Flow Cytometric Analysis of Extracellular Vesicles from Cell-Conditioned Media

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Abstract

Flow cytometry (FC) is the method of choice for semi-quantitative measurement of cell-surface antigen markers. Recently, this technique has been used for phenotypic analyses of extracellular vesicles (EV) including exosomes (Exo) in the peripheral blood and other body fluids. The small size of EV mandates the use of dedicated instruments having a detection threshold around 50-100 nm. Alternatively, EV can be bound to latex microbeads that can be detected by FC. Microbeads, conjugated with antibodies that recognize EV-associated markers/Cluster of Differentiation CD63, CD9, and CD81 can be used for EV capture. Exo isolated from CM can be analyzed with or without pre-enrichment by ultracentrifugation. This approach is suitable for EV analyses using conventional FC instruments. Our results demonstrate a linear correlation between Mean Fluorescence Intensity (MFI) values and EV concentration. Disrupting EV through sonication dramatically decreased MFI, indicating that the method does not detect membrane debris. We report an accurate and reliable method for the analysis of EV surface antigens, which can be easily implemented in any laboratory.

Introduction

Cells secrete extracellular vesicles (EV) of different sizes including microvesicles (MV) and exosomes (Exo). The latter can be distinguished from MV by both size and the subcellular compartment of origin. MV (200-1,000 nm in size) are released from parent cells by shedding from the plasma membrane. Conversely, Exo (30- 150 nm) originate from endosomal membranes and are released into the extracellular space when the multivesicular bodies (MVB) fuse with the cell membrane[1,2].

EV are increasingly used as diagnostic biomarkers as well as, potentially, therapeutic tools in many fields including oncology, neurology, cardiology, and musculoskeletal diseases[3,4,5]. A vast majority of ongoing studies using EV as therapeutic agents exploit the isolation of vesicles from cell-conditioned medium (CM) of in vitro cultured cells. Mesenchymal stem cells (MSCs) exert beneficial effects in several contexts, and MSC-derived EV have shown benefits in models of myocardial ischemia/reperfusion injury[6] and brain injury[7]. MSC-derived EV also exhibit immune modulatory activities that can be exploited to treat immune rejection, as demonstrated in a model of therapy-refractory graft-versus-host disease[8]. Amniotic fluid stem cells (hAFS) actively enrich CM with MVs and Exo, heterogeneously distributed in size (50-1,000 nm), which mediate several biological effects, such as proliferation of differentiated cells, angiogenesis, inhibition of fibrosis, and cardioprotection[4]. We have recently shown that EV, and particularly Exo, secreted by human cardiac-derived progenitor cells (Exo-CPC) reduce myocardial infarct size in rats[5,9].

Exo share a common set of proteins on their surface, including tetraspanins (CD63, CD81, CD9) and major histocompatibility complex class I (MHC-I). In addition to this common set of proteins, Exo also contain proteins specific for the EV subset of the producer cell type. Exo markers are gaining paramount importance because they play a crucial role in inter-cellular communication, thereby regulating many biological processes[5,10]. Because of their small size, finding an easy way to analyze EV using classical flow cytometry (FC) remains a challenging task.

Here, we present a simplified protocol for EV analysis using FC, which can be applied to pre-enriched samples obtained through ultracentrifugation or directly to CM (**Figure 1**). The method uses beads coated with a specific antibody that binds canonical Exo-associated surface epitopes (CD63, CD9, CD81) without additional washes. FC analyses can be performed using a conventional cytometer with no need for adjustments prior to measurements. Methods for the characterization of antigens on individual small particles using flow cytometers have been described by other groups with respect to various applications[11,12,13]. Here, we used functionalized magnetic beads for the capture of small particles and Exo, followed by phenotyping of captured particles by FC. Although this method can be used to characterize the antigenic composition of small vesicles released by any cell type in vitro, here we provided specific cell culture conditions that apply for the culture of human cardiac progenitor cells (CPC) and the most appropriate environment for the production of EV by these cells.

