

# Journal of Visualized Experiments

## Isolation and characterization of microvesicles from peripheral blood

--Manuscript Draft--

<b>Manuscript Number:</b>	JoVE55057R1
<b>Full Title:</b>	Isolation and characterization of microvesicles from peripheral blood
<b>Article Type:</b>	Invited Methods Article - JoVE Produced Video
<b>Keywords:</b>	extracellular vesicles; microvesicles; blood; flow cytometry; biomarkers; liquid biopsy; cancer
<b>Manuscript Classifications:</b>	1.11.284.430.214.190.875.190.880: Transport Vesicles; 1.11.284.430.214.190.875.190.880.495: Exosomes; 4.23.101: Biological Markers; 5.1.370.225: Clinical Laboratory Techniques; 5.5.200.500.386.350: Flow Cytometry
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<b>Abstract:</b>	The release of extracellular vesicles (EVs) including small endosomal-derived exosomes (Exos, diameter <100 nm) and large plasma membrane-derived microvesicles (MVs, diameter >100 nm) is a fundamental cellular process that occurs in all living cells. These vesicles transport proteins, lipids and nucleic acids specific for their cell of origin and in vitro studies have highlighted their importance as mediators of intercellular communication. EVs have been successfully isolated from various body fluids and especially EVs in blood have been identified as promising biomarkers for cancer or infectious diseases. In order to allow the study of MV subpopulations in blood, we present a protocol for the standardized isolation and characterization of MVs from peripheral blood samples. MVs are pelleted from EDTA-anticoagulated plasma samples by differential centrifugation and typically possess a diameter of 100 - 600 nm. Due to their larger size they can easily be studied by flow cytometry, a technique that is routinely used in clinical diagnostics and available in most laboratories. Several examples for quality control assays of the isolated MVs will be given and markers that can be used for the discrimination of different MV subpopulations in blood will be presented.
<b>Author Comments:</b>	
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**TITLE:**

**Isolation and characterization of microvesicles from peripheral blood**

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**KEYWORDS:**

extracellular vesicles, microvesicles, blood, flow cytometry, biomarkers, liquid biopsy, cancer

**SHORT ABSTRACT:**

Extracellular vesicles present in blood have been suggested as novel biomarkers for various diseases. Here, we present a protocol for the isolation of large plasma membrane-derived

microvesicles from peripheral blood samples and their subsequent analysis by conventional flow cytometry and Western Blotting.

#### **LONG ABSTRACT:**

The release of extracellular vesicles (EVs) including small endosomal-derived exosomes (Exos, diameter <100 nm) and large plasma membrane-derived microvesicles (MVs, diameter >100 nm) is a fundamental cellular process that occurs in all living cells. These vesicles transport proteins, lipids and nucleic acids specific for their cell of origin and *in vitro* studies have highlighted their importance as mediators of intercellular communication. EVs have been successfully isolated from various body fluids and especially EVs in blood have been identified as promising biomarkers for cancer or infectious diseases. In order to allow the study of MV subpopulations in blood, we present a protocol for the standardized isolation and characterization of MVs from peripheral blood samples. MVs are pelleted from EDTA-anticoagulated plasma samples by differential centrifugation and typically possess a diameter of 100 - 600 nm. Due to their larger size, they can easily be studied by flow cytometry, a technique that is routinely used in clinical diagnostics and available in most laboratories. Several examples for quality control assays of the isolated MVs will be given and markers that can be used for the discrimination of different MV subpopulations in blood will be presented.

#### **INTRODUCTION:**

In the last years many *in vitro* studies have demonstrated that extracellular vesicles (EVs) play an important role in intercellular communication. Living cells constantly shed vesicles which differ in size, content and biogenesis. The best studied EVs are exosomes which originate from the endosomal system where they are stored as intraluminal vesicles in multivesicular bodies. Once the latter fuse with the plasma membrane, the contained vesicles are released as exosomes (Exos, diameter 30-100 nm<sup>1</sup>). A second population of EV which has gained increasing attention in the last years are large microvesicles (MVs, diameter 100-1000 nm) which bud off directly from the plasma membrane <sup>2</sup>.

Both types of vesicles are surrounded by a lipid bilayer and contain nucleic acids *e.g.* DNA, mRNA or miRNA <sup>3-5</sup>, and a plethora of proteins which they can transfer to neighboring cells. While in general the protein composition of the vesicles reflects the state of the cell of origin, some proteins seem to be selectively targeted and enriched on EVs <sup>1</sup>. A major research interest is to characterize EVs from abnormal and diseased cells in order to define specific EV signatures which might allow the use of EVs as novel biomarkers. Especially in cancer, in which often the tumor itself is not easily accessible, liquid biopsies targeting tumor-specific EVs in blood might allow monitoring of therapy responses or help characterizing the primary tumor without the need for invasive procedures <sup>6</sup>.

Indeed, EVs have already been successfully isolated from various body fluids including urine <sup>7</sup>, CSF <sup>8</sup>, breast milk <sup>9</sup> or blood <sup>10</sup>. Several studies identified changes in EV counts and composition in different human diseases. For example, in sepsis patients the number of pro-coagulant MVs is significantly increased compared to healthy individuals <sup>11</sup>. Also in patients with severe cerebral malaria an increase in total MVs in blood can be observed and counts of platelet-



derived MVs correlate with coma depth and thrombocytopenia <sup>12</sup>. Other studies report elevated numbers of endothelium-derived vesicles in patients with systemic lupus erythematosus or heart failure and in the case of the latter, this correlates with a higher probability of cardiovascular events <sup>13,14</sup>.

Especially in cancer, EVs in blood are currently discussed as novel biomarkers with diagnostic and prognostic value. Levels of MVs expressing tumor-associated proteins such as MUC1, EGFR or FAK seem to be elevated in the blood of breast cancer patients <sup>15,16</sup>. Also for Exos, recent studies have shown that blood-derived Exos carrying tumor-specific antigens such as Glypican-1 for pancreatic cancer or Del-1 for breast cancer allow early disease detection with high specificity and sensitivity <sup>17,18</sup>. Additionally, serum-derived tumor Exos may contain DNA which can be used for detection of mutations such as KRAS and p53 which suggests their use for therapy prediction <sup>19</sup>. Recent advances have shown that analysis of Exos in blood of glioblastoma patients using a specific microfluidic chip allows monitoring of therapy <sup>20</sup>. Taken together, these findings imply that analysis of disease-specific subpopulations of vesicles gives valuable information about diagnosis, prognosis as well as therapeutic options and success.

