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Isolation of Extracellular Vesicles from Murine Bronchoalveolar Lavage Fluid Using an Ultrafiltration Centrifugation Technique

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Phillip Steindel, PhD.

Review Editor, Journal of Visualized Experiments

August 6, 2018

Dear Dr. Steindel,

We appreciate the comments of the editors on our manuscript entitled “Isolation of Extracellular Vesicles from Murine Bronchoalveolar Lavage Fluid Using an Ultrafiltration Centrifugation Technique”. We have made all changes per editorial suggestion. Our responses to editorial comments are enclosed with this letter.

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agree with submission to Journal of Visualized Experiments. The authors have no conflicts of interest to declare.

We would like to thank you for this opportunity. We look forward to hearing from you at your earliest convenience.

Yours sincerely,

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TITLE:

Isolation of Extracellular Vesicles from Murine Bronchoalveolar Lavage Fluid Using an Ultrafiltration Centrifugation Technique

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KEYWORDS:

Extracellular vesicles, exosomes, ultrafiltration centrifugation, ultracentrifugation, isolation technique, bronchoalveolar lavage fluid

SUMMARY:

Here, we describe two extracellular vesicle isolation protocols, ultrafiltration centrifugation and ultracentrifugation with density gradient centrifugation, to isolate extracellular vesicles from murine bronchoalveolar lavage fluid samples. The extracellular vesicles derived from murine bronchoalveolar lavage fluid by both methods are quantified and characterized.

ABSTRACT:

Extracellular vesicles (EVs) are newly discovered subcellular components that play important roles in many biological signaling functions during physiological and pathological states. The isolation of EVs continues to be a major challenge in this field, due to limitations intrinsic to each technique. The differential ultracentrifugation with density gradient centrifugation method is a commonly used approach and is considered to be the gold standard procedure for EV isolation. However, this procedure is time-consuming, labor-intensive, and generally results in low scalability, which may not be suitable for small-volume samples such as bronchoalveolar lavage fluid. We demonstrate that an ultrafiltration centrifugation isolation method is simple and time- and labor-efficient yet provides a high recovery yield and purity. We propose that this isolation method could be an alternative approach that is suitable for EV isolation, particularly for small-volume biological specimens.

INTRODUCTION:

Exosomes are the smallest subset of (EVs), 50 - 200 nm in diameter, and have multiple biological functions across a diverse array of signaling processes[1-5]. They govern cellular and tissue homeostasis primarily by facilitating intercellular communication through cargo molecules such as lipids, proteins, and nucleic acids[6-9]. One critical step in EV research is the isolation process. Differential ultracentrifugation (UC), with or without density gradient centrifugation (DGC), is considered the gold standard approach, but this method carries major limitations, including inefficient EV recovery rates and low scalability[10-12], that restrict its best utilization to larger volume samples, such as cell culture supernatant or high exosome production specimens. The advantages and disadvantages of other methods, such as size exclusion by ultrafiltration or chromatography, immunoaffinity isolation by beads or columns, and microfluidics, are well described, and modern supplemental procedures have been developed to overcome and minimize technical limitations in each approach[11-15]. Others have shown that an ultrafiltration centrifugation (UFC) with a nanoporous membrane in the filter unit is an alternative technique that provides comparable purity to a UC method[16-18]. This technique could be considered as one of the alternative isolation methods.

Bronchoalveolar lavage fluid (BALF) contains EVs that possess numerous biological functions in various respiratory conditions[19-22]. Studying BALF-derived EVs entails some challenges due to the invasiveness of the bronchoscopy procedure in humans, as well as a limited amount of lavage fluid recovery. In small laboratory animals such as mice, only a few milliliters can be recovered in normal lung conditions, even less in inflamed or fibrotic lungs[23]. Consequently, collecting a sufficient amount of BALF for EV isolation by a differential ultracentrifugation for downstream applications may not be feasible. However, isolating correct EV populations is a crucial factor for studying EV biological functions. The delicate balance between efficiency and efficacy continues to be a challenge in well-established EV isolation methods.

In this current study, we demonstrate that a centrifugal ultrafiltration approach, utilizing a 100-kDa molecular weight cut-off (MWCO) nanomembrane filter unit, is suitable for small-volume biological specimen such as BALF. This technique is simple, efficient, and provides high purity and scalability to support the study of BALF-derived EVs.

PROTOCOL:

The utilization of animals and all animal procedures were approved by the Institutional Animal Care and Use Committees (IACUC) at Cedars-Sinai Medical Center (CSMC).

1. Murine Bronchoalveolar Lavage Fluid (BALF) Collection and Preparation

1.1. BALF collection

1.1.1. Euthanize mice with a cocktail of ketamine (300 mg/kg) and xylazine (30 mg/kg) *via* the intraperitoneal route followed by cervical dislocation.

1.1.2. Insert a 22-G angiocatheter into the trachea. Attach an insulin syringe containing 1 mL (mL) of ice-cold sterile Dulbecco's phosphate buffer saline (DPBS) and instill 1 mL of DBPS into both lungs through the angiocatheter.

1.1.3. Slowly withdraw the syringe plunger to retrieve BALF and dispense the BALF into a 50-mL conical tube. Keep the BALF on ice.

