

# Chapter 14

## Microvesicles: Isolation, Characterization for In Vitro and In Vivo Procedures

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### Abstract

Microvesicles and exosomes are released to the extracellular milieu and are detectable in body fluids. They act as unique vehicles for cargo transfer to other cells/tissues. They contain a set of membrane receptors, intracellular proteins and nucleic acids. Here, we describe basic techniques for their isolation and characterization from cell culture media or body fluids. We also describe critical techniques for characterizing their cargo (miRNAs) and their protein content. Finally, we present labeling methods for their use in in vitro procedures and for their in vivo delivery and subsequent analysis of their cargo transfer to multiple cell types.

**Key words** Exosome, Microvesicle, Nanoparticle

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### 1 Introduction

Exosomes are small (30–150 nm) membrane vesicles of endocytic origin that are released by a variety of cells to the extracellular milieu by fusion of multivesicular bodies with the plasma membrane [1, 2]. The composition of exosomes depends on the cell of origin, although the membrane composition remains distinct from its parental cell as a result of a selective process of assembly, enabling specialized functions [3]. Exosomes contain a set of nucleic acids including miRNAs and mRNAs which could be transferred to target cells [4] inducing regulatory changes [5] under physiological conditions. Current estimate of microvesicle concentration in the peripheral blood of healthy individuals is 5–50 µg/ml; the majority of it has its origin in platelets [6]. Yet, this process could be deeply altered in different pathological settings such as immune response, inflammation, tumor progression and tissue repair. Exosome-mediated cargo transfer to adjacent or remote cells could deeply influence disease progression. This mechanism of intercellular communication is especially relevant in cancer. Accumulated evidence

indicates the potential role of exosomes as non-invasive biomarkers with prognostic or diagnostic role, as they can be detected in body fluids [7, 8].

A lot of information about exosome-specific signatures, especially disease related can be obtained from the ExoCarta complement of exosomal proteins and RNA [9].

In this chapter we have assembled and adapted several protocols for (a) the isolation of exosomes from conditioned medium, (b) the characterization of their nucleic acid cargo and hallmark proteins, (c) the measurement of their size and particle number, and (d) the fluorescent labeling for in vitro and in vivo tracking purposes [10].

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## 2 Materials

1. Ultracentrifuge plastic tubes were 25 × 89 mm, Ref. #326823 for a SW40 rotor of a Beckman Optima™ LE-80K ultracentrifuge. For the recovery of exosomes we used conical 35 ml ultracentrifuge tubes (Beckman Ref #358126) with their adaptors Ref #358156.
2. The transmission electron microscope was from Philips CM120 BioTwin. The copper grids carbon coated were EMS CF300-Cu from Aname.
3. The 4 μm latex beads were purchased from Invitrogen (Ref# A37304).
4. Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a resistivity of 18 MΩ cm at 25 °C) and analytical grade reagents.
5. DEPC water was obtained by dissolving for 12 h at 37 °C 0.1 g in 100 ml of deionized water and subsequently autoclaved.
6. PBS and molecular grade BSA were purchased from Sigma. To prepare PBS/5 % BSA solution, 5 g of BSA were dissolved in 100 mL of PBS prewarmed at 37 °C by gently stirring and filtered by 0.22 μm.
7. Penicillin/streptomycin solution (Sigma, Ref # P0781-100 ML).
8. RIPA lysis buffer 1×: for 50 mL: 5 ml of 10 % NP-40 detergent solution, 1.5 ml of 5 M NaCl, 2.5 ml of 1 M Tris pH:7.4, 200 μl of 0.25 M EDTA and ~41 ml of ultrapure H<sub>2</sub>O. Add protease inhibitors as follows: 10 μl of PMSF (20 mg/ml), 10 μl of 0.5 M NaF, 5 μl of 200 mM NaVO<sub>3</sub>, and a Protease inhibitor cocktail tablet (Roche, Ref# 11 836 170 001).
9. TRIZOL LS Reagent® was from Life Technologies.

