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Rapid fluorescence-based characterization of single extracellular vesicles in human blood with nanoparticle-tracking analysis

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Dear Mr Myers,

Thank you for the invitation to publish our work in JOVE. Herewith, we would like to submit our manuscript entitled 'A novel method for rapid fluorescence-based characterization of single extracellular vesicles in human blood as an original article.

In January 2015, we already published the manuscript "An Innovative Method for Exosome Quantification and Size Measurement in your journal which gained a lot of attention in the world of science and reached more than 15.000 views and as of yet 23 citations.

The protocol that is presented addresses the common problem of many researchers in this field and provides the complete workflow for rapid isolation of extracellular vesicles from whole blood and characterization of specific markers by fluorescence-based nanoparticle-tracking analysis.

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.

We hope that our work will find the interest of the editorial office and meet the high standards of *JOVE*.

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TITLE:

Rapid Fluorescence-based Characterization of Single Extracellular Vesicles in Human Blood with Nanoparticle-tracking Analysis

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KEYWORDS:

Exosomes, nanovesicles, particle tracking analysis, CD63, CD9, vimentin, serum

SUMMARY:

In this protocol, we describe the complete workflow for rapid isolation of extracellular vesicles from human whole blood and characterization of specific markers by fluorescence-based nanoparticle-tracking analysis. The presented results show a high level of reproducibility and can be adjusted to cell culture supernatants.

ABSTRACT:

Extracellular vesicles (EVs), including exosomes, are specialized membranous nano-sized vesicles found in bodily fluids that are constitutively released from many cell types and play a pivotal role in regulating cell-cell communication and a diverse range of biological processes. Many different methods for the characterization of EVs have been described. However, most of these methods have the disadvantage that the preparation and characterization of the samples are very time-consuming, or it is extremely difficult to analyze specific markers of interest due to their small size and due to the lack of discrete populations. While methods for analysis of EVs have been considerably improved over the last decade, there is still no standardized method for characterization of single EVs. Here, we demonstrate a semi-automated method for characterization of single EVs by fluorescence-based nanoparticle-tracking analysis. The protocol that is presented addresses the common problem of many researchers in this field and provides the complete workflow for rapid isolation of EVs and characterization with PKH67, a general cell membrane linker, as well as with specific surface markers such as CD63, CD9, vimentin, and lysosomal-associated membrane protein 1 (LAMP-1). The presented results show a high level of reproducibility, as confirmed by other methods, such as Western blotting. In the

conducted experiments, we exclusively used EVs isolated from human serum samples, but this method is also suitable for plasma or other body fluids and can be adjusted for characterization of EVs from cell culture supernatants. Irrespective of the future progress of research on EV biology, the protocol that is presented here provides a rapid and reliable method for rapid characterization of single EVs with specific markers.

INTRODUCTION:

Extracellular vesicles (EVs), including exosomes, are specialized membranous nano-sized vesicles (20-150 nm) containing certain combinations of lipids, adhesion and intercellular signaling molecules, as well as other functional cytosolic components like microRNA (miRNA) and mRNA, and play a pivotal role in regulating cell-cell communication[1,2]. EVs are released in their environment from many different cell types, *e.g.,* endothelial cells, immune cells, and tumor cells, and can be detected in body fluids such as serum semen, urine, breast milk, saliva, or cerebrospinal fluid[3,4]. Increasing numbers of studies highlight the diverse contribution of EVs as potential biomarkers for early diagnosis of several diseases and/or prediction of disease progression[5,6]. Exosomes are often described by the presence of molecules that they are specifically associated with, regardless of the cell type they derive from[7]. For example, exosomes contain different tetraspanins (CD9, CD63, CD81), major histocompatibility complex class I (MHC I) molecules, various transmembrane proteins, typical cytosolic proteins (tubulin and actin), molecules involved in multivesicular body (MVB) biogenesis (TSG101 and alix), heat shock proteins (HSP 70 and HSP 90), and proteins that participate in signal transduction (protein kinases)[8].

Many different methods have been described for the characterization of EVs[9]. The most common and prevalent methods used for EV analysis are flow cytometry[10], scanning electron microscopy (SEM), and transmission electron microscopy (TEM)[11]. The best-established and commonly used method for the biochemical characterization of EV content is Western blotting[12,13]. While SEM and TEM allow for the detection of EVs across the entire size spectrum, the very limited identification of specific surface proteins is a particular disadvantage of these methods. In contrast, flow cytometry is a powerful tool for identification of specific EV surface markers, but the threshold of this method limits the analysis to EVs with a size greater than 500 nm. Hence, analysis of isolated EVs with detection of specific surface markers is currently not accessible through any of these three well-established methods. We previously described another highly sensitive method for visualization and analysis of EVs, nanoparticle-tracking analysis (NTA)[14]. Briefly, this method combines two different physical principles. First, particles scatter light when they are irradiated with a laser beam, and the second principle, known as Brownian motion, implies that diffusion of different particles in a liquid suspension is inversely proportional to their size. The semi-automated desktop nanoparticle analysis instrument for liquid samples consists of the particle tracking analyzer with a software-based analysis, where digital images of scattered light from single particles are recorded. The particles and the movement of the particles are detected by a laser scattering microscope with a video camera. The laser beam is oriented vertically, while the optical axis is horizontal and focused into the cell channel filled with the sample. The data provided by plots of scattered light spots and their speed of motion enable the determination of total particle count and size distribution. After irradiation by the laser, the particles scatter the light, which is recorded by a digital video camera *via* the microscope[14]. The advancement to our former method is the insertion of a 500 nm long wave-pass (LWP) cut-off filter between the laser (wavelength of 488 nm) and the cell channel, which enables the direct analysis of fluorescence-labeled particles (**Figure 1**). Our protocol addresses the common demand of many researchers in this field for a fast characterization of single EVs, *e.g.,* according to their parental origin. In this protocol, we describe the complete workflow for rapid isolation of EVs from human whole blood and fast characterization of specific markers by fluorescence-based nanoparticles-tracking analysis. EVs can be detected by staining with PKH67, a general cell membrane linker, as well as with specific exosomal markers, *e.g.,* CD63, CD9, and vimentin. Our protocol is also suitable for EDTA and citrated plasma, as well as other body fluids and cell culture supernatants.