1.1.4. Repeat steps 1.1.2 and 1.1.3 3x (4x in total in each mouse).

Note: Approximately 0.8 mL is generally retrieved per milliliter of instillation. Also, the following steps can be performed for individual mice (*i.e.*, 3 mL of BALF), but pooling multiple BALF samples will allow the isolation of a larger batch of EVs for consistency in downstream experiments.

1.2. BALF preparation

1.2.1. Pool BALF from 25 mice and divide it into two equal sets (~35 mL per aliquot).

1.2.2. Centrifuge the BALF at 400 x g, at 4 °C for 5 min, to remove cells and other cellular debris and collect the supernatant.

1.2.3. Centrifuge the supernatant at 1,500 x g, at 4 °C for 10 min, to remove cell debris. Collect the supernatant and proceed to the EV isolation steps.

2. Isolation of Extracellular Vesicles from Murine Bronchoalveolar Lavage Fluid

Note: In this study, two EV isolation techniques, namely UFC and ultracentrifugation with buoyant density gradient centrifugation (UC-DGC), are used to isolate EVs from BALF. The detailed protocol of each method is described below.

2.1. Ultrafiltration centrifugation (UFC) enrichment method

Note: This method was modified from a previously described protocol[10].

2.1.1. Filter the supernatant from step 1.2.3 through a 0.2-µm sterile syringe filter and keep the filtered BALF on ice.

Note: This is a size exclusion step whereby only vesicles smaller than 200 nm are collected.

2.1.2. Equilibrate the 100-kDa MWCO centrifugal filter unit with sterile DPBS for 10 min. Centrifuge the centrifugal unit at 1,500 x g for 10 min at 4 °C to discard the DPBS.

CAUTION: Once the membrane in the filter device is equilibrated with DPBS, the membrane must be kept wet at all time until the device is used.

2.1.3. Fill the filter unit with 15 mL of BALF sample from step 2.1.1 and centrifuge at 3,000 x g for 30 min at 4 °C. The flow-through BALF can be discarded or collected into a separate canonical tube and stored at (-)80 °C for future use.

2.1.4. Repeat step 2.1.3 for the remaining 0.2-µm-filtered BALF.

Note: It took three repetitions of centrifugations to sufficiently concentrate the BALF EVs from the original starting volume of 35 mL. This resulted in 1 - 1.5 mL of retentate.

2.1.5. Wash the retentate with 14 mL of sterile DPBS by a gently pipetting repetitively. Centrifuge the filter unit at 3,000 x g, at 4 °C for 30 min, to remove the DPBS and to concentrate the EV retentate.

2.1.6. Collect the concentrated BALF-derived EVs from the filter device by inserting a pipettor into the bottom of the filter device and withdrawing the sample using a side-to-side sweeping motion to ensure total recovery.

2.1.7. Aliquot the BALF-derived EVs and store them at (-)80 °C for further particle quantification and characterization (see step 3).

2.2. Ultracentrifugation (UC) with buoyant density gradient centrifugation (DGC)

Note: The following protocol was modified from the previously described protocol[24].

2.2.1. Transfer the supernatant from step 2.1.1 into a 37-mL ultracentrifuge tube and centrifuge the sample at 10,000 x g for 30 min at 4 °C using ultracentrifuge. Collect the supernatant and centrifuge at 100,000 x g, at 4 °C for 60 min. While the EV pellets are centrifuged, prepare different concentrations of buoyant density gradient buffers (**Table 1**) for step 2.2.3.

2.2.2. Discard the supernatant and resuspend the EV pellets in 200 µL of DPBS.

2.2.3. Mix the EV suspension with 300 µL of 50% iodixanol working solution (**Table 1**) and transfer it to the 15-mL ultracentrifuge tube. On top of the 50% iodixanol-EV mixture suspension, sequentially layer the following buffer solution in the order from the bottom to the top: 30% iodixanol (4.5 mL), 25% of iodixanol (3 mL), 15% iodixanol (2.5 mL), and 5% iodixanol (6 mL). Centrifuge at 100,000 x g, at 4 °C for 230 min.

Note: The buoyant density gradient is based on the percent of iodixanol scaling with the highest concentration (50%) at the bottom to the lowest concentration (5%) at the top. To generate different concentrations of iodixanol, various amounts of homogenization medium (**Table 1**) were mixed with working solution (50% iodixanol).

2.2.4. Collect the 15% and 25% fraction and dilute them in sterile DPBS to bring up the volume to 15 mL. Transfer them to a new small ultracentrifuge tube and centrifuge at 100,000 x g, at 4 °C for 60 min.

2.2.5. Discard the supernatant and resuspend the EV pellets in 50 µL of sterile DPBS for further characterization.

3. Extracellular Vesicle Quantification

Note: The BALF-derived EVs recovery yield is quantitated with two metrics.

3.1. Nanoparticle tracking analysis (NTA) measurement

3.1.1. Dilute the EV sample at 1:200 - 1:500 in 1 mL of DPBS and load the sample into an insulin syringe. Attach a sample syringe to a syringe pump and begin to measure the particle numbers and size (see **Table of Materials**).

3.1.2. Set the camera level at 14 and the detection threshold at 1 for all sample measurements. Five repetitive measurements with 1,500 frames in 30 s were recorded for each sample, with a delay of 20 s between reads. Combine and average the data for final concentration and size reports.