However, isolation and analysis of Exos from blood is time-consuming, requires special lab equipment and therefore is not yet suited for routine clinical diagnostics. In contrast, MVs can be isolated much faster and, due to their larger size, can be easily analyzed by flow cytometry without the need to couple them to latex beads as it is necessary for Exos <sup>18,21</sup>. Thus, here we present a protocol that can be used for the standardized isolation of MVs from blood samples and the subsequent characterization of MV subpopulations by flow cytometry. This protocol will allow the further study and in depth characterization of MV profiles in large patient groups which will be required in order to use MVs for everyday clinical diagnostics.

## **PROTOCOL:**

All experiments including human subjects have been approved by the local ethics committee (approval no. 3/2/14). For the choice of patients it should be noted that several factors such as age, sex, current therapy regimens and many more may influence MV composition in blood and therefore should be taken into consideration prior to sample collection <sup>22,23</sup>.

### **1. Preparation of plasma samples**

1.1) Draw 1-2 tubes of blood per donor through a 21-gauge butterfly needle into a vacutainer blood collection tube containing EDTA (1.6 mg/mL blood). Make sure to invert the tube(s) several times to guarantee efficient blood anticoagulation.

Note: The recommended volume of blood for subsequent flow cytometry and Western Blot analysis is 5-15 mL. In order to prevent MV degradation and loss, blood samples should be handled <30 min after blood withdrawal.

1.2) Prepare plasma samples by centrifuging the samples for 15 min at 1,200 x g at room temperature (RT).

1.3) Apply a valve filter in order to help the separation of plasma (= upper layer) from remaining blood cells (= lower layer).

1.4) Transfer the plasma into a 15 mL tube.

1.5) Centrifuge for 15 min at 1,500 x g, RT to pellet larger cell debris and remove remaining platelets.

1.6) Transfer the supernatant into a 15 mL tube and directly proceed with MV isolation or store samples for up to 6 months at -20 °C.

Note: The presented protocol can also be used to isolate MVs (and Exos) from cell culture supernatants. In order to do so, cultivate cells at 60-80% confluency for 24-48 h in culture medium supplemented with vesicle-depleted FCS, and then collect the supernatant. Centrifuge for 5 min at 750 x g, 4°C to deplete residual floating cells, fill the supernatant into a new 15 mL tube and centrifuge again for 5 min at 1,500 x g, 4°C to pellet larger cell debris. This supernatant can then be used for the isolation of MVs as described in Step 2.1-2.16.

## 2. Isolation of MVs

2.1) Transfer the plasma sample in a suitable centrifugation tube. If necessary, fill up tube with PBS in order to dilute the sample and prevent collapse of thin-walled tubes during the centrifugation procedure.

2.2) Centrifuge for 35 min at 14,000 x g, 4 °C.

2.3) Decant supernatant, keep tubes turned upside-down and put on a paper towel. Wait 3-5 min until all remaining supernatant has been soaked into the towel and thereby removed from the sample.

2.4) Resuspend the MV pellet in 1000 µL PBS, transfer to a 1.5 mL tube and centrifuge for 35 min at 14,000 x g, 4 °C in a tabletop centrifuge.

2.5) Aspirate supernatant.

2.6) Resuspend the MV pellet in 50-500 µL PBS, depending on the size of the pellet. Alternatively, lyse MVs directly *e.g.* in RIPA buffer (150 mM NaCl/ 0.1% SDS/ 0.5% Na-deoxycholate/ 1% Triton X-100/ 50 mM Tris, pH 7.2) for subsequent Western Blot analysis. Store MVs at -20 °C. They will remain stable for several months, but avoid repeated freeze-thaw-cycles.

2.7) Optional: Determine the MV protein concentration with a protein assay (*e.g.* Bradford or Lowry method) in order to assess MV yields or dose MVs for subsequent experiments.

2.9) If additional Exos are to be isolated from the plasma samples, decant the supernatant from step 2.3 into an ultracentrifugation tube and centrifuge for 2 h at 110,000 x g, 4 °C. Decant the supernatant as described in step 2.3, resuspend Exo pellet in 1000 µL PBS and transfer into small (1.5 mL) ultracentrifugation tubes.

2.9.1) Ultracentrifuge for 2 h at 110,000 x g, 4 °C, aspirate supernatant and resuspend Exo pellet in 50-75 µL PBS or RIPA buffer.

### **3. Characterization of MVs by flow cytometry**

3.1) Transfer 15 µL PBS+1% vesicle-depleted fetal calf serum (FCS) in a flow cytometry tube.

Note: Vesicle-depleted FCS is prepared by centrifuging heat-inactivated (30 min at 56 °C) FCS for 18 h at 110,000 x g and filtrating the supernatant through a 0.2 µm filter as described previously <sup>24</sup>.

3.2) Add 5 µg (in case of low yields 3 µg are also applicable) of MVs in PBS.

3.3) Incubate samples for 30 min at RT in order to block unspecific binding sites at the MV surface and thereby reduce background staining.

3.4) Add a fluorescently-labeled antibody against the protein-of-interest. Titrate the amount of antibody used for the staining prior to use in order to determine the optimal concentration and guarantee a low signal-to-noise ration. Make sure to also include one tube with unstained MVs as negative control and one tube of MVs stained with the matching isotype control antibody at the same concentration (e.g. if 1 µg of antibody is used, also use 1 µg of the isotype control antibody) to quantify background staining.

Note: It is also possible to perform multicolor flow cytometry by adding multiple antibodies coupled to different fluorochroms.

3.5) Incubate for 20 min at RT in the dark.

3.6) Add 250 µL PBS and proceed with measurement of the sample using a flow cytometer.

3.6.1) In case that samples cannot be measured immediately, add 150 µL PBS and 50 µL 4% paraformaldehyde (PFA) to fix samples and store at 4 °C. CAUTION: PFA is toxic. Use gloves and suitable personal protective equipment.