Protocol

1. Collection and processing of conditioned media

1. Coat 55 cm² Petri dishes with 0.02% porcine skin gelatin in PBS.
2. Plate CPC (8,000/cm²) in pre-coated dishes with 7 mL of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS (Fetal Bovine Serum) and 1% Penicillin/Streptomycin (P/S).
   NOTE: The term "CPC" refers to human explant derived cells that have been described elsewhere[14]. CM can be collected from different cell types cultured in specific culture conditions. Wear gloves and work under a biological hood.
3. Once cells reach about 80% confluence, remove the culture medium, wash cells twice with Dulbecco's PBS (PBS) Ca- and Mg-free and replace it with 10 mL of serum-free Exo-production medium (Dulbecco's Modified Eagle's Medium [DMEM] High Glucose, 4.5 g/mL).
   NOTE: Proceed with a progressive decrease of serum concentration in culture medium, to gradually adapt cells to serum-free condition (SF). For cells that are sensitive to serum-free medium prepare a medium containing FBS but depleted of EV. Prepare medium supplemented with all nutrients, plus 10%–20% (v/v) FBS. Centrifuge the medium overnight at 100,000 × g, 4 °C. Be aware that this procedure does not guarantee 100% removal of FBS-derived EV.
4. After 7 days, collect the CM in a polypropylene centrifuge tubes.
   NOTE: The time for medium conditioning depends on the cell type. For cells that are sensitive to serum-free medium, increase the initial volume of medium and decrease the time for CM collection to 24-48 h.
5. Clear CM from cell debris by centrifuge at 3,000 x g for 20 min at 4–10 °C. Collect the supernatant in a 100 kDa centrifuge filter tube.
6. Concentrate cleared CM by spinning the tube at 2,000 x g for 20 min at 4–10 °C.
   NOTE: This step will reduce the initial volume of CM allowing the use of small-volume tubes for high speed centrifuge steps and will contribute to eliminate small protein aggregates.
7. Collect the concentrate in a microcentrifuge tube. Centrifuge at 10,000 x g for 15 min at 4–10 °C.
8. Collect the supernatant and proceed to section 2 (EV enrichment). If an ultracentrifuge is not available proceed directly to section 3 (Nanoparticle Tracking Analysis (NTA) quantification).
   NOTE: Pre-enrichment of EV fraction improves final read-out in fluorescence intensity (see representative results and **Figure 4**). Cleared CM (from point 1.10) can be stored at 4 °C for no longer then 1-2 days or alternatively at −80°C for several months.[5] CM should be pre-cleared to remove any cellular debris before storage at -80°C in order to ensure that exosome-sized debris is not formed in the freeze-thaw process.

2. Extracellular vesicles enrichment by ultracentrifuge (optional)

1. Place the supernatant from step 1.8 in a polycarbonate thick wall ultracentrifuge tube. Fill the tube with 1x phosphate buffer saline (PBS) until it reaches the maximal capacity (3.2 mL).
2. Load samples in a titanium fixed-angle rotor (8 x 3.2 mL, k-Factor 13). Load the rotor in a tabletop ultracentrifuge. Ultracentrifuge at 100,000 x g for 3 h at 4–10 °C.
3. Discard the supernatant and resuspend the pellet in 100 µL of 1x PBS.

3. Nanoparticle tracking analysis (NTA) quantification

1. Dilute 1 µL of sample (from either step 2.3 or 1.8) in 999 µL of 1x PBS. Load the sample in a 1 mL syringe, avoiding bubble formation. Load the syringe in the inlet port of the examination chamber.
2. Turn on the laser. Open the camera with the **Capture** button. Adjust the focus.
3. Record at least 3 different frames of 60 s each.
4. Analyze the 3 different acquisitions using the **Batch Process** option in the software.
   NOTE: If the NTA technology is not available, perform a protein quantification by the Bradford assay (1 x 10⁸ particles correspond to 1-2 µg of total protein after ultracentrifugation or to 50-60 µg total protein if quantification is performed directly for CM as it includes proteins contaminants different from EV; see **Table 1**)[5].