Note: For accurate capturing of all particles, adjust the camera level as appropriate to visualize all particles and use similar settings for all sample measurements in each experiment.

3.2. Protein quantification

Note: The bicinchoninic acid (BCA) protein assay was used to measure the protein concentration of the BALF-derived EVs.

3.2.1. Solubilize the EV samples in 1x lysis buffer.

3.2.2. Quantify the amount of protein in the BALF-derived EVs per BCA standard protocol using colorimetric detection by measuring the absorbance at 560 nm in a plate reader.

4. Detection of BALF-derived Extracellular Vesicles

Note: Commonly known exosome surface marker proteins (TSG101, CD63, CD81, and CD9) were used to verify EV recoveries by SDS-PAGE and immunoblotting and flow cytometry analysis.

4.1. SDS-PAGE and immunoblotting

4.1.1. Dissolve an equal amount of EV proteins of each sample with a blotting loading buffer (lithium dodecyl sulfate) and 50 mM dithiothreitol (DTT) in a 1.6-mL tube. Heat the samples at 70 °C for 10 min.

4.1.2. Load the samples from step 4.1.1 into a 4% - 12% Bis-Tris Plus acrylamide gel and run electrophoresis (150 volts, 35 mA) for 35 min.

4.1.3. Transfer proteins to a nitrocellulose membrane using a dry transfer method.

4.1.4. Block the membrane with 5% skimmed milk for 60 min, rocking at room temperature (RT).

4.1.5. Incubate the membrane with an antibody to an EV surface protein marker, Tumor Susceptibility Gene 101 (TSG101), at 1:500 dilution in 5% BSA in Tris-buffered saline Tween-20 (TBST) at 4 °C, rocking overnight.

4.1.6. The next day, wash the membrane 3x, 10 min each wash, in TBST buffer, and incubate it with anti-rabbit IgG, an HRP-linked antibody, at 1:5,000 dilution for 60 min at RT.

4.1.7. Wash the membrane 3x, 10 min each wash, in TBST buffer. Develop the membrane with chemiluminescent HRP antibody detection reagent and image (see **Table of Materials** for imaging system).

4.2. Flow cytometry

4.2.1. Dilute BALF-derived EVs in 49 µL of PEB staining buffer (PBS + 5 mM EDTA + 0.5% BSA, filtered through a 0.1-µm syringe filter membrane).

4.2.2. Add each of the following antibodies into each individual sample: 1) rat anti-mouse PE CD63 antibody (100 ng per reaction); 2) rat anti-mouse PE CD81 (500 ng); 3) rat anti-mouse FITC CD9 (200 ng). Incubate at 4 °C, rocking for 60 min in the dark.

4.2.3. Dilute the samples with 450 µL of membrane-filtered PEB staining buffer and subject the samples to flow cytometry analysis (see **Table of Materials**)[25].

4.2.4. Adjust the flow cytometer settings as follows: 1) set all channels on hyper log (hlog); 2) set the trigger on SSC at 4; 3) turn off the secondary trigger. Run the samples in a low-speed setting and acquire at least 10,000 events in each sample.

4.2.5. Perform low cytometry data analysis in each sample using analysis software (see **Table of Materials**).

REPRESENTATIVE RESULTS:

We performed EV isolation from mouse BALF using UFC and UC-DGC isolation methods on the same day. The UFC method required approximately 2.5 - 3 h, whereas the UC-DGC technique

required 8 h of processing time. This did not include buffers and reagent preparation time. It should be noted that some other tasks could be performed during the long centrifugation periods. Nevertheless, the entire procedure lasted nearly an entire day for the UC-DGC isolation technique.

BALF-derived EVs from normal mice isolated by the UFC method displayed a smaller size and a more uniform size distribution (148.8 ± 1.1 nM, **Figure 1A**) compared to UC-DGC EVs (176.7 ± 7.8 nM, **Figure 1B**). The UFC technique had a profound 65-fold greater total particle counts when compared to UC-DGC isolation (29.4 ± 18.4 *vs.* 0.5 ± 0.1 x 10^{10} particles; $p < 0.05$; **Table 2** and **Figure 1C**). The total protein recovery (in μg) of the UFC EVs was also higher (3,136 ± 1,860 *vs.* 73.7 ± 38.3 μg; $p < 0.05$; **Table 2** and **Figure 1D**). Thus, UFC is time- and effort-efficient and provides a higher EV yield.

To further phenotypically characterize BALF-derived EV populations, we examined the presence of commonly known exosome surface protein markers: CD63, CD9, and CD81 by flow cytometry and TSG101 by immunoblotting. Using flow cytometry analysis, we demonstrated that UFC EVs and UC-DGC EVs both expressed CD63 (**Figures 2A** - **2C**), CD9 (**Figures 2D** - **2F**), and CD81 (**Figures 2G** - **2I**). The geometric mean expression (gMFI) of CD63, CD9, and CD81 was quantified and not statistically different between the two conditions (**Figures 3A** - **3F**).

Next, we examined another EV protein marker, TSG101, by immunoblotting. We showed that the 20 μg of the UFC flow-through (UFC-FT) sample did not contain TSG101 proteins, suggesting that the UFC isolation technique efficiently selected and retained the EV population from the BALF sample (**Figure 4**). When equal amounts of total protein (20 μg) from the BALF-EV sample was loaded, we found that UFC EVs expressed a higher level of TSG101 than UC-DGC EVs (**Figure 4**). We also showed that the purity of UFC-EV protein was acceptable, demonstrated by a single isolated protein band.