3.7) Reduce the threshold of the flow cytometer to the lowest value possible and search for the MV population using a forward scatter (FSC) versus side scatter (SSC) plot in logarithmic scale. Gate on the MV population and evaluate the fluorescent signal in a corresponding histogram.

### **4. Characterization of MVs by Western Blotting**

4.1) Resuspend the MV pellet directly in a suitable lysis buffer (e.g. RIPA buffer).

- 4.1.1) In case the MV pellet has already been resuspended in PBS, dilute it at least 1:1 in a suitable lysis buffer (*e.g.* RIPA buffer).
- 4.2) Determine the protein concentration of the MV sample *e.g.* by Lowry assay.
- 4.3) Prepare 10-20 µg of MVs in 22.5 µL RIPA buffer. Then add 7.5 µL 4x Laemmli loading buffer and heat for 5 min at 95 °C.
- 4.4) Load samples on a polyacrylamide gel and perform electrophoresis and immunoblotting according to standard protocols.
- 4.5) After transferring the proteins onto the membrane, perform a Ponceau staining as a loading control according to standard protocols.
- 4.6) Destain membrane in TBST for 5 min at RT.
- 4.7) Block membrane for 30 min up to 1 h at RT in 5% BSA in TBST.
- 4.8) Incubate membrane with the primary antibody at 4 °C overnight or for 2 h at RT.
- 4.9) Wash the membrane with TBST 3x 5 min.
- 4.10) Incubate membrane with the hP-coupled secondary antibody at a dilution of 1:10,000 in 5% BSA. Note: In case of high background signals, use milk powder instead of BSA.
- 4.11) Wash the membrane with TBST 3x 5 min.
- 4.12) Develop membrane with an ECL detection reagent and detect signals on chemiluminescence films or a chemiluminescence imaging system.

Note: In order to discriminate MVs from Exos, proteins like Tubulin, actinin-4 or mitofilin can be used which should mainly be present on MVs<sup>16,25</sup>. Pay attention that most tetraspanin antibodies (*e.g.* CD9, CD81), used as markers for Exos, do not work under reducing conditions and should therefore be prepared in non-reducing loading buffer followed by heating for 10 min at 70 °C.

#### **REPRESENTATIVE RESULTS:**

In order to quantify the yield of MVs that can be isolated following the described protocol, we calculated the amount of MVs isolated from blood samples of 10 donors. The MV yield, which was assessed in a Lowry protein assay, ranged from 10 up to 30 µg with a mean of 19.2 µg MVs per mL blood (Table 1). The particle concentration determined by nanoparticle tracking analysis (NTA) ranged from  $1.66 \times 10^9$  to  $2.36 \times 10^{10}$  with a mean of  $5.9 \times 10^9$  particles per mL plasma sample (Table 2). Further characterization of the MVs by transmission electron microscopy

revealed a population of vesicles with a diameter > 100 nm that were surrounded by a lipid bilayer and did not contain any cell organelles (Figure 1A). NTA confirmed that the size of the isolated MVs ranged from 100 up to 600 nm (Figure 1B) and the mean MV size was 201 nm (Figure 1C). Staining for typical MV and Exo markers by Western Blotting demonstrated that the isolated MVs were positive for Tubulin and only showed a slight expression of CD9 and CD81, while Exos were negative for Tubulin and enriched in CD9 and CD81 (Figure 2).

Analysis of the isolated MVs by flow cytometry (Figure 3A) revealed a defined vesicle population that could be gated using the same parameters normally used for MVs isolated from cell culture supernatants and that was clearly different from the background signal obtained by the measurement of PBS + 1% vesicles-depleted FCS without addition of MVs (Figure 3B). In order to analyze the different MV populations present in blood, MVs were stained with established markers for the different blood cell populations *e.g.* CD62P for platelet-derived MVs, CD45 for leukocyte-derived MVs, CD235a for red blood cell-derived MVs and CD62E for endothelial cell-derived MVs (Figure 4). This characterization showed that the percentage of MV subpopulations differed among the investigated donor blood samples, while the majority of MVs seemed to be shed by platelets in all samples.

**Figure 1: Size distribution of MVs isolated from peripheral blood.**

**A**, Isolated MVs were visualized by transmission electron microscopy. **B**, Representative nanoparticle tracking analysis (NTA) of MVs illustrating the size distribution of the vesicles. **C**, The mean MV size from 10 independent preparations was measured by NTA (mean).

**Figure 2: Characterization of isolated MVs by Western Blotting.**

Differential protein expression on the isolated MVs and Exos from two donors was visualized by Western Blotting.

**Figure 3: Analysis of MVs by flow cytometry.**

**A**, MVs are first visualized on forward (FSC) versus sidescatter (SSC) plots to gate on the respective MV population. Subsequently, these MVs are characterized for the antigen of interest by using fluorescently-labeled antibodies directed against the antigen. **B**, Typical FSC versus SSC plots for MVs isolated from the plasma of two donors. As comparison, a typical plot for tumor cell-derived MVs from A549 lung cancer cells isolated from cell culture supernatant as well as a negative control using only PBS + 1 % vesicle-depleted FCS without MVs are shown on the right.

**Figure 4: Characterization of isolated MVs by flow cytometry.**

MVs from three donors were characterized for the expression of established blood cell markers (red) by flow cytometry. The respective isotype controls are shown in grey.

**Table 1: MV protein yield from peripheral blood samples.**

Shown is the amount of MVs per mL peripheral blood that was drawn from 10 donors. MV yields were quantified by Lowry assay.

**Table 2: MV particle yield from peripheral blood samples.**

MV were isolated from plasma samples of 10 donors and particle counts were determined by nanoparticle tracking analysis. Shown is the mean value from three independent measurements.

**DISCUSSION:**

Recent studies on EVs in blood have demonstrated that EV composition and counts change during the course of several diseases. Therefore, the analysis and further characterization of these EVs are of high interest to further assess their potential use as disease biomarkers for diagnosis and prognosis or to evaluate therapy responses. The protocol we present here allows the isolation of vesicles with a diameter of up to 600 nm which do not contain any cell organelles. These observations are in line with the current definition of MVs and exclude the presence of apoptotic bodies<sup>2</sup>. Using Western Blotting, we were able to demonstrate that the isolated MVs show a high expression of Tubulin, while the tetraspanins CD9 and CD81 that are often used as Exo markers were only slightly expressed. This confirms that MVs differ from Exos and fits to recent in-depth characterization and comparison of both EV populations by proteomics<sup>25</sup>.

