Miltenyi Biotec

Characterization of exosome populations using the MACSQuant® Analyzer

Characterization of exosomes

Background

Exosomes are small membrane vesicles with endocytic origin that are released by many cell types, such as T cells, B cells, dendritic cells, platelets, neurons, and epithelial cells.[1] Depending on the originating cell, exosomes are loaded with a specific set of proteins, lipids, and nucleic acids (including small RNAs)[2] and are released constitutively or after stimulation. They are taken up by other cells via membrane fusion or ligand-receptor interactions.[3] Exosomes are involved in various biological processes, including immune surveillance, blood coagulation, neuronal communication, stem cell maintenance, and tissue repair. Their impact on tumor progression, neurodegeneration, autoimmune disorders, and other diseases is under investigation.[2]

Flow cytometry is a powerful technique to characterize cells according to their light scattering properties or their expression of certain cell surface epitopes which can be stained by specific fluorescently labeled antibodies. Due to their small size, single exosomes cannot be detected by light scattering using standard flow cytometry. However, screening for the presence of epitopes on the surface of exosomes is possible, as described in this application note focusing on bulk exosome populations. Flow cytometry is therefore a valuable tool to study cell-type specific surface proteins or activation markers on exosomes.

Materials

Reagents and solutions

- Cell culture supernatant or plasma
- PBS
- PEB (PBS + 5 mM EDTA + 0.5% BSA) filtered through membrane (0.1 µm pore size)
- Protein quantification assay
- CD63-APC, human (# 130-100-182)
- (Optional) Mouse IgG1-APC (# 130-092-214) – isotype control antibodies

Instruments and disposables

- Centrifuge (up to 10,000×g)
- Ultracentrifuge (up to 100,000×g)
- MACSQuant® Analyzer 10 (# 130-096-343)
- MACSmix™ Tube Rotator (# 130-090-753)
- Membrane filter (0.22 µm pore size)

Procedure

Exosome isolation from cell culture supernatant

(modified according to reference 4)

1. Incubate the cells of interest in serum-free medium for 12–72 hours depending on the cell line. Adjust the incubation conditions to an apoptosis rate of less than 5%.
2. Remove cells, cell debris, and larger vesicles by serial centrifugations at 300×g for 10 minutes, 2,000×g for 30 minutes, and 10,000×g for 45 minutes.
3. (Optional) Filter the supernatant through a 0.22 µm membrane.
4. Isolate the exosomes by ultracentrifugation of the supernatant at 100,000×g for 2 hours. Resuspend and pool the pellets in PBS and repeat the ultracentrifugation step.
5. Resuspend the exosome pellet in PBS (1/2000 of the original supernatant volume) and determine the exosome concentration indirectly by quantifying the protein concentration.
6. Store the exosomes at –20 °C or –80 °C.

Exosome isolation from plasma

(modified according to reference 4)

1. Draw blood into EDTA or citrate tubes (minimum: 10 mL).
2. Separate plasma by centrifugation at 1,000×g for 10 minutes.
3. Dilute plasma with an equal volume of PBS.
4. Remove cells and cell debris by serial centrifugations at 2,000×g for 30 minutes and 10,000×g for 45 minutes.

5. Isolate the exosomes by ultracentrifugation of the supernatant at 100,000×g for 2 hours. Resuspend and pool the pellets in PBS.
6. (Optional) Filter the resuspended pellet through a 0.22 µm membrane.
7. Repeat the ultracentrifugation step and resuspend the exosome pellet in PBS (1/250 to 1/500 of the initial volume). Determine the exosome concentration. indirectly by quantifying the protein concentration
8. Store the exosomes at –20 °C or –80 °C.

Exosome staining

1. For each staining dilute an exosome sample, containing a minimum of 500 ng of protein, in 49 µL of membrane-filtered PEB. Prepare another sample in 48 µL for an unstained control.
2. Add 5 ng of CD63-APC (in 1 µL) to one exosome sample and (optionally) 5 ng of Mouse IgG1-APC (in 2 µL) to a second exosome sample. Incubate for 1 hour at 4 °C in a MACSmix Tube Rotator, protected from light. Set the MACSmix Tube Rotator to maximum speed.
3. Dilute each of the labeled samples with 450 µL of membrane-filtered PEB.