For all results, Student's *t*-test was used for two-group comparison. The results are presented as mean ± SEM (standard error of the mean), and $p < 0.05$ was considered significant.

TABLE AND FIGURE LEGENDS:

Figure 1: Ultrafiltration centrifugation of murine bronchoalveolar lavage fluid (BALF)-derived EVs demonstrated a more homogenous size distribution than ultracentrifugation with density gradient centrifugation of murine BALF-derived EVs and had a significantly higher total particle count and protein content. The distribution of EV particle sizes was measured by nanoparticle tracking analysis (NTA), and the total protein content was measured by the bicinchoninic acid assay. (**A**) UFC-BALF EVs' NTA size distribution graph. (**B**) UC-DGC-BALF EVs' size distribution graph. (**C**) Total particle count by NTA (mean ± SEM x 10^{10} particles; * $p < 0.05$). (**D**) Total protein content (in μg, * $p < 0.05$). The data were derived from three independent experiments. UFC = ultrafiltration centrifugation; UC-DGC = ultracentrifugation with density gradient centrifugation; EVs = extracellular vesicles; SD = standard deviation; Conc = concentration.

Figure 2: Murine bronchoalveolar lavage fluid-derived EVs isolated by ultrafiltration centrifugation and ultracentrifugation with density gradient centrifugation methods expressed tetraspanin proteins CD63, CD9, and CD81. Shown are percentages of EVs stained positive by the UFC and UC-DCG isolation techniques, illustrated by pseudocolor plots, for (**A** - **C**) PE-CD63, (**D** - **F**) FITC-CD9, and (**G** - **I**) PE-CD9. The data are derived from three independent experiments. UFC = ultrafiltration centrifugation; UC-DGC = ultracentrifugation with density gradient centrifugation; EVs = extracellular vesicles; SSC-A = side scatter analysis; PE = phycoerythrin; FITC = fluorescein isothiocyanate.

Figure 3: Ultrafiltration centrifugation-isolated murine bronchoalveolar lavage fluid-derived EVs expressed a similar fluorescent density of tetraspanin proteins to ultracentrifugation-isolated EVs. (**A** and **D**) Histogram and geometric mean expression (gMFI) of PE-$CD63^+$-stained EVs. (**B** and **E**) Histogram and gMFI of FITC-$CD9^+$-stained EVs. (**C** and **F**) Histogram and gMFI of PE-$CD81^+$-stained EVs. These data are derived from three independent experiments. UFC = ultrafiltration centrifugation; UC-DGC = ultracentrifugation with density gradient centrifugation; EVs = extracellular vesicles; SSC-A = side scatter analysis; PE = phycoerythrin; FITC = fluorescein isothiocyanate.

Figure 4: Murine bronchoalveolar lavage fluid-derived EVs isolated by ultrafiltration centrifugation and ultracentrifugation with density gradient centrifugation methods expressed the exosome surface protein, TSG101. This panel shows the immunoblotting of murine BALF-derived EVs for the TSG101 antibody (47 kDa). UFC = ultrafiltration centrifugation; UC-DGC = ultracentrifugation with density gradient centrifugation; EVs = extracellular vesicles; FT = flow-through; TSG = tumor susceptibility gene.

Table 1: Buoyant density gradient buffers. This table gives the composition and buffer ratio of each gradient solution that was used to purify murine bronchoalveolar lavage fluid-derived extracellular vesicle populations isolated by the ultracentrifugation technique.
* The working solution was 50% iodixanol (25 mL of density gradient medium [see **Table of Materials**] + 5 mL of diluent solution [pH 7.4 of 0.25 M sucrose + 120 mM HEPES + 0.9 M NaCl]).
** Homogenization medium (pH 7.4 of 0.25 M sucrose + 20 mM HEPES + 150 mM NaCl)

Table 2: The ultrafiltration centrifugation isolation method provided a high murine bronchoalveolar lavage fluid-derived extracellular vesicle yield. The particle concentration and total particle count were measured by nanoparticle tracking analysis (NTA). The protein concentration and total amount of protein were measured by a bicinchoninic acid protein assay.
* BALF EVs' particle concentration (mean ± SEM x 10^8/µL from three independent experiments).
† BALF EVs' total particle (mean ± SEM x 10^{10} particles from three independent experiments).
‡ BALF EVs' protein concentration (mean ± SEM µg/µL from three independent experiments).
§ BALF EVs' total protein (mean ± SEM µg from three independent experiments).
UFC = ultrafiltration centrifugation; UC-DGC = ultracentrifugation with density gradient centrifugation; Conc = concentration; NTA = nanoparticle tracking analysis; SEM = standard error of the mean.

DISCUSSION:

In the past few decades, scientists have unraveled the significances of EVs in cellular homeostasis. More importantly, EVs play major roles in many disease processes by modulating neighboring and distant cells through their bioactive cargo molecules[1,21,22,26-30]. Future development and advancement in this field profoundly rely upon reliable and efficient methods that not only identify and separate correct subsets of EV populations but also preserve their biological functions for downstream applications[10,11,14,31]. In the current study, we described a nanomembrane ultrafiltration centrifugation (UFC) method to isolate EVs from mouse BALF. In concordance with other reports, we showed that UFC is simple and results in a high recovery yield and purity and, therefore, is suitable for small biological samples[10,17,18,32].