10. Red Blood Lysis Buffer, MINI26 cell linker kit and PKH67 Green Fluorescent Cell Linker Mini Kit (ref #MINI67) were purchased from Sigma.
11. Glycogen was from Ambion.
12. ExoQuick™ was from Systems Biosciences.
13. Phycoerythrin was purchased from Jackson Immuno Research, (cat# 016110-084).
14. Anti-CD63 anti-human antibody was purchased from BD Biosciences (Ref #556019).
15. Anti-IG2 goat biotinylated anti-mouse antibody was from Dako.
16. INMMUNO solution was contained PBS, 1 % BSA, 0.1 % Tween 20 and 0.2 % DAPI.
17. Vectashield® was purchased from Vector Laboratories.
18. Automacs buffer was obtained by dissolving 2.5 ml FBS, 2.5 ml of 0.5 M EDTA, 5 ml of penicillin/streptomycin solution (Sigma, Ref # P0781-100 ML) in 500 ml of PBS.

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## 3 Methods

### 3.1 Isolation of Exosomes from Cells in Culture

#### 3.1.1 Preparation of Exosome-Depleted Medium

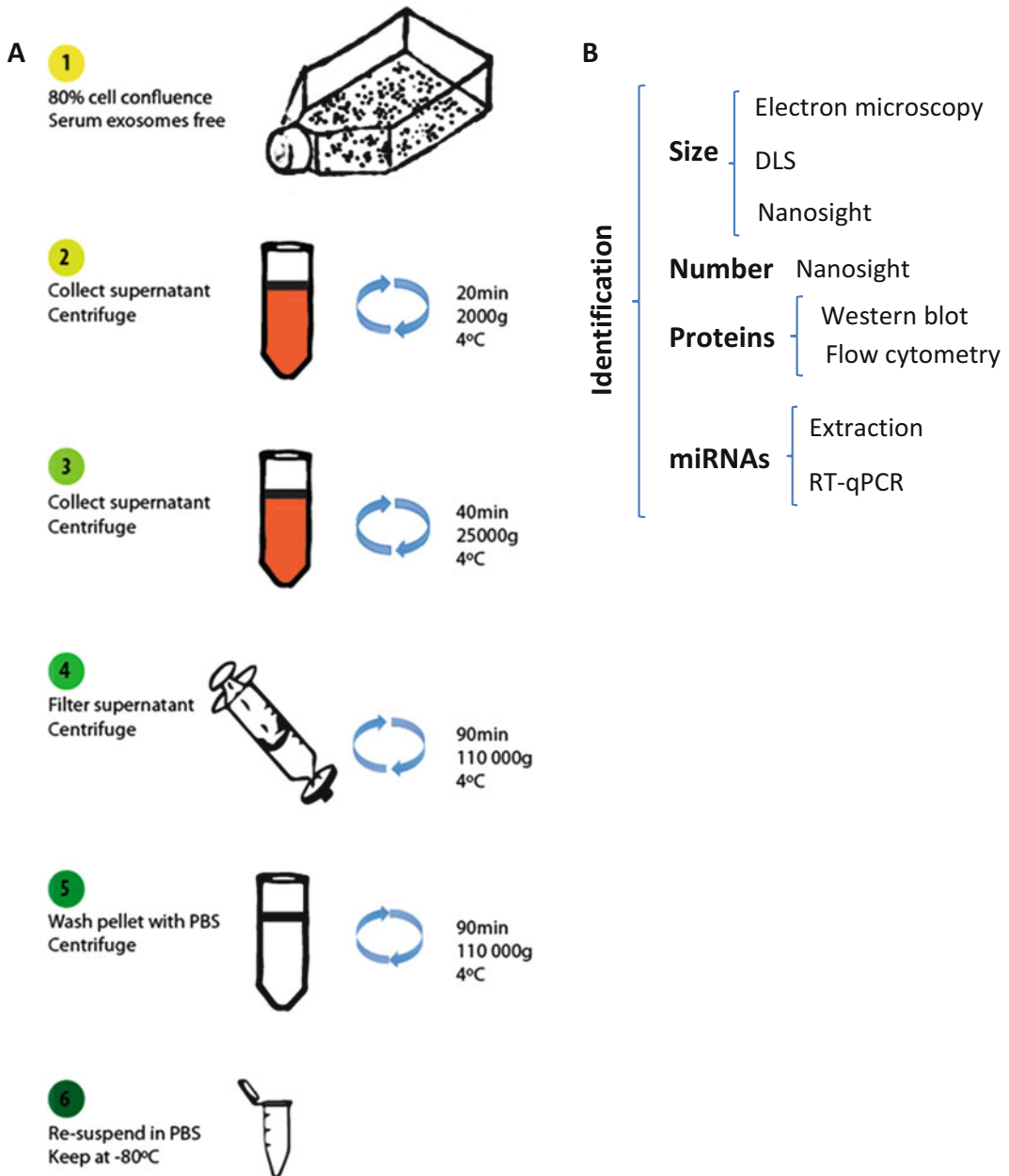
Work under sterile conditions all along these procedures (*see Note 1*).