Note: Multiple stainings with non-overlapping dyes are possible (e.g. APC, FITC, and VioBlue®). Use 5 ng per antibody conjugate. To obtain the same total volume for incubation (50 µL), adjust the PEB volume in step 1 accordingly.

Exosome analysis on the MACSQuant Analyzer 10

1. Before measurement select the following settings on the tab "channels": Set all channels on hyper log (hlog), set the trigger on SSC to 4, click on the button "advanced", and shut off the secondary trigger by removing the tick.
2. Measure an unstained control of membrane-filtered PEB to evaluate the buffer background noise.
3. Measure the unstained exosome sample to evaluate its autofluorescence.
4. (Optional) Measure the isotype control sample labeled with Mouse IgG1-APC to evaluate non-specific binding of the conjugate.
5. Measure your exosome sample stained with the CD63-APC conjugate.

Results and conclusion

Exosomes tend to stick together. These exosome aggregates scatter light and can be visualized by conventional flow cytometry (figs. 1 and 2). The MACSQuant Analyzer 10 enables the detection of exosome surface proteins labeled with fluorochrome-conjugated antibodies (fig. 2).

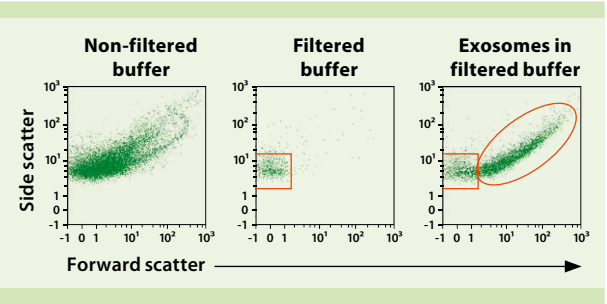

Figure 1: Comparison of non-filtered and filtered PEB buffer. Exosomes are indicated by the oval.

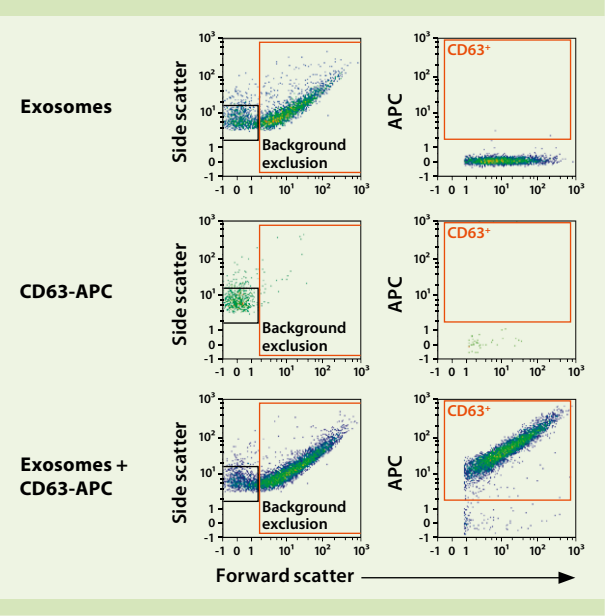

Figure 2: Gating strategy for the analysis of CD63-positive exosomes and gating controls.

References

1. Raposo, G. and Stoorvogel, W. (2013) Extracellular vesicles: Exosomes, microvesicles, and friends. J. Cell Biol. 200: 373–383.
2. El Andaloussi, S. *et al.* (2013) Extracellular vesicles: biology and emerging therapeutic opportunities. Nat. Rev. Drug Discov. 12: 347–357.
3. Théry, C. *et al.* (2009) Membrane vesicles as conveyors of immune responses. Nat. Rev. Immunol. 9: 581–593.
4. Théry, C. *et al.* (2006) Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr. Protoc. Cell Biol. 3.22.1–3.22.29.

Miltenyi Biotec

Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.