UC-DGC is commonly used and is considered to be the gold standard technique for EV isolation because it provides highly purified EV particles[10,14]. However, this method is technically cumbersome, time-consuming, labor-intensive, and has low scalability. The newly developed microfluidics-based techniques overcome these limitations, but this approach requires further validation before it can be fully implemented as an alternative method[33,34]. Thus, appropriate methods that accommodate those difficulties without compromising the purity and scalability of samples are sorely needed, particularly for small-volume biological fluid.

We demonstrated that UFC using a nanomembrane filter device was effective for the EV isolation from BALF specimens. The findings presented here highlight the superiority of the UFC procedure in comparison to the gold standard UC-DGC method due to its simplicity and higher scalability. The ultrafiltration-based approach has become widely adopted to isolate EV from a variety of biological specimens: urine[35], cell-conditioned media[17], and fetal bovine serum[36]. The other modular size-based EV isolation technique that uses ultrafiltration as a platform is exosome total isolation chip (ExoTIC)[31]. This method is also suitable for small sample-size specimens. A few factors, such as filter material and the pore size of the nanomembranes, need to be considered when using the UFC technique because they may affect the properties of recovered EVs. For example, different types of filter membranes resulted in different EV-associated RNA recovery yields from urine[18]. In this study, we showed that regenerated cellulose (RC) with a 10-kDa MWCO provided the highest mRNA expression of NOP10, OST4, SNRPG, and TOMM7 compared to Hydrosart 10 kDa, or polyethersulfone (PES) of 10 kDa[18]. We further demonstrated that the RC with 10 kDa had a higher retentate EV recovery than the 100 kDa. Others have characterized urine EVs cargo contents that were affected by the type of isolation techniques[37]. Our study showed that the 100-kDa MWCO RC membrane provided a satisfactory BALF-derived EV yield with the advantage of much less unwanted proteins in the retentate due to the larger MWCO.

This study demonstrated that the sizes and size distribution of UFC EVs were smaller and more homogeneously distributed than those of UC-DGC EVs. Vesicle aggregation, which is common with UC techniques, may explain the dimension heterogeneity of UC-DGC EVs[38]. We assessed BALF-derived EV purity by detecting the EV membrane proteins TSG101, CD63, CD9, and CD81, which confirms the presence of exosomes in the retentates. We and others have also used TEM to demonstrate the morphology of the UFC EVs[35,39-41]. Liu *et al.* used a similar ultrafiltration-based approach to isolate EVs and, when compared to EVs isolated by ultracentrifugation, the

proteomic and transcriptomic profiles were similar[31]. Thus, we describe an ultrafiltration centrifugation method using a regenerated cellulose membrane with an MWCO of 100 kDa as an alternative EV isolation method that is suitable for small biological fluid samples such as bronchoalveolar lavage fluid.

The critical steps using this UFC approach to isolate EVs from biological fluid include the initial nanoporous membrane 0.2-µm filtration to ensure that the enriched particles are in the exosomal size range and the avoidance of applying additional force that can damage or deform the exosome morphology and structures[10,42]. EVs can adhere to the filtration membrane, which results in lower scalability. Therefore, the volume of the sample should not exceed the recommended amount in each type of filter unit. We chose to use regenerated cellulose, which provided a higher mRNA yield from urine-derived EVs[18]. The type of filter membranes used can alter the recovery yield and type of EVs. Lastly, even though an MWCO of 100 kDa should eliminate the majority of proteins in the biological fluids, some protein contaminants that were larger than 100 kDa or protein aggregates were observed[43]. In this case, a washing step is critical to minimize EV and protein aggregation. Moreover, functional studies must be properly controlled in order to fully interpret the results, as non-EV-associated proteins will be present in the UFC EVs.

We conclude that UFC is an alternative approach that is feasible for EV isolation for small- or larger-volume samples. The currently available microfilter units can accommodate up to 15 mL of samples.

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DISCLOSURES:
The authors have nothing to disclose.