During acquisition of blood samples, it is critical to keep the time between venipuncture and plasma preparation as short as possible in order to prevent MV degradation. Moreover, prolonged storage of blood samples could lead to the activation of blood cells causing enhanced MV shedding and ultimately apoptosis which results in the release of apoptotic bodies. Another important consideration for MV isolation is to prevent contaminations of MV preparations with plasma proteins or smaller Exos. Therefore, it is critical to remove as much of the supernatant as possible after spinning down the MVs at 14,000 x g. Since the pellet is normally visible and tightly attached to the wall of the tube, the supernatant can be easily removed with a pipette tip. In contrast to Exos which tend to form aggregates during preparation by high speed ultracentrifugation and are often hard to resuspend, this problem does not occur with MVs.

Our study shows that it is possible to characterize MV subpopulations present in blood by flow cytometry. Although the detection limit of most flow cytometers is around 200 - 300 nm, MVs were reproducibly measured in donor as well as cell culture samples with the same parameters of analysis and gates that clearly allowed their distinction from background signals. It is important to verify prior to the measurements that the PBS used for the analyses does not contain any contaminating particles that could cause a high background during flow cytometry (Figure 3). Although some smaller MVs might not be captured in a flow cytometric approach, we detected MVs from all major blood cell populations (*e.g.* platelets, red blood cells, leukocytes, endothelial cells). In our analyses we used standard markers for the different blood cells that have been previously found on MVs<sup>26-29</sup>. It should be noted that in order to obtain the best possible results by flow cytometry, the amount and concentration of all antibodies should be titrated on a MV sample expressing the antigen of interest. If specific MV subpopulations in blood shall be identified with higher specificity, it is possible to perform double staining against two different antigens present on the respective MVs and only consider

all double positive MVs for subsequent analyses<sup>23</sup>. Currently, there are efforts to define a standard range of MV subpopulations in blood of healthy individuals<sup>23,30</sup>. These studies have already shown that platelet-derived MVs constitute the largest population of MVs in blood which is in correspondence with our observations.

One advantage of flow cytometry to characterize MV samples is that this method is already well established for diagnostic purposes in most clinical centers which would allow the possible use of MVs as biomarkers in everyday clinical diagnostics. Previous studies on EVs in blood which have mostly focused on smaller Exos, rely on either specific sorting procedures to selectively analyze the desired Exo target population<sup>31,32</sup> or require a time-consuming (2 days) isolation process with coupling of Exos to Latex beads prior to analysis<sup>18</sup>. Our own unpublished observations suggest that the flow cytometric analysis of MVs from whole blood preparations even allows the detection of *e.g.* tumor-derived MVs without any such selection processes.

Taken together, the protocol presented here allows the fast isolation of MVs from peripheral blood samples with standard lab equipment and their subsequent characterization using flow cytometry and Western Blotting. The whole process can be performed in around 2 hours which will facilitate future studies on MV profiles in patients' blood that are required to assess the potential of MVs as disease biomarkers.

#### **ACKNOWLEDGMENTS:**

The authors acknowledge Meike Schaffrinski for excellent technical assistance. We would like to thank the following people for their help in the collection of peripheral blood samples (all from University Medical Center Göttingen): Henrietta Vida (Dept. of Transfusion Medicine), Kia Homayounfar, Lena-Christin Conradi (Dept. of General, Visceral and Pediatric Surgery), Leila Siam, Bawarjan Schatlo (Dept. of Neurosurgery), Hendrik A. Wolff, Martin Canis (Dept. of Otorhinolaryngology, Head and Neck Surgery) as well as all employees of the interdisciplinary short-term oncology. We acknowledge Dirk Wenzel (Max Planck Institute for Biophysical Chemistry, Göttingen) for his help with the electron microscopy of MVs.

The study was funded by the German Ministry of Education and Research (BMBF) project MetastaSys (grant no. 0316173) as well as the German Cancer Aid (grant no. 109615).