1. Thaw 50 ml aliquot of complement inactive fetal calf serum (FCS) and mix it with 160 ml of regular serum-free medium used in cell culture. This will allow filling six tubes (35 ml per tube).
2. Use the Beckman plastic tubes.
3. Complete to 35 ml each tube and equilibrate with a balance.
4. Centrifuge  $110,000\times g$  at 4 °C overnight (12–16 h) on an ultracentrifuge (*see Note 2*).
5. Recover carefully the supernatant. The pellet is invisible.
6. Filter through a 0.22 µm filter on sterile conditions.
7. Add serum-free medium and antibiotics (if needed) to obtain the regular complete medium used in cell culture.

This complete medium is used to culture the cells and can be stored at 4 °C for 1 month.

#### 3.1.2 Purification of Exosomes from Cultured Cells (Fig. 1) (See Note 3)

1. Cells should be growing exponentially until 80 % confluence. The medium should contain exosome-free FCS prepared as previously described in Subheading 3.1.1. Use the best culture conditions to minimize cellular debris as much as possible (*see Notes 4 and 5*).



**Fig. 1** Procedure for exosome isolation and characterization techniques. (a) Schematic of exosome isolation. (b) Basic techniques required for the physical characterization and their miRNA and protein cargo

2. Collect 48–96 h -supernatant after the last medium change performed in complete medium. We use 10 ml per T-75 flask. Usually we collect six T-75 flasks to obtain 60 ml of conditioned medium (*see Note 6*).
3. Centrifuge supernatant at  $2000 \times g$  for 20 min at 4 °C to spin down cell debris and death cells.

4. Recover supernatant.
5. Ultracentrifuge supernatant at  $25,000 \times g$  for 40 min at  $4^\circ\text{C}$  to eliminate large microvesicles on an SW40 rotor or similar (*see Note 7*).
6. Recover and filter the supernatant through  $0.22\ \mu\text{m}$  pore filters.
7. Ultracentrifuge supernatant at  $110,000 \times g$ , 90 min,  $4^\circ\text{C}$  using conical tubes and adaptors. This is crucial to ensure a good recovery of the invisible pellet.
8. Discard supernatant and wash the pellet with PBS, first resuspend in 1 ml and then add 29 ml. Note that the pellet is not visible.
9. Ultracentrifuge supernatant at  $110,000 \times g$ , 90 min, at  $4^\circ\text{C}$ .
10. Discard supernatant and resuspend pellet in  $50\text{--}100\ \mu\text{l}$  of sterile PBS in each tube and then collect the content of exosomes from six tubes on a regular 1.5 ml tube.
11. Store and keep at  $-80^\circ\text{C}$  (**Notes 7 and 8**).