4. Sample preparation

1. Prepare a pool of the 3 types of capture beads (CD9 beads, CD63 beads, and CD81 beads at a 1:1:1 ratio; see **Table of Materials**) and vortex.
   NOTE: The pool has been tested to be stable for 1 month. A single type of capturing bead may be used, this will allow the detection of EV sub-populations not discriminable with pool of beads. By using single antigen capturing beads, it will be possible to contemporary demonstrate the presence of tetraspanin proteins, such as CD9, CD81, or CD63, and the antigen of interest detected by fluorescent antibody. Alternative aldehyde-sulfate latex beads are also commercially available. These beads present hydrophobic surface for the adsorption of MV and EV.
2. In a round bottom tube, add the amount of volume corresponding at 1 x 10⁸ particles plus 1 µL of the bead pool, corresponding to 1.2 x 10⁵ beads in total (for each test).
3. Prepare a tube containing 1 µL of beads without EV that will serve as negative control, hereby referred as "Beads".
4. Adjust the volume to 100 µL with 1x PBS. Place the tube in a thermo-mixer, and shake at 400 rpm overnight at 4-10 °C.
5. The next day, move the samples to a round bottom 96-well plate (or FC tubes).
6. Add the antibody (10 µg/mL of CD9_FITC, 10 µg/mL of CD63_PE, and 5 µg/mL of CD81_PE).
7. Incubate 1 h at 4–10 °C.
8. Add 100 µL of 1x PBS to each well.

9. Proceed to acquisition.

5. Acquisition

1. Open the acquisition software and start up the instrument (**Cytometer > System Startup Program**). Open a new experiment.
2. Create an experimental template, then select **Plate** and then **Add Plate**. Select the position of the samples on the plate and then press **Set As Sample Well**.
3. Enter the sample name (i.e. CPC#1_CD9FITC) in the **Naming Rules**. Open the **Channel** Tab and switch on the FITC and PE Channels.
4. Load the plate in the instrument.
5. Press **Dot Plot** and create a new dot plot (P1). Set Forward Scatter Area (FSC-A) versus Side Scatter-Area (SSC-A).
6. Select **Initialize** to start the laser and the fluidics. Press **Acquire** to start the acquisition.
7. Adjust the scale to show the population in the middle of the P1. In P1 draw the "BEADS" gate around the whole population (excluding debris) (**Figure 2A**). Press **Stop**.
8. Press **Dot Plot** and create a new dot plot (P2). Set Forward Scatter-Height (FSC-H) versus FSC-A. Gate the new dot plot on "BEADS." In P2 draw a new gate around the bigger population and name it "SINGLETS" (**Figure 2A**).
   NOTE: This strategy is designed in order to avoid as much as possible the inclusion of beads aggregates in both acquisition and analysis steps.
9. Press **Dot Plot** and create two different dot plots; one FITC versus SSC-A and one PE versus SSC-A. Gate the new dot plot on "SINGLETS."
10. Select the number of **Events to record** (e.g., 20,000). Select **Record** and start the experiment.

6. Data analysis

1. Load the acquisition files into the analysis software.
   NOTE: The following steps have to be applied to every sample including beads only and every unknown sample.
2. Open a new protocol and create dot plots following the same strategy described in step 5.
3. Set all scatter axes to linear scale and all the fluorescence axes to log scale.
4. Show the Fluorescence Geometric Mean (X-Gmean-MFI) in FITC and PE channels.
5. Calculate the ratio X-G_{mean} of the samples / X-G_{mean} of the beads stained with antibody without exosome.
6. Compare the fold change MFI of different EV preparations.

7. Extracellular vesicle number titration

NOTE: Sections 7 to 10 can be performed to set up the number of particles, antibodies concentration and specificity, but can be skipped if the cell type, from which EV are derived, and antibodies remain the same.