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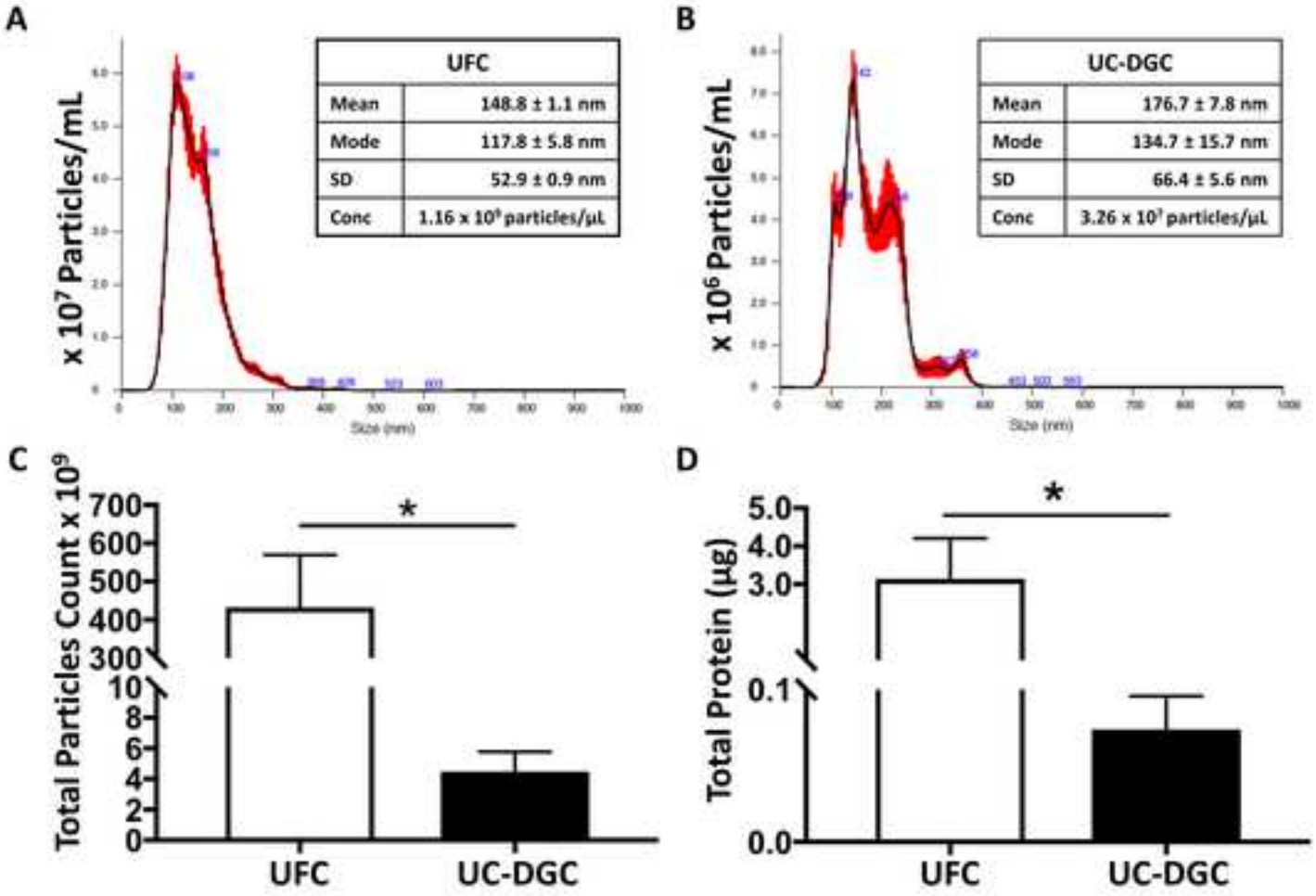
Figure



Figure

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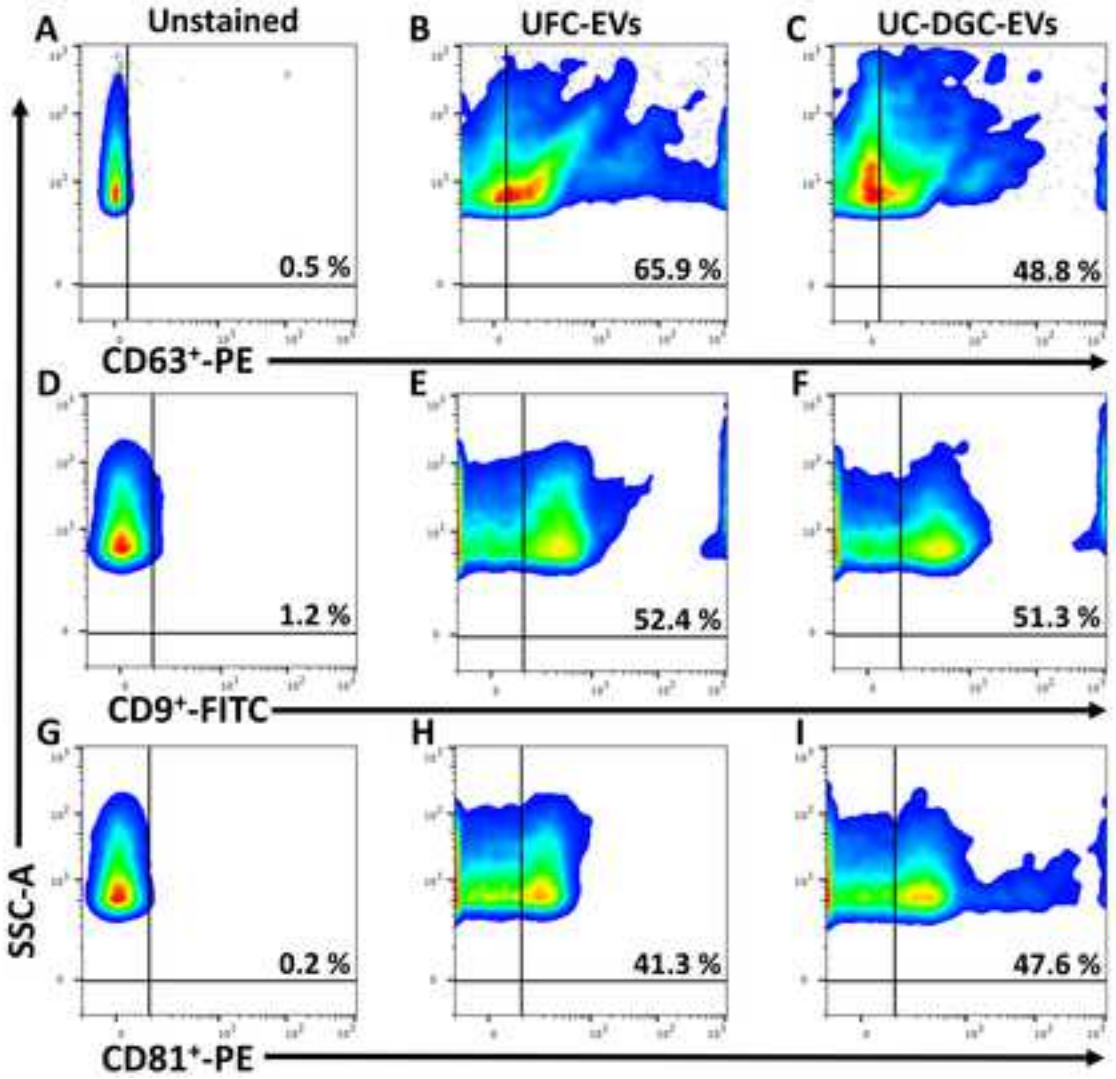