### 3.1.3 *Micovesicle Imaging by TEM*

1. Use transmission electronic microscopy (TEM) copper grids carbon coated.
2. Perform vacuum electrical discharge (glow discharge technique) so grids become temporarily hydrophilic.
3. Put a  $5\ \mu\text{l}$  drop of each sample at the right concentration on a parafilm and invert grid on it.
4. Leave 1 min at room temperature to allow adherence.
5. Dry the drop with a Whatman paper and invert the grid over a  $5\ \mu\text{l}$  drop of 2 % uranyl acetate.
6. Leave 30 s at room temperature.
7. Repeat dye if necessary.
8. Remove uranyl acetate and let the grid dry.
9. Samples were assessed using a transmission electron microscope. Images were captured by a digital Olympus SIS camera.

### 3.1.4 *Assessment of Particle Size and Number*

A couple of techniques are important to measure the number and the size of the particles. It is important to carefully measure the exact number of cells and/protein from which exosomes were isolated for normalization purposes. These measurements are becoming mandatory in any research involving microvesicles or exosomes.

**Step 1:** Dynamic light scattering (DLS) and zeta potential determinations can be performed using a Zetasizer nanoseries instrument (Malvern Nano-Zetasizer). Add always same exosome concentration diluted in PBS (around  $50\ \text{ng}/\mu\text{l}$ ). \* Pick should appear between 30 and 120 nm (Fig. 1).

**Step 2:** Nanoparticle tracking analysis (NTA) measurements can be performed with a NanoSight LM20 instrument (NanoSight). The samples should be diluted at 2–5 ng/ml and injected with a sterile syringe until the liquid reaches the tip of the nozzle.

### 3.2 Exosome Characterization

#### 3.2.1 By Flow Cytometry: CD63

This protocol should be performed at 4 °C (*see Note 9*).

1. 5 µl exosomes + 5 µl latex beads 4 % w/v. \*Less amount of exosomes is possible. Keep [exosome: bead] at 1:1 ratio.
2. Incubate 30 min and resuspend every 5 min by gentle pipetting.
3. Add 400 µl of [PBS/5 % BSA] solution.
4. Incubate 30 min and resuspend every 5 min by gentle pipetting.
5. Centrifuge 1000×g for 10 min at 4 °C.
6. Discard supernatant and resuspend pellet in 50 µl de [PBS/5 % BSA] solution.
7. Incubate with first antibody diluted at 1/50 1 h, 4 °C (anti-CD63 anti-human antibody).
8. Wash twice with 400 µl [PBS/5 % BSA] solution.
9. Centrifuge at 1000×g for 10 min at 4 °C.
10. Discard supernatant and leave exosomes with beads resuspended in 200 µl of PBS/5 % BSA.
11. Incubate with secondary biotinylated antibody diluted at 1/200 for 30 min at 4 °C (anti-IG2 goat biotinylated anti-mouse antibody).
12. Wash twice with 400 µl [PBS/5 % BSA] solution.
13. Centrifuge at 1000×g for 10 min at 4 °C.
14. Discard supernatant and resuspend pellet in 50 µl solution [PBS/5 % BSA].
15. Add streptavidin with phycoerythrin (×28) 1/50 and incubate for 30 min in darkness.
16. Wash twice with 400 µl PBS (without BSA).
17. Centrifuge at 1000×g for 10 min at 4 °C.
18. Resuspend in 300 µl PBS and perform FACS analysis.

#### 3.2.2 By Western Blot

Use 25 µg of proteins from the isolated exosomes and add 10× RIPA buffer to a final 1× concentration. Add protein loading buffer, boil the samples, and run a regular SDS-PAGE gel.

#### 3.2.3 miRNA Extraction from Conditioned Media

Before engaging into large experiments, we suggest comparing the protocol below with other commercially available extraction methods such as ExoQuick™ (*see Note 10*)

Similarly, the analysis of total miRNAs in the conditioned medium should be compared with the miRNA cargo of isolated exosomes. In our case, the free miRNA is negligible as compared to the miRNA cargo in exosomes.

Here, we describe the analysis in the conditioned media.