1. Dilute particles, obtained from step 2.3, in 1x PBS to obtain a series of suspensions ranging from 5×10^5 to 2.5×10^8 particles.
2. For each suspension add in a round bottom tube 1 µL of capture bead pool.
3. Follow the protocol from step 4.3.

8. Antibody titration

NOTE: Antibody titration is usually performed by adding a single antibody for each tube.

1. Keep the number of EV (total particle or protein content) constant in all samples.
   NOTE: The appropriate number is empirically determined as described above in section 7.
2. Follow the protocol from steps 4.1 to 4.5.
3. Test a range of antibody concentrations above and below the amount recommended by the supplier. For example, for an antibody with a suggested concentration of 10 µg/mL per test, test 1, 2, 5, 10, 20 and 50 µg/mL. Include a sample with "beads only", adding the highest concentration of antibody.
4. Incubate for 1 h at 4–10 °C.
5. Add 100 µL of 1x PBS for each sample.
6. Acquire the samples.

9. Incubation time

1. Verify the incubation time performing acquisitions at different time points (e.g., 1 h, 2 h, and 5 h).
2. Follow the protocol starting from section 4.
3. Incubate samples at 4–10 °C for 1 h.
4. Acquire samples.
5. Incubate samples at 4–10 °C for 3 h.
6. Acquire samples.

10. Protocol validation

1. EV binding
   1. In a round bottom tube, add the amount of volume corresponding to 1×10^8 particles or the corresponding amount of total protein.

2. Adjust the volume to 100 µL with 1x PBS.
3. Disrupt the EV membrane by sonication (alternatively a heat shock step can be applied) according to manufacturer's instructions. Set frequency and intensity. The sonication period can be empirically established setting a time-course experiment (i.e., 0 s, 30 s, 1 min, 5 min, etc.).
4. Add 1 µL of the bead pool.
5. Follow the protocol starting from step 4.4.

2. Antibody specificity
   1. Prepare one tube for each fluorochrome-conjugated IgG and add the complex of beads-EV as described in step 4.6.
      NOTE: The fluorochrome-conjugated Isotype must match with the IgG of the used antibody.

Representative Results

Total number of particles for single staining

Since a single bead can bind more than one particle, we tested different conditions to set the smallest amount of total EV (single antibody per tube) to reach the early exponential phase of MFI curve. A fixed concentration of antibody was used while the total number of particles ranged from 5×10^5 to 2.5×10^8. As shown in **Figure 3A**, the number of particles that allows us to ensure that the antibody performs within an acceptable MFI, avoiding the use of an excess of EV, is 1×10^8 particles/staining.

Antibody titration

We selected the proper concentration of antibody resulting in the highest signal preventing the nonspecific antibody binding. This test has been optimized for 1×10^8 particles, as determined in the previous setting. Anti-CD9_FITC, anti-CD63_PE, and anti-CD81_PE were tested with concentrations ranging from 1 to 50 µg/mL (**Figure 3B**). Anti-CD9_FITC and anti-CD63_PE antibodies gave a good resolution of signal (7.5 and 130-fold change of MFI vs. beads alone, respectively) when used at concentration of 10 µg/mL while the selected concentration for the anti-CD81_PE antibody was 5 µg/mL (465.3 Fold Change MFI).

Method validation

In order to confirm that our method is suitable to analyze only "cup-shaped" extracellular vesicles and not membrane debris, we applied different sonication steps, at 10% of amplitude, to the solution containing particles. 100 µL of 1x PBS solution containing 1×10^8 particles underwent different sonication steps from 30 seconds to 5 minutes and the resulting preparation containing both broken and well-shaped EV were analyzed as described (experimental protocol from point 3.3). As a result, we found that 30 seconds of sonication decrease MFI that was completely dumped after 1 minute. At this timepoint, no fluorescence is detectable for any of the EV markers (**Figure 3D**).