#### **DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1

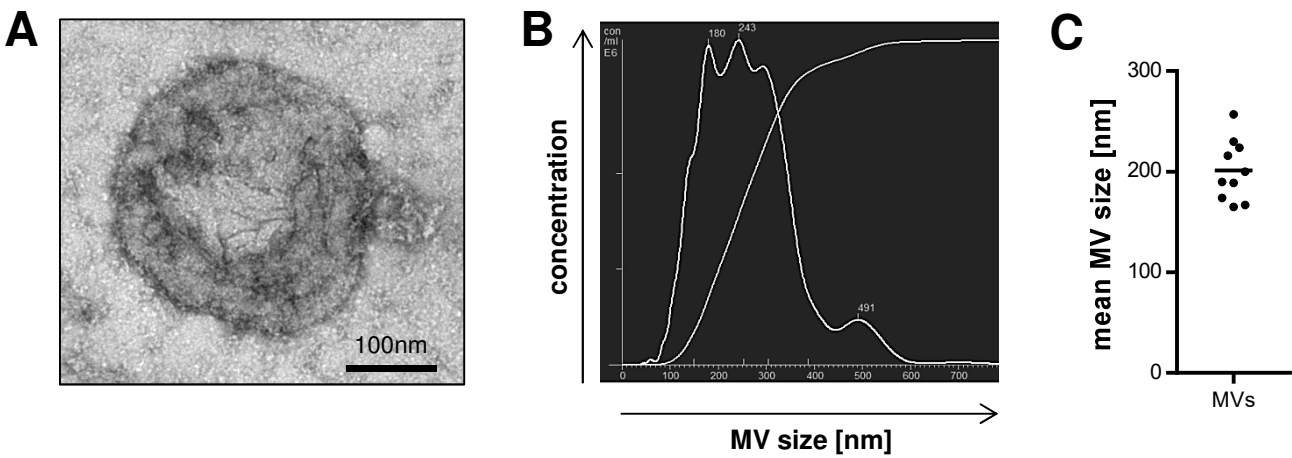


Figure 2

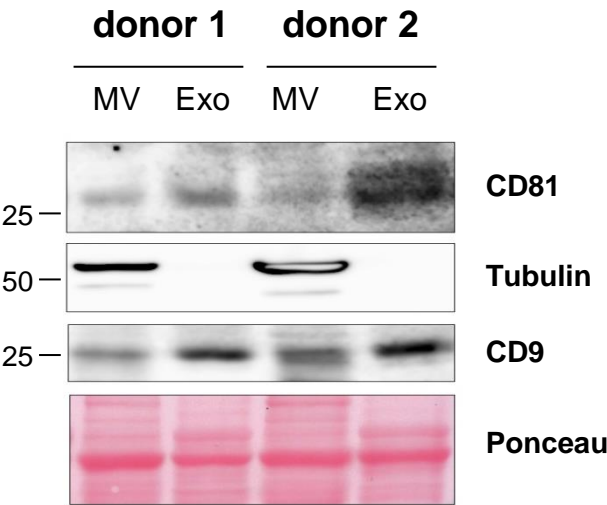


Figure 3

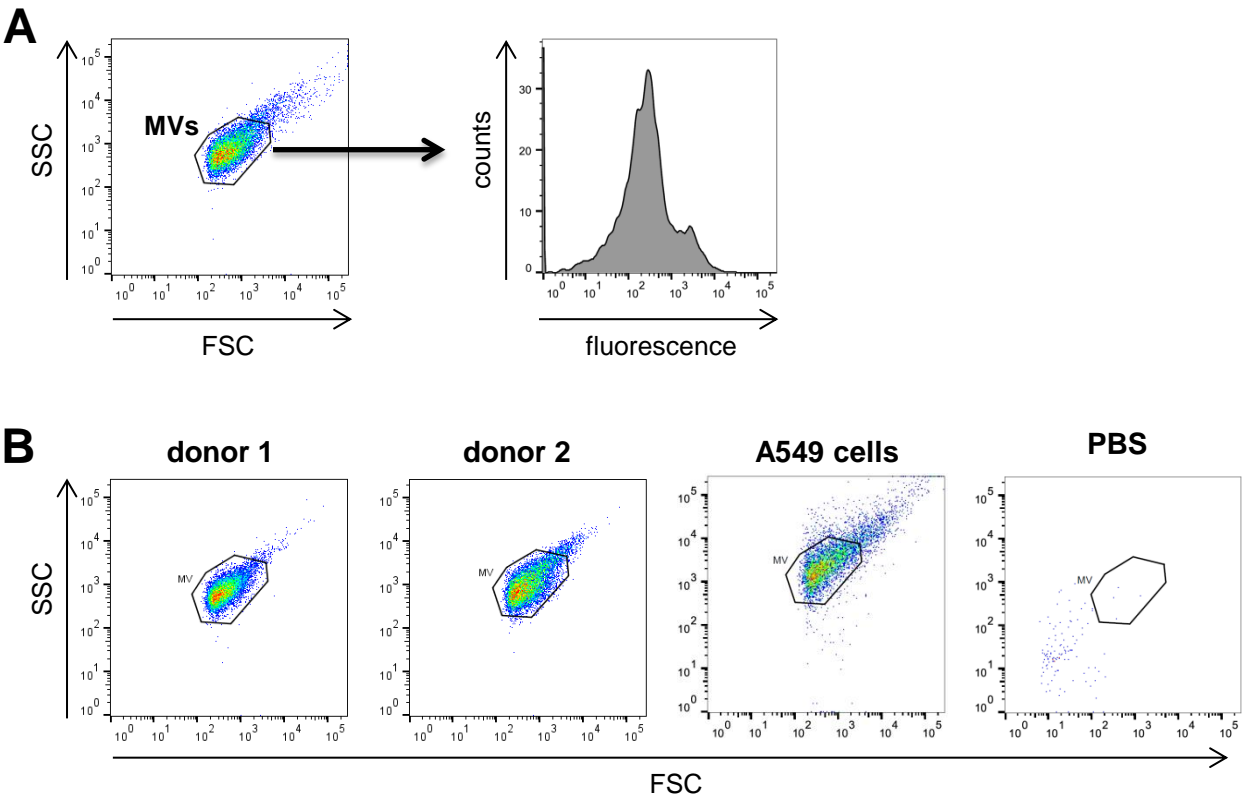
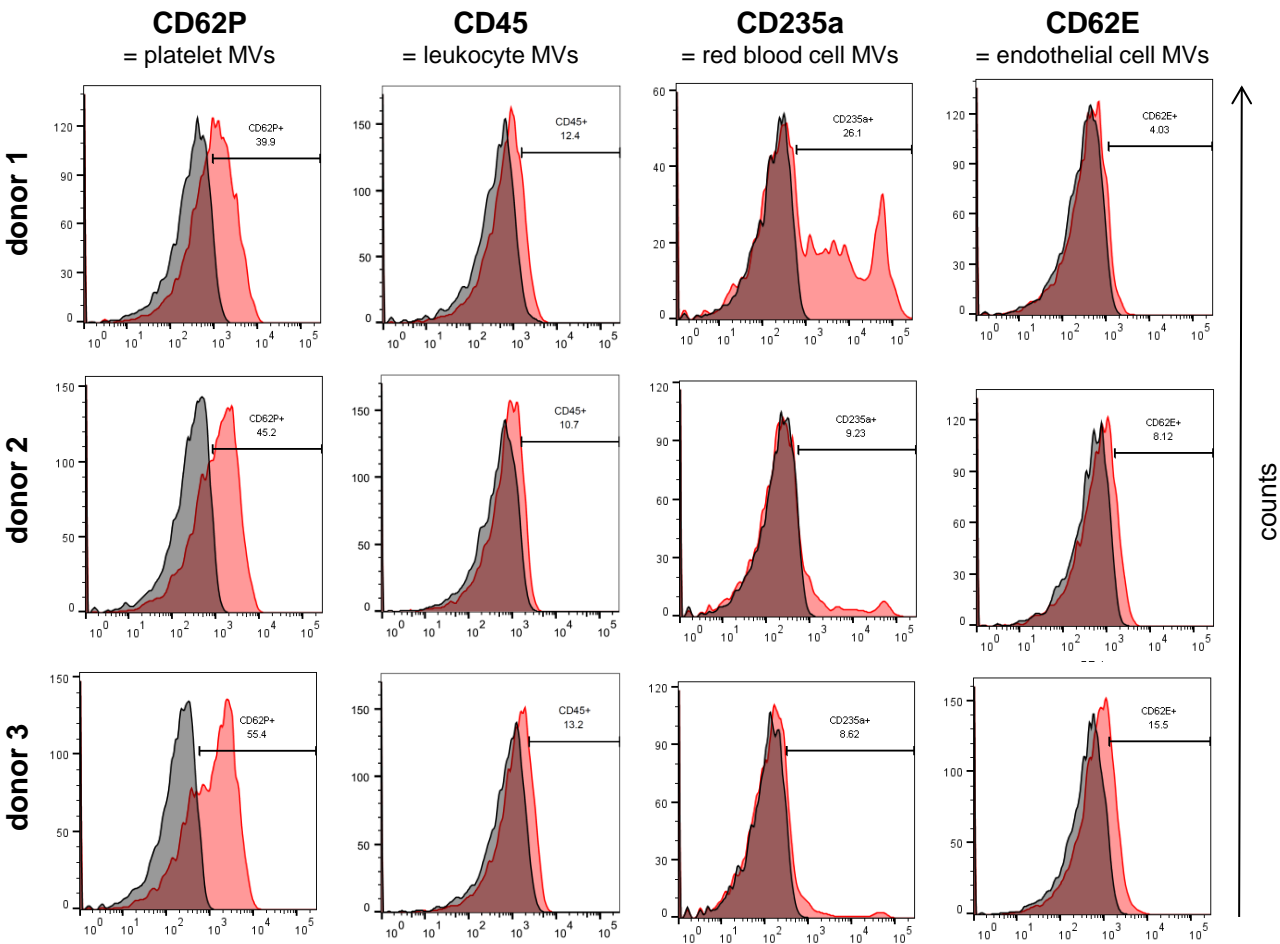