1. Use 15 ml tubes for the miRNA extraction of 48–96-h cultured cells as previously stated. Use three tubes per cultured sample, and add 1.6 ml conditioned medium in each tube.
2. Add 2 ml of TRIZOL LS Reagent<sup>®</sup>. Vortex and leave 5 min at room temperature.
3. Add 2  $\mu$ l spike for each miRNA (each miRNA at 25 fmol concentration) of three different miRNAs per sample for normalization purposes. A total 6  $\mu$ l per tube.
4. Add two volumes of chloroform (400  $\mu$ l per tube) and leave for 3 min at room temperature.
5. Centrifuge at  $3500\times g$  for 15 min at 4 °C.
6. Carefully take the upper aqueous phase (~2 ml) to another clean 15 ml tube.
7. Add 3  $\mu$ l of glycogen at a concentration of 5 mg/ml in RNase-free water.
8. Add one volume of isopropanol (2 ml).
9. Mix and distribute in 1.5 ml tubes.
10. Centrifuge  $12,000\times g$  for 10 min at 4 °C.
11. Wash the pellet with 1 ml of cold ethanol 75 % and spin for 5 min,  $7500\times g$  at 4 °C.
12. Discard the supernatant.
13. Dry the pellet at room temperature.
14. For each original sample (which is distributed in three tubes), resuspend in 30  $\mu$ l of DEPC water and store at  $-80$  °C (*see Note 11*).

### **3.3 Tracking Microvesicles in Functional Assays (Fig. 2)**

#### **3.3.1 Fluorescence Microvesicle Labeling**

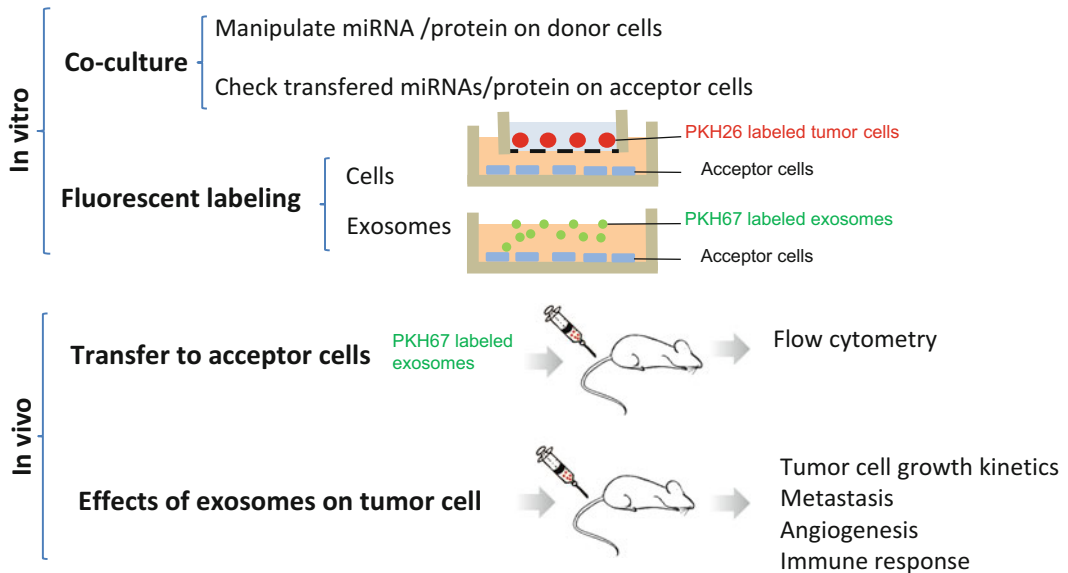
##### **Method 1: Exosome Labeling with PKH 26**

For in vitro applications, we use the MINI26 cell linker kit, which incorporates a red fluorescent dye into lipid regions of the cell membrane.

Perform the protocol after **step 7**, Subheading **3.1.2**, right before PBS washing step (*see Note 12*).

1. Add diluent C to exosomes to reach a final concentration  $2\times 10^7$  particles/ml, i.e., derived from  $1\times 10^6$  cells and add 50  $\mu$ l diluent C.
2. Immediately prior to staining, prepare a volume of  $2\times$  PKH26 ( $4\times 10^{-6}$  M) in diluent C.
3. Rapidly add volume of  $2\times$  Dye to exosomes.