Flow cytometry characterization of HEK293- and CPC-derived exosomes

These results were generated following the protocol presented above with the ultracentrifuge isolation method. The isolated exosomes are quantified by NTA technology and loaded overnight with 1 µL of mixed beads (1x anti-CD9:1x anti-CD63:1x anti-CD81). The complex beads + Exo were stained with the proper amount of antibody anti-CD9_FITC, anti-CD63_PE, and anti-CD81_PE (**Figure 3E,F** and **Table 2**).

Furthermore, by using EV-CPC we compared FC analysis with or without pre-enrichment by ultracentrifugation. **Figure 4** shows that both methods are suitable to profile Exo surface markers. Pre-enrichment of extracellular vesicles fraction greatly improves fluorescence intensity especially for CD63_PE and CD81_PE staining (**Figure 4** and **Table 3**).
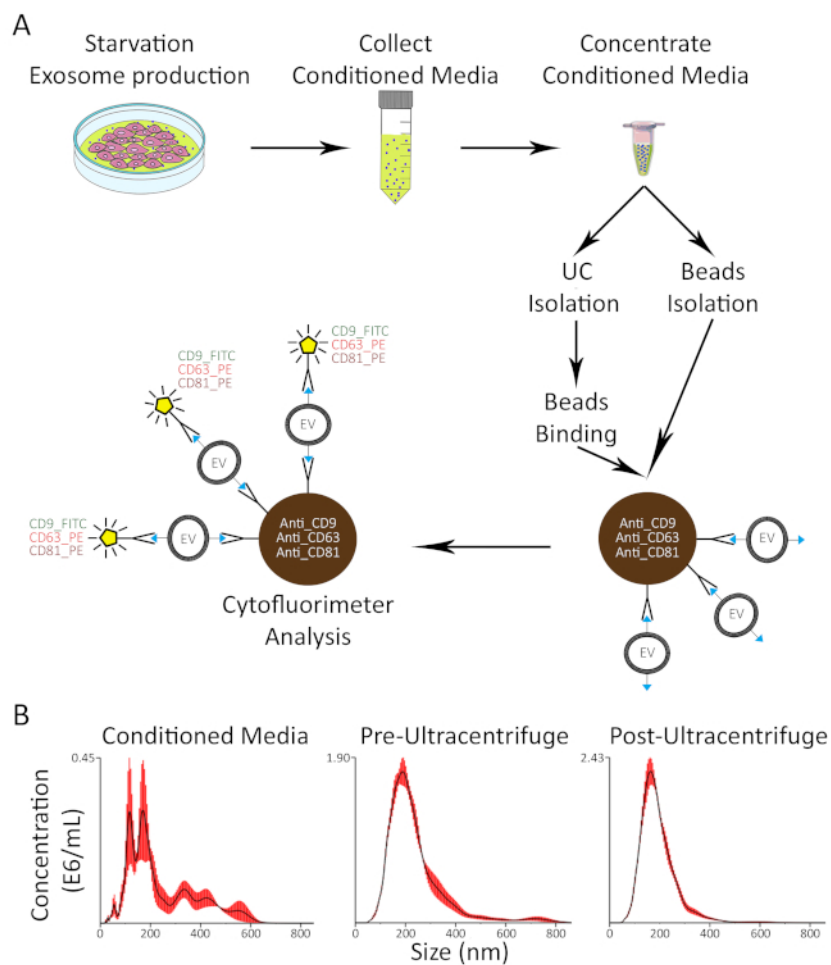
Figure 1: Protocol and NTA plots. (**A**) Schematic representation of the experimental protocol. (**B**) Representative NTA plots for Conditioned Media, Pre-Ultracentrifuge and Post-Ultracentrifuge step. Please click here to view a larger version of this figure.
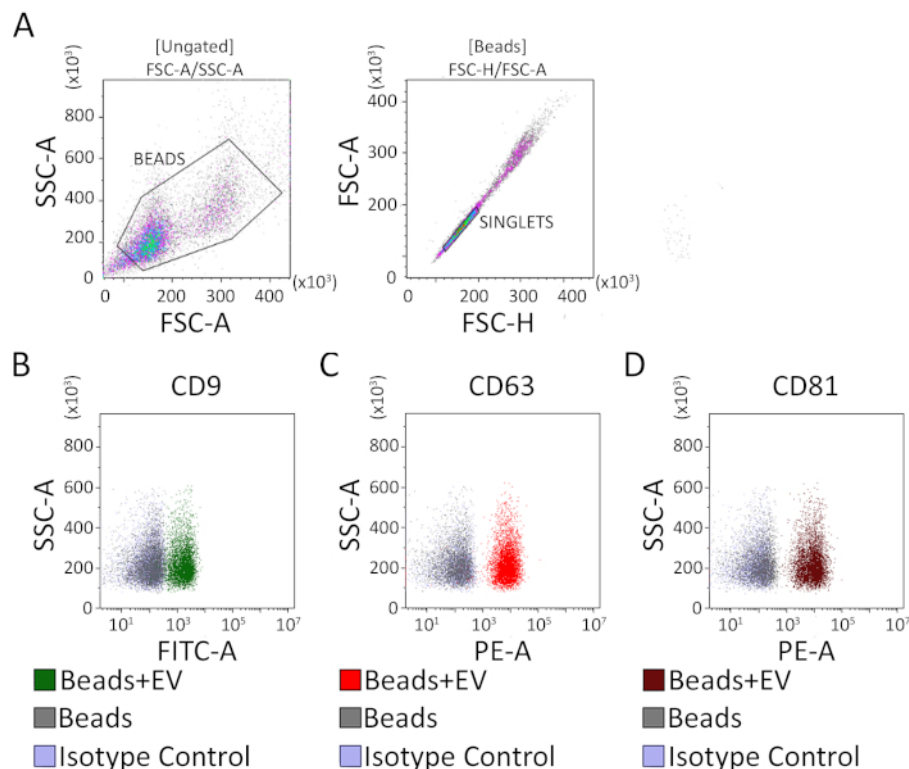
Figure 2: Acquisition and data analysis. (**A**) Flow cytometry analysis begins with creating a first gate to the whole beads population "beads" (excluding debris) and then a second gate to distinguish "singlets" events. Singlets are gated on a plot set up with FSC-H as x-axis and FSC-A as y-axis. (**B-D**) Representative dot plots showing right-shift of fluorescence intensity for the positive populations of beads-exosomes complexes (green, CD9+; red CD63+; brown CD81+). Isotype control (violet) overlap negative gray colored beads. Please click here to view a larger version of this figure.