Figure 4



Sample	MVs/mL blood [µg]
#1	29.4
#2	10.3
#3	15.2
#4	31.1
#5	18.8
#6	22.7
#7	19.1
#8	18.7
#9	15.0
#10	11.9

Sample	MVs/mL plasma [particle count]
#1	6.42E+09
#2	2.36E+10
#3	1.88E+09
#4	6.51E+09
#5	3.48E+09
#6	4.57E+09
#7	2.09E+09
#8	1.66E+09
#9	2.54E+09
#10	6.20E+09

Name of Reagent/ Equipment	Company	Catalog Number
butterfly needle (21 gauge)	Hospira Deutschland GmbH	490P29201
Bovine serum albumin Fraction V	Roth	8076.3
CD235a-PE	Beckman Coulter	A07792
CD45-FITC	Beckman Coulter	7782
CD62E-PE	Biolegend	336008
CD62P-PE	Biolegend	304905
CD81 antibody	Biolegend	349501
CD9 antibody	Immunotools	21270091
Chemiluminescence imager ImageQuant LAS-4000	Fujitsu Life Sciences	
DC protein assay kit II	Bio-Rad	5000112
ECL detection reagent	GE Healthcare	RPN2232
EDTA vacutainers for blood collection	Sarstedt	01.1605.001
FACSCanto II	BD Biosciences	
fetal calf serum (FCS)	Invitrogen	10091148
filter 0.22µm	Sarstedt	83.1826.001
HRP-coupled anti-mouse secondary antibody	santa cruz	sc-2005
HRP-coupled anti-rabbit secondary antibody	santa cruz	sc-2004
4x Laemmli loading buffer, Roti-Load 1	Roth	K929.1
microfuge SIGMA 1-15K	Sigma Laborzentrifugen	
milk powder (Blotting-Grade Blocker, nonfat dry milk)	BioRad	170-6404
multifuge 3 L-R	Heraeus	



NanoSight LM10	NanoSight Ltd.	
PBS, w/o Ca and Mg	Pan biotech	P04-36500
perfusor syringe 50 mL	Braun	8728844F
Ponceau-S staining solution	PanReac AppliChem	A2935,0500
rotor Sw32.1 Ti for ultracentrifugation (6x 17 mL)	Beckman Coulter	
rotor TLA-120.2 for ultracentrifugation (10x 1.5 mL)	Beckman Coulter	
tubes for flow cytometry (5 mL, round- bottom)	BD Biosciences	352054
tubes for ultracentrifugation (15 mL)	Beckman Coulter	344061
tubes for ultracentrifugation (11* 34 mm)	Beckman Coulter	343778
Tubulin antibody	Millipore	05-829
ultracentrifuge Optima L-80 XP	Beckman Coulter	
ultracentrifuge TL-100	Beckman Coulter	
valve filter Seraplas V15	Sarstedt	53,428

## Comments/Description

For 10x TBS weigh 24.2 g Tris base and 80 g NaCl, ad 1 L H<sub>2</sub>O  
and adjust pH to 7.6

For 1x TBS-T mix 100 mL 10x TBS with 900 mL H<sub>2</sub>O and add 1  
mL Tween-20

use 5µl for staining

use 5µl for staining

use 0.8µg for staining

use 0.1µg for staining

1:2000 in 5%BSA in TBS-T; use non-reducing conditions for Western Blotting

1:1000 in 5%BSA in TBS-T; use non-reducing conditions for Western Blotting

heat-inactivated (30 min, 56°C)

use 1:10,000 in 5% milk powder in TBS-T

use 1:10,000 in 5% milk powder in TBS-T

1:5000 in 5%BSA in TBST



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To the Editors and Reviewers  
of *Journal of Visualized Experiments*

Manuscript revision:

Isolation and characterization of microvesicles from peripheral blood

Dear Editor,

We appreciate the opportunity to resubmit our manuscript entitled “Isolation and characterization of microvesicles from peripheral blood”. We have read the helpful comments/reviews very carefully and improved our manuscript based on the suggestions. Consecutively, you find our point-to-point responses to the comments raised by the editors/reviewers.

### Comments of the Editor:

**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.**

The manuscript has been thoroughly proofread by the authors.