Figure

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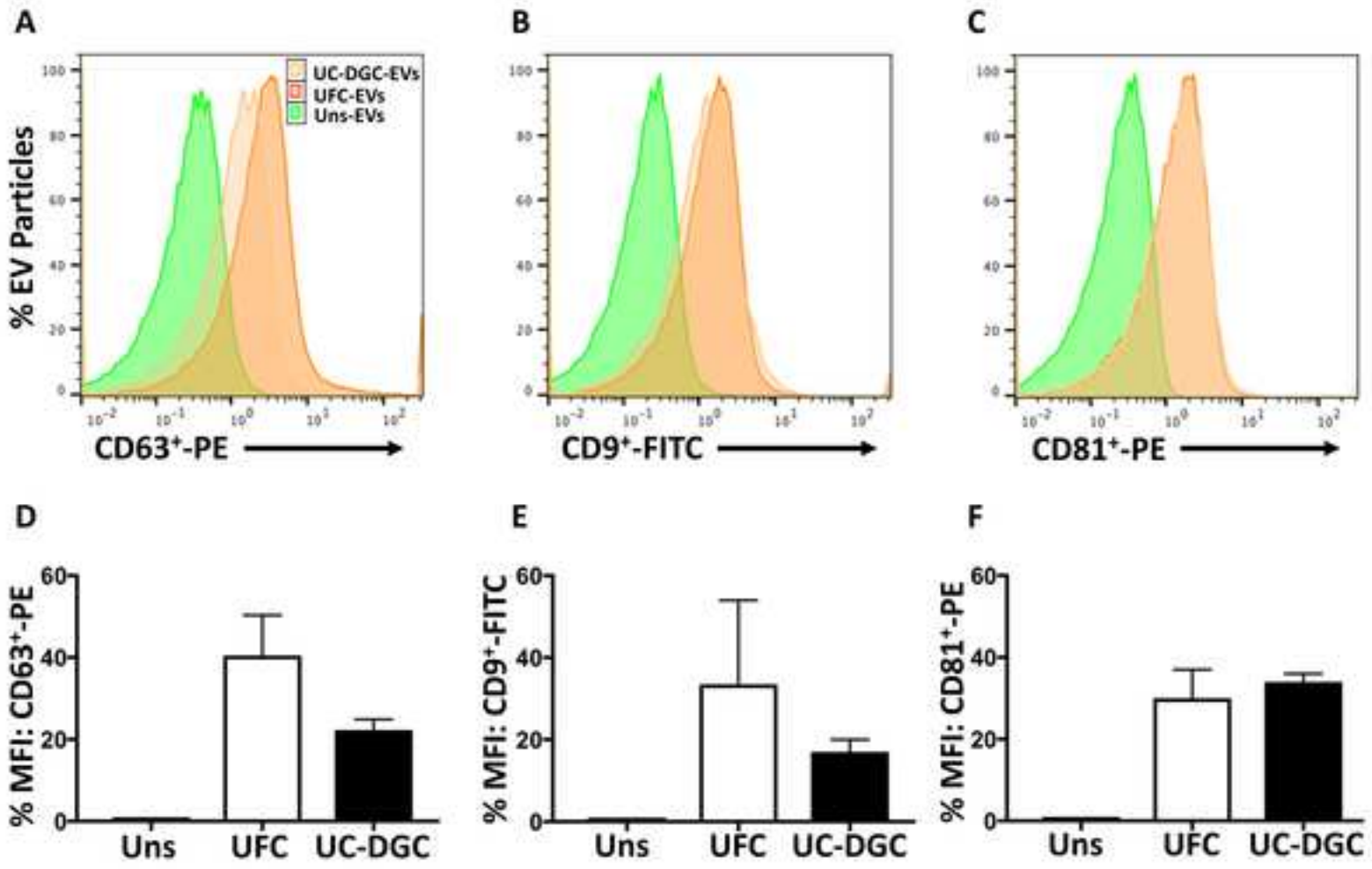
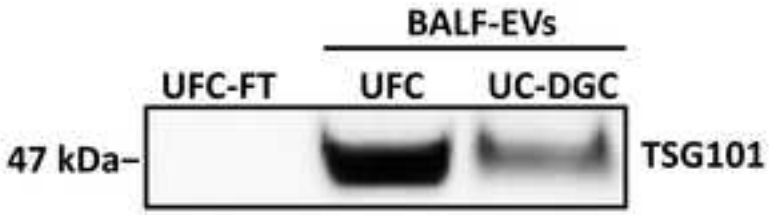