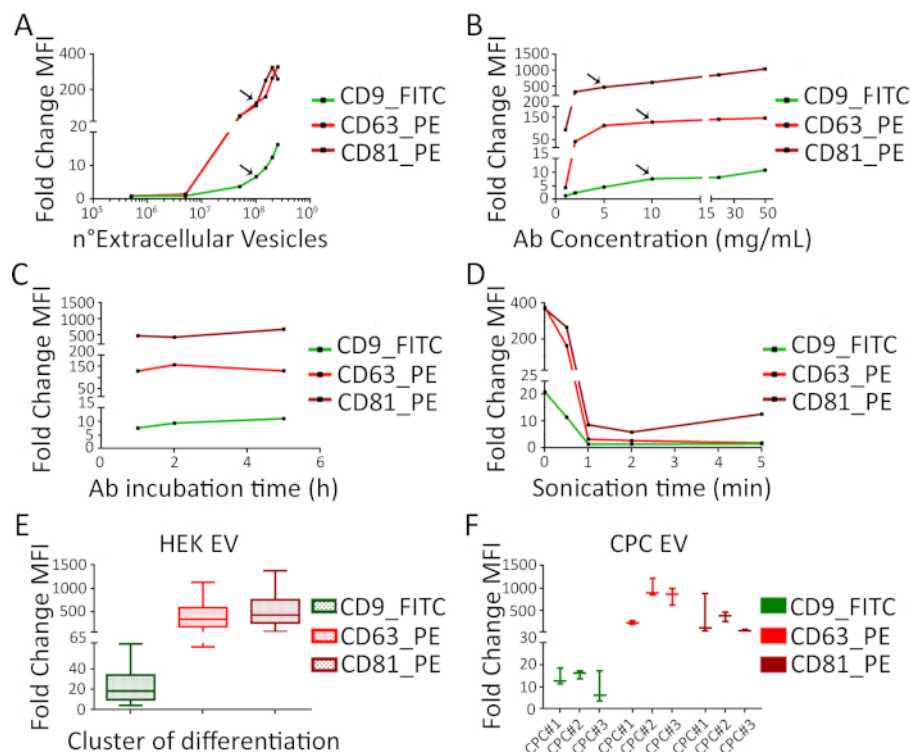
Figure 3: Titration curves for number of particles (arrows show the selected amount of particles, 1 x 10^8). (**A**) Antibody concentrations (arrows show the selected concentration for each used antibody). (**B**) Both number of particles and antibody concentration are plot vs. mean fluorescence intensity (MFI). (**C**) Curve showing 3 different acquisition of the preparation with 1 h - 2 h or 5 h of antibody incubation. (**D**) Curve showing the decrease of fluorescence following sonication at different time-points. (**E-F**) Fold change (mean ± SD) of MFI for CD9, CD63 and CD81 vs negative control (beads + antibodies, no EV) are shown for HEK293 EV (n = 3 independent replicates) and for CPC EV (n = 3 primary cell lines from 3 different patients) Please click here to view a larger version of this figure.
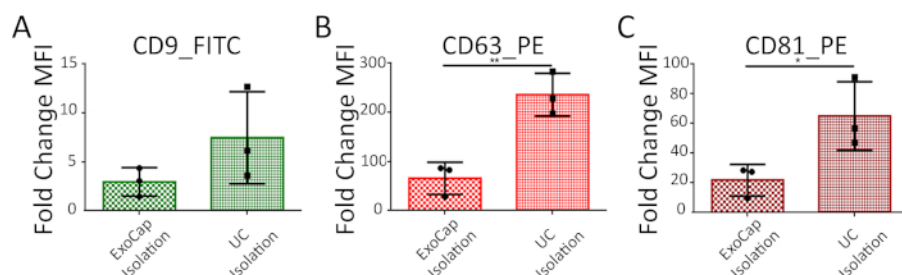
Figure 4: MFI analysis and comparison between two procedures: direct EV-binding with beads (Capture Beads Isolation) and pre-enrichment with ultracentrifuge (Ultracentrifuge Isolation). Data are shown as fold change (mean ± SD) of MFI for (**A**) CD9, (**B**) CD63, and (**C**) CD81 vs. negative control (beads + antibodies, no EV). N = 3 primary cell lines from 3 different patients. Please click here to view a larger version of this figure.