**2. Please abbreviate all journal titles.**

All journal titles in the reference section were abbreviated.

**3. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.**

Table 1 was now uploaded as xlsx instead of a pdf file.

### **4. Formatting:**

**-Please use periods to indicate decimal points rather than commas, including in the materials table.**

The comma in the materials table was changed to a period.

**-3.8 should be a note under step 3.1, and should not be highlighted for filming.**

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As suggested, point 3.8 was moved as a note to point 3.1 and the highlighting removed so that it will not be filmed.

### **-3.6.1 – Paraformaldehyde is toxic and requires a caution statement.**

A toxicity statement was added in the text: "In case that samples cannot be measured immediately, add 150 µL PBS and 50 µL 4% paraformaldehyde (PFA) to fix samples and store at 4 °C. **CAUTION: PFA is toxic. Use gloves and suitable personal protective equipment.**"

### **-References – Please abbreviate all journal titles.**

All journal titles in the reference section were abbreviated.

### **5. Grammar: Line 55 – Please remove "e.g."**

In line 55 "e.g." was removed.

**6. Visualization: Section 4 should not be highlighted for filming as insufficient detail is provided in lieu of citing standard protocols. Significant additional detail would be required (gel running conditions, transfer conditions, what buffers are used). This would likely take the protocol above the length limit for filming. If the authors wish to film this method, the detail can be added and alternative methods mentioned in sections 1 & 2, which likely would not be filmed anyway, should be unhighlighted.**

As suggested by the Editor, we removed section 4 from filming.

### **7. Additional detail is required:**

#### **-4.3 – What volume of loading buffer?**

The following details were added in point 4.3: "Prepare 10-20 µg of MVs **in 22,5µl RIPA buffer, add 7,5µl 4x Laemmli loading buffer...**"

#### **-4.6 – Please provide a citation. Alternatively, please provide detail regarding how this is performed.**

Based on the suggestion of the Editor, we added more details on this procedure (see new points 4.6-4.12 and Materials table).

#### **-4.7 – Which antibodies are used here? What are they diluted in?**

Details on antibodies (CD9, CD81 and Tubulin primary antibodies as well as HRP-coupled secondary antibodies) as well as the recipe for the TBS-T buffer in which the antibodies are diluted in have been included in the Materials table. Moreover, the former point 4.7 was instead placed as a note at the end of Protocol section 4.

### **8. Branding should be removed: Nanosight – Results, Figure 1 legend**

The brand name "Nanosight" was replaced by "nanoparticle tracking analysis" in the Results section and legend of Figure 1.

### **9. Discussion: Please discuss the critical steps of the protocol.**



We added one paragraph in the discussion describing the critical steps for MV isolation and also highlighted several important points in the paragraph about the flow cytometry analyses.

#### **Comments of Reviewer #1:**

***When extracellular vesicles are abbreviated as EV - please use plural "s". Eg. EVs, which also counts for MV / MVs.***

The plural "s" was added for abbreviations of EVs, Exos and MVs in the text and Figures 1, 3 and 4.

#### **In protocol:**

**Add 1.1: A note should be given to discard the first tube of blood drawn to avoid the stressed cells from the penetration.**

We thank the reviewer for his point. In order to clarify this question, we have discussed it with several physicians and haematologists in our large university medical center. However, the conclusion from these discussions was that it is not usual to discard the first tube of blood when drawing peripheral blood from the crook of the arm. In contrast, the first tube of blood is the most precious one that is always used for the most important tests. Maybe the reviewer is referring to drawing blood from a central venous catheter where indeed the first 3-5ml of blood are discarded which is solely due to the architecture of the catheter and not due to biological reasons. Since most of the readers will be drawing blood from the arm, we refrained from including this note in the protocol.

**Add 1.4: it is not serum but plasma you transfer.**

"Serum" was changed to "plasma".

**Add 1.6 note: Which temperature is used for centrifugation?**

The temperature for centrifugation was added to the protocol.

**Add 2.1: It is irrelevant which size the centrifugation tube has (13 mL) and the speed of 14.000g is not normally termed "ultra".**

We agree with the reviewer that it is better to formulate this step in a more general way and changed the text in point 2.1 and 2.2 accordingly. The term "ultra" was removed.

**Add 2.8: unnecessary information - should be erased.**

Point 2.8. was erased.

**Add 3.3: Explain what is being blocked at this stage.**

Unspecific binding of the antibody or the attached fluorochrome to the MV surface can be caused by e.g. electrostatic, glycolipid or protein-protein interactions. These unspecific interactions should be largely covered by incubation with protein (=FCS). Therefore, MV are incubated in PBS+1%FCS in order to block all these unspecific binding sites on the MV surface. In order to clarify this point, 3.3 was slightly modified: "Incubate samples for 30 min at RT in order to block unspecific binding sites at the MV surface and thereby reduce background staining."

**Add 3.8: Unnecessary information at this stage - eventually move it to paragraph 3.1.**

As already suggested by the Editor, point 3.8 was moved as a note to point 3.1.

#### **Discussion:**

**line 300: Proteomics should be proteomics.**

See "track changes" in the manuscript.

**line 304: donor should be donors.**

Since "donor" refers to donor "samples", the addition of an "s" would not be correct.

**Figure 4: It is not correct to use "=platelets"; "=leukocytes" etc. It is not cells analysed. It is markers for EVs originating from "platelets" etc...**

In order to make clear that we are dealing with MVs, we changed platelets to platelet MVs, leukocytes to leukocyte MVs etc. A new version of Figure 4 with the changes was uploaded.

#### Comments of Reviewer #2:

**It is suggested to give more details, especially in the following sections:**

**1. preparation of plasma samples - there is no information about the starting sample volume of blood (for this certain procedure) and the sample handling temperature (even if it take less than 30 min. ) is not specified.**

We agree that detail on the exact volume of blood was missing in point 1.1, therefore we added the following specification in the note below: "The recommended volume of blood for subsequent flow cytometry and Western Blot analysis is 5-15 mL." Furthermore, detailed information on typical yields is displayed in Table 1 and new Table 2.