Figure



Working Solution Iodixanol (%)	5	15	25
Working solution (mL)*	2	6	10
Homogenization medium (mL)**	18	14	10

30
12
8

Methods	Starting Volume (mL)	NTA* ($x10^8/\mu L$)	Total Particles† ($x10^{10}$)
UFC	35	7.69 ± 2.6	29.4 ± 18.4
UC-DGC	35	0.5 ± 0.05	0.5 ± 0.1

Protein Conc[‡] (µg/µL)	Total Proteins[§] (µg)
3.7	3,136 ± 1860
0.6	73.7 ± 38.3

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials-UFC BALF-derived EV isolation 081318.xlsx

Name of Material/ Equipment
Material
Amicon Ultra-15 centrifugal filters Ultracel-100K
Dulbecco's Phosphate Buffered Saline (DPBS)
Sucrose
HEPES
EDTA
Sodium Chloride
OptiPrep
Ketamine
Xylazine
Syringe 1 mL
Angiocatheter 20G
Centrifuge tubes 15 mL
Centrifuge tubes 50 mL
Bicinchonic acid (BCA) protein assay
Rabbit anti-mouse TSG101 Antibody
Rat anti-mouse PE-CD63 Antibody
CD81
CD9
Anti-rabbit IgG, HRP-linked antibody
4x LDS
10x Reducing agent (Bolt)
10x Lysis buffer (Bolt)
Bolt 4-12% Bis-Tris Plus acrylamide gel
iBlot 2 Nitrocellulose mini stacks
Chemiluminescent HRP antibody detection reagent HyGLO

Company	Catalog Number	Comments/Description
Sigma-Millipore, St. Louis, MO	UFC910024	
Corning Cellgro, Manassas, VA	21-031-CV	
Sigma-Millipore, St. Louis, MO	EMD8550	
Research Products International, Prospect, IL	75277-39-3	
Corning Cellgro, Manassas, VA	46-034-CI	
Sigma-Millipore, St. Louis, MO	S3014-1KG	
Sigma-Millipore, St. Louis, MO	MKCD9753	Density Gradient Medium
VetOne, Boise, ID	13985-702-10	
Akorn Animal Health, Lake Forest, IL	59399-110-20	
BD Syringe, Franklin Lakes, NJ	309656	
BD Syringe, Franklin Lakes, NJ	381703	
VWR, Radnor, PA	89039-666	
Corning Cellgro, Manassas, VA	430828	
Pierce, Thermo Fischer Scientific, Rockford, IL	23235	
AbCam, Cambridge, MA	AB125011	
Biolegend, San Diego, CA	143904	
Cell Signaling Technology, Danvers, MA	7074S	
Cell Signaling Technology, Danvers, MA		
Invitrogen, Thermo Fisher Scientific, Waltham, MA	NW04120	
Invitrogen, Thermo Fisher Scientific, Waltham, MA	IB23002	
Denville Scientific, Holliston, MA	E2400	

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Author(s): Tanyalak Parimon, Noman E Garrett III, Peter Chen, Travis Antes

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1. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: The 2.75-page protocol for filmable content was highlighted.

2. 4.2.2: How much of these antibodies is added?

Response: The amount of each antibody in each reaction were the followings;

- Anti-mouse CD63-PE was 100 ng
- Anti-mouse CD9-FITC was 500 ng
- Anti-mouse CD81-PE was 200 ng

3. Figure 1A, B: It looks like the units in the boxes should be nm, not nM. Also, where do the errors for these values come from?

Response: The true meaning of nM is nanometer not nanomole. The unit abbreviation was changed to nm per editorial suggestion. A revised figure 1A is attached.

4. Figure 1C, D: What statistical test is used to produce p-values?

Response: Student t-test was used. Statistics section was added into the Protocol (line 200-201).

5. Figure 2: Please either combine A and B (and adjust panel labelling accordingly) or split this into Figures 2 and 3.

Response: Figure 2A and 2B were split into 2 separate figures, specifically, figure 2A is now figure 2 and figure 2B is now figure 3.

6. Figure 2A: What is the scale for the psuedocolor plots?

Response: The scale for the pseudocolor plots was hyper log (hlog) scale.

7. Figure 2B: What are the error bars for the gMFI plots?

Response: The error bars for the gMFI plots was the standard error of the mean (SEM).

8. Table 1: What are the stars here? What are the working and homogenization media? They do not seem to be explained elsewhere, including in the Table of Materials.

Response: One-star (* asterixis symbol) referenced the composition of working solution. Two-star (**) referenced the composition of homogenization medium. The explanation of these symbols was present in the original submission but was mistakenly left behind in the revised version. The definitions of * and ** were added in Table 1 legend (line 288-290). For further clarification, the use of these buffers were added in the protocol section as "Note" (line 139-142).

9. References: Please ensure all journal names are written out (e.g., see ref. 8).

Response: Changes were made per editorial suggestion.