a TMEM192 IP and a control IP (BSA-beads).
- Add 400 µL of KPBS to each FACS tube and label them accordingly

Flow cytometry and analysis

7
- Turn on the cytometer.
- Prepare windows and gates according to the lysotracker used. LysoTracker™ Deep Red has the excitation at 647nm and emission at 668nm (APC channel)
- Dilute the samples. We used a dilution of 1/10, by adding 40 µL of stained beads to 400 µL of KPBS into FACS tube.
- Run the samples on a flow cytometer.

Note

On a BD LSR Fortessa we used the following voltages:
FSC: 600
SSC: 240
APC: 700

7.1 FlowJo analysis of FCS files
- Isolate the beads from the debris by gating on the SSC vs FSC plot.
- Isolate positive beads either on the APC histograms or on the APC vs FSC plot

Note

Use the appropriate control to gate for the positive beads (TMEM192 pulldown without tracker)

Protocol references

https://www.protocols.io/view/purification-of-tag-less-lysosomes-from-cells-isol-b5ziq74e
dx.doi.org/10.17504/protocols.io.e6nvwk1d2vmk/v1