## Functional assays



**Fig. 2** In vitro and in vivo functional assays for donor–acceptor transfer

4. Final concentration: particles:  $2 \times 10^7$  exosomes/ml PKH26:  $2 \times 10^{-6}$  M.
5. Immediately mix the sample by pipetting. Rapid and homogeneous mixing is critical for uniform labeling because staining is nearly instantaneous.
6. Incubate at room temperature for 7 min in the darkness.
7. Stop the staining reaction by adding an equal volume of serum or compatible protein solution (i.e., 1 % BSA). Incubate 1 min.
8. Add complete medium to the top of the tube.
9. Centrifuge exosomes at  $11,000 \times g$  for 90 min at  $4^\circ\text{C}$ .
10. Discard supernatant and wash pellet with PBS.
11. Centrifuge exosomes at  $11,000 \times g$  for 90 min at  $4^\circ\text{C}$ .
12. Discard supernatant and resuspend stained exosomes in 50  $\mu\text{l}$  de PBS.  
Keep exosomes at  $-80^\circ\text{C}$ .

### Method 2: Exosome Labeling with PKH67

We use the PKH67 Green Fluorescent Cell Linker Mini Kit.

Perform the protocol after **step 7**, Subheading [3.1.2](#), right before PBS washing step (*see* **Note 12**).

1. Take 100  $\mu\text{l}$  resuspended exosomes in PBS.
2. Add 500  $\mu\text{l}$  of solution C [2  $\mu\text{l}$  PKH67 + 448  $\mu\text{l}$  solution C].



3. Add PKH67 to exosomes and mix by pipetting up and down.
4. Incubate for 2 min at room temperature.
5. Neutralize with 1 ml [PBS/20 % BSA].
6. Incubate for 1 min at room temperature.
7. Add PBS to fill the centrifuge tube (~25 ml) in conical tubes and adaptors recommended.
8. Centrifuge  $11,000 \times g$  for 90 min at 4 °C.
9. Remove supernatant.
10. Wash with PBS.
11. Centrifuge at  $11,000 \times g$  for 90 min at 4 °C.
12. Remove supernatant.
13. Resuspend exosomes in 100  $\mu$ l.
14. Store exosomes at -80 °C.

### 3.3.2 Evaluation of In Vitro Uptake of Exosomes

Day 1

1. Treat a rounded cover slide for a p-24 with 0.2 % gelatin over 20 min at 37 °C.
2. Remove gelatin and let dry under the hood.
3. Plate  $2 \times 10^5$  of recipient cells per well.

Day 2

1. Treat cells with 5–10  $\mu$ g exosomes for 24 h (or other timing: 1, 5, 24 h).

Day 3

1. Carefully wash the cells twice with PBS by slowly dropping the PBS through the tissue culture wall and fix them in 1 % paraformaldehyde for 15 min.
2. Wash twice with PBS and plate can be kept for 1 week at 4 °C.
3. Incubate for 30 min at 4 °C with INMUNO solution in dark 40  $\mu$ l Immuno solution drops on a parafilm®.
4. Wash cover slide twice with PBS.
5. Mount with Vectashield®. Evaluate experiment on a fluorescence microscope.

### 3.3.3 Evaluation of In Vivo Uptake of Exosomes

After in vivo injection of exosomes, we used this protocol to evaluate the fluorescent in vivo uptake into several cell subpopulations of the bone marrow (Fig. 2) (*see Note 13*).

1. Label exosomes with PKH67, as detailed previously.
2. Quantify exosome protein of isolated exosomes.
3. Intravenously inject (i.v.) the mice with 10  $\mu$ g in 100  $\mu$ l fluorescently labeled exosomes and wait 4 h. Control mice will be i.v. injected with 100  $\mu$ l PBS.