**1.2) this reviewer would like to know why centrifugation is performed at 4°C since plasma (platelet rich plasma) preparation is usually done at RT**

We agree with the reviewer that based on the recommendations of the International Society for Extracellular Vesicles (ISEV) the plasma preparation should be done at room temperature (Witwer et al, J Extracell Vesicles, 2013). We have changed the protocol accordingly.

**1.3) it should be specified what kind of "serum filter" authors use, and why authors transfer serum instead of plasma into the 15ml tube (1.4).**

We thank the Reviewer for his point, in 1.4 the term "serum" was wrongly used and was replaced by "plasma". As indicated in the text, the serum filter (name changed to "valve" filter) is used to separate the plasma from any remaining blood cells and is specified in the Materials table.

**A second centrifugation step of plasma should be considered to avoid platelets contamination.**

We agree with the reviewer that platelet removal is very important when isolating MVs. However, we already have a second centrifugation step of the plasma included (see point 1.5). In order to clarify this point, we added the following statement in point 1.5: "Centrifuge for 15 min at 1,500 x g, 4° C to pellet larger cell debris and remove remaining platelets."

#### **2. isolation of MV**

**2.1) authors pay attention to vesicle -depleted FCS which they used for MV preparation, however, in this reviewer's opinion, they also should specify if vesicle-depleted PBS was used for MV/exosomes washing and FACS analysis.**

Since we show in Figure 3B that our routinely used PBS does not contain any MVs, we are not concerned about vesicle contaminations from the PBS and used non-depleted PBS throughout the whole protocol, as indicated. However, to draw the reader's attention to this point, we included a statement in the discussion that the PBS should be validated not to contain any contaminating vesicles.

### **3. Characterization of MV by flow cytometry**

#### **Flow cytometry analysis of MV is limited by their size, a threshold setting of the FL parameter/channel may address this problem**

It is true that flow cytometry analysis of MVs is limited by their size and that especially smaller MVs might be lost in the described analysis. We are also aware of several studies that have been presented this year at the annual meeting of the ISEV showing that modulating the threshold settings of the fluorescence channels can allow detection of smaller particles, even exosomes. However, we are concerned that exactly for this reason the modulation might lead to the detection of mixed signals coming from pure, larger MVs and smaller vesicles (i.e. exosomes). Since so far we obtained very reliable results with our flow cytometry settings that we also were able to confirm by other methods such as Western Blotting, and never observed any problems with too few particle counts during the flow cytometry measurements, we would like to refrain from adding these modulations to our protocol.

#### **3.5) information about multicolor staining possibility is lacking**

We thank the reviewer for his point and added a respective note in point 3.4: "Note: It is also possible to perform multicolor flow cytometry by adding multiple antibodies coupled to different fluorochroms." Moreover, the possibility of doing multicolour analyses has already been mentioned in the discussion: "If specific MV subpopulations in blood shall be identified with higher specificity, it is possible to perform double stainings against two different antigens present on the respective MVs and only consider all double positive MVs for subsequent analyses."

#### **3.7) information about the log scale for FSC and SSC is lacking**

Information about the logarithmic scale was added in point 3.7: "Reduce the threshold of the flow cytometer to the lowest value possible and search for the MV population using a forward scatter (FSC) versus side scatter (SSC) plot in logarithmic scale."

#### **Additional Comments to Authors:**

**Comments/description section need clarification (page 19), there is no information what kind of "serum filters" were used (1.3)**

See point 1.3 above.

**Besides the concentration of MV (protein) also information about the MV numbers, concentration, etc. (e.g. NTA analysis) should be included (Table 1).**

As suggested by reviewer 2 and 3, we calculated the particle concentration in the MV samples from 10 donors by NTA and included this information in the Results section as new Table 2.

#### **Comments of Reviewer #3:**

**The authors state the size of Exosomes as <100nm. Authors should give also a minimum size, eg 30-100nm. The field in general uses 70-150 nm.**

We thank the Reviewer for his point and have specified the size of exosomes as 30-100 as shown in Raposo&Stoorvogel, 2013, J Cell Biol.

**The authors did Nanosight analysis for size analysis of the particles. Besides, the concentration could be given as well, to compare the yields of the samples and changes of concentrations as a potential diagnostic tool.**

As mentioned above, we included this information as new Table 2.

**Only limited data is available in the literature that proofs DNA-content of EVs. The statement needs to be cited.**

We agree with the reviewer that the literature on the DNA content of EV is sparse, however, there are some

studies that have already demonstrated the presence not only of RNA, but also of DNA, in EV. The according references have been added to the introduction: "Both types of vesicles are surrounded by a lipid bilayer and contain nucleic acids e.g. DNA, mRNA or miRNA <sup>3-5</sup>,..."

**The authors performed Western Blot analysis to show MV and Exosome specific marker expressions. Tubulin is stated as a MV marker, whereas CD9 and CD81 are stated as Exosome markers. Tubulin as a MV marker could be shown different from other groups (Elham Hosseini-Beheshti et al, 2012, Clotilde Théry et al, 2002) other markers have been described to discriminate between Exosomes and MV in Kowal et al, 2016.**

In our manuscript we state that Tubulin, actinin-4 or mitofilin can be used as markers for MVs to discriminate them from Exos (final note in point 4 of the protocol section). The two examples mitofilin and actinin-4 are already taken from the recent study of Clotilde Thery's lab (Kowal et al, PNAS, 2016) and are also referenced in the manuscript.

In the Results section we used the established standard markers CD9, CD81 and Tubulin to show that with the protocol it is possible to isolate these two different vesicle populations. Since by using these markers, we can clearly confirm this assumption, we do not think that this point needs further confirmation by testing other markers.

With regard to Tubulin, we agree with the reviewer that previous studies sometimes found Tubulin also in exosomes. However, these studies focused on exosomes only and thereby did not pay attention to the separation of exosomes from MVs like we did in our study by an additional filtration step (Menck et al, J Mol Cell Biol, 2015). Therefore, we are convinced that the tubulin signals found in the exosome pellet originally stem from contaminations with tubulin-expressing MV and that tubulin, in our hands, is a good marker for separating MV from Exo.

We are grateful for the thorough evaluation of our protocol by the editor and the reviewers. We feel that the manuscript has now been greatly improved by following the suggestions and hope that the revised version will be suitable for publication.

With kindest regards,  
Claudia Binder