CPC	CONC NTA (part/µL)	CONC (µg/µL)
CPC#1 Pre-Ultracentrifuge	5.02E+06	2.01
CPC#1 Post-Ultracentrifuge	6.10E+07	1.04
CPC#2 Pre-Ultracentrifuge	5.74E+06	2.30
CPC#2 Post-Ultracentrifuge	7.43E+07	0.79
CPC#3 Pre-Ultracentrifuge	2.02E+06	1.90
CPC#3 Post-Ultracentrifuge	2.91E+07	0.42

Table 1: Comparison between NTA concentration and protein concentration for 3 different patient derived CPC before and after ultracentrifuge.

FOLD CHANGE OF MFI ± SD	CD9	CD63	CD81
HEK293	24.44 ± 19.17	430.7 ± 344.9	535.2 ± 410.3
CPC#1	14.15 ± 3.72	236.05 ± 43.40	353.30 ± 452.43
CPC#2	15.76 ± 1.87	983.06 ± 195.63	374.45 ± 108.05
CPC#3	8.94 ± 7.19	830.50 ± 184.73	60.05 ± 23.18

Table 2: Value of fold change (mean ± SD) of MFI for HEK293 EV (n = 3 independent replicates) and CPC EV (n = 3 primary cell lines from 3 different patients).