4. Sacrifice mice and isolate the bone marrow by bone marrow “flushing” technique as follows:
  - (a) Damped briefly death mice in ethanol 70 %.
  - (b) Excised long bones from the hind limbs, perfectly clean from surrounding tissue and keep bones on  $\alpha$ -MEM with no FCS.
  - (c) Cut with scissors both bone ends. Handle the bones with tweezers.
  - (d) Use a 27 G needle for flushing the bone marrow with a syringe containing 5 ml of serum-free  $\alpha$ -MEM medium and collect it on a 50 ml tube. The bone will appear as a vivid white color.
  - (e) Spin cells to  $600\times g$  for 10 min at 4 °C and discard supernatant.
  - (f) Gently resuspend the pelleted cells in complete medium [ $\alpha$ -MEM/10 % FBS/1 % penicillin/streptomycin solution] ten times with a 18 G needle to disaggregate cell clumps, and filter the cell suspension through a 70  $\mu$ m cell strainer to retain cellular debris.
  - (g) Spin cells at  $600\times g$  for 10 min at 4 °C.
5. Resuspend cells on 5 mL of red blood lysis buffer and leave 5 min at 37 °C.
6. Neutralize lysing buffer with 5 mL complete medium.
7. Spin cells at  $600\times g$  for 10 min at 4 °C.
8. Resuspend cells in PBS and count the cells.
9. Spin cells to  $900\times g$  for 10 min at 4 °C.
10. Resuspend in Automacs Buffer and maintain at 4 °C until use.
11. For different subpopulation sorting, plate 100  $\mu$ l ( $2\times 10^6$  cells) in a U-bottom 96-well plate.
12. Add diluted antibody that works for flow cytometry (*see Note 14*).
13. Incubate for 15 min at 4 °C in the dark.
14. Centrifuge at  $800\times g$  for 2 min at 4 °C.
15. Discard supernatant and resuspend pellet in 100  $\mu$ l Automacs buffer.
16. Take volume to a cytometer tube and raise volume to 300  $\mu$ l.
17. Use flow cytometer.

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## 4 Notes

1. Sterilize ultracentrifuge tubes and adaptors under UV light for 15 min. To avoid contamination be careful with the tissue culture technique. Serum used to complement cell medium used all along this procedure must be exosome-depleted.
2. Before operating the centrifuge, operators should be carefully instructed on its use. Several key points should scrupulously be observed. For instance, the way of handling the rotor, the type of tubes to be used, filling the tubes almost to the top, and the equilibration of the tubes using a balance are critical measures to be taken before each ultracentrifugation.
3. Before choosing the cell line of study it is worthy to check the amount of exosomes secreted by each cell line. Please note that the amount of secreted exosomes is cell-line dependent.
4. Since stress conditions could increase the exosome secretion, it is important to keep and regularly check that all cell culture conditions (CO<sub>2</sub>, humidity, pH, mycoplasma-free, etc.) are exquisitely maintained all along different extraction procedures.
5. We use a six conical 35 ml ultracentrifuge tubes (Beckman Ref #358126) with their adaptors Ref #358156. We distributed 60 ml of CM, leaving 30 ml of conditioned medium per tube.
6. The amount of T-75 flasks will depend on the amount of exosomes secreted by the chosen cell line. For some cell lines, one flask could be enough.
7. It is recommended to check the quality of the exosome purification with other methods commercially available such as ExoQuick® or Dynabeads®.
8. It is recommended to perform protein quantification with small aliquot in each round of isolation (we used 3 µl of exosome suspension).
9. Use a negative control running the same protocol but skipping **step 7**.
10. Before starting to work with RNA, clean all the bench surfaces with ethanol 70 % and an RNase solution (RNase away, Molecular BioProducts). Special care should be put during isolation and analysis of miRNA. All solutions should be prepared using RNase-free glassware, autoclaved water and chemicals reserved to work with RNA. Disposable gloves should be worn all along these procedures. Diligently follow all waste disposal regulations when disposing waste materials. DEPC should be handled with care in a fume hood.

11. For each sample, add the DEPC water in the first tube, resuspend and transfer to the second tube, and resuspend and pass to the third tube.
12. Work in low light conditions since fluorescent dyes are photosensitive.
13. This protocol could be adapted to other cell types as long as the antibody specific of this cellular phenotype works on flow cytometry.
14. The dilution of each antibody should experimentally adjusted.

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