FOLD CHANGE OF MFI ± SD	CD9	CD63	CD81
Capture Beads Isolation	2.96 ± 1.45	65.65 ± 18.87	21.85 ± 6.12
Ultracentrifuge Isolation	7.47 ± 2.71	236.00 ± 25.06	65.05 ± 13.38

Table 3: Value of fold change of MFI (mean ± SD) for CPC EV (n = 3 primary cell lines from 3 different patients) isolated by capture beads or ultracentrifuge.

Table 4: Single product specification.

Discussion

Conventional FC technique remains the most straightforward analytic method to characterize markers expressed onto the surface of EV. In this regard, selecting the most appropriate protocol is crucial to obtain useful information on individual particle fractions of interest by avoiding limitations due to instrument sensitivity. We described a method using magnetic particles coupled with antibodies that recognize Exo and small EV surface antigens which are suitable for downstream FC application. We validated the method using two different cell types: primary human CPC that are emerging as a major cell source for Exo-based therapeutic approaches for heart disease; and HEK293 cells, an immortalized cell line widely used in cell biology research because of reliable cell growth and plasticity.

The method can be applied to ultracentrifuge-enriched EV and, for faster analysis, also directly on in vitro cell-derived CM with no pre-enrichment by ultracentrifugation. The starting material is critical when comparing samples. Adding capturing beads directly to the CM will speed up the procedure but at the same time decrease fluorescence intensity, as shown in **Figure 4A**. It is also critical to use an appropriate amount of PBS to mix beads and EV during the "capturing" step 4.4. When using a constant incubation time, an increased volume will decrease fluorescence intensity due to inefficient EV coupling.

A limitation of the protocol is that a single capture bead can bind multiple EV/Exo particles on its surface. This will limit the possibility of identifying subsets of EV expressing peculiar combination of antigens using a multiple staining. The bead-based method therefore yields semi-quantitative data. Using beads carrying a single capturing Ab (CD9, CD63 or CD81) will allow at maximum the characterization of particles that express two epitopes: the one present on the bead and recognized by the capturing antibody and the one detected by the antibody that is subsequently added.

The current gold standard for Exo analyses using FC is a protocol developed by van der Vlist et al. in 2012[15]. It allows for a high-resolution analysis of EV using an optimized configuration of the commercially available high-end FC (e.g., BD Influx). This protocol is extremely detailed and useful but still needs a complex hardware setting with specific FC calibration before use. Three years later, Pospichalova et al.[16] proposed a simplified protocol for FC analysis of Exo using a dedicated cytometer specifically developed for analysis of small particles (e.g., Apogee A50 Micro)[17]. With respect to this protocol and others that have used special threshold setting[11], here we propose a basic protocol to perform small EV phenotyping using magnetic binding beads that is suitable for conventional FC instruments and does not require any special setting. Different protocols have described bead-based methods to characterize small EV found in bodily fluids by FC[12]. Here, we show the immunocapture of discrete sub-populations of vesicles positive for CD9, CD63, and CD81 that are commonly used as Exo markers[18]. Aldehyde-sulfate latex[19,20] and polystyrene[12] beads remain valid alternatives for binding of EV present in CM and blood plasma fluid; however, aldehyde groups exposed on the surface of the polymer particle enable coupling of unspecific proteins and other materials to the latex particles, thus increasing the risk of contamination by lipoprotein or apoptotic bodies during isolation and detection process[21,22].

Beads used in the protocol bind only entire, well-shaped EV. We proposed to disrupt EV structure by sonication to quench the signal (section 4, "protocol validation"). Indeed, one minute of sonication results in diminished fluorescence intensity, thus showing that a positive signal cannot be affected by membrane debris adsorbed on beads.

Disclosures

Nothing to declare.

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