PROTOCOL:

The institutional ethical board of the University of Dusseldorf has approved the experiments presented in this work (Reference number: 3381).

1. EV Isolation from Human Whole Blood

1.1. Isolate EVs with exosome precipitation solution.

1.1.1. Collect 2 mL of human whole blood in serum-separating tubes (SST) *via* venipuncture and incubate the tube for 15 min at room temperature (RT) until coagulation is finished.

1.1.2. Centrifuge the SST at 1,700 x g for 10 min at RT to separate cells from serum and transfer 1 mL of the serum to a 1.5 mL reaction tube. Centrifuge the platelet-rich plasma (PRP) at 3,000 x g for 15 min at 4 °C to remove platelets and transfer 100 µL of the platelet-poor plasma (PPP) to a new 1.5 mL reaction tube.

1.1.3. Add 25 µL of exosome precipitation solution (4 parts PPP, 1 part exosome precipitation solution) and vortex thoroughly. Incubate the sample for 30 min on ice. Keep the tube upright and do not rotate or mix the tube during the incubation period.

1.1.4. Centrifuge the sample at 1,500 x g for 30 min at 4 °C to pellet the EVs. After centrifugation, the EVs appear as a beige or white pellet at the bottom of the vessel. Aspirate the supernatant and centrifuge the sample at 1,500 x g for 5 min at 4 °C.

1.1.5. Remove all traces of fluid and re-suspend the pellet in 100 µL of phosphate-buffered saline (PBS) by frequently pipetting up and down. Store the EV suspension at -80 °C when analysis is not performed immediately.

Note: When isolating EVs from plasma, fibrinogen and fibrin can impede efficient recovery and resuspension is heavier and takes more time.

1.2. Isolate EVs with ultracentrifugation.

1.2.1. Take the pre-cooled rotor (TLA-55 fixed-angled) from the fridge and cool down the ultracentrifuge before use.

1.2.2. Transfer 1.25 mL of PPP (prepared in step 1.1) to a suitable 1.5 mL ultracentrifugation tube with cap and centrifuge the sample at 110,000 x g for 90 min at 4 °C. Make sure that the rotor load is balanced before starting.

1.2.3. Decant the supernatant and place tube upside-down on a paper towel for 2 min. Re-suspend the pellet in 500 µL of PBS and centrifuge the sample at 110,000 x g for 90 min at 4 °C.

1.2.4. Aspirate the supernatant and re-suspend the pellet in 50 µL of PBS.

Note: Use only rotors and accessories designed for the ultracentrifuge that is in use. Pretest the tubes in the rotor by using water because the strength of the tubes can vary between lots.

2. Staining of the Samples

2.1. Stain samples with PKH67.

2.1.1. Prepare the staining solution by adding 1 µL of the PKH67 ethanolic dye solution to 50 µL of diluent C (included in the kit) in a 1.5 mL reaction tube and mix thoroughly.

2.1.2. Transfer 10 µL of the staining solution to 20 µL of the EV suspension (prepared in step 1.1) to the reaction tube, mix thoroughly and incubate for 5 min at RT in the dark.

2.1.3. Dilute 50 µL of the stained EV suspension with 2.5 mL distilled water in a 15 mL reaction tube, mix thoroughly, and use this final suspension for particle measurement.

Note: It is crucial to use water as diluent for the EV suspension, because other diluents (*e.g.,* PBS) can impair the measurement. When using stored EV-suspension, thaw the samples on ice and mix thoroughly before staining.

2.2. Stain samples with specific antibodies.

2.2.1. Dilute 10-20 µL of the EV suspension (prepared in step 1.1) with 50 µL of distilled water in a 1.5 mL reaction tube and mix thoroughly.

2.2.2. Add 2.5-5 µL of the specific antibody (**Table 1**) to the reaction tube, mix thoroughly, and incubate for 30 min at RT in the dark.

2.2.3. Transfer 50 µL of the stained EV suspension to 2.5-10 mL of distilled water (**Table 1**) in a 15 mL reaction tube, mix thoroughly, and use this final suspension for particle measurement.

Note: Under some circumstances, the concentration of the used antibody must be adjusted (**Table 1**).

3. Processing of the Samples

Note: The fundamentals of this method were extensively described previously and below the protocol focuses on the specific steps needed for analysis of fluorescence-labeled EVs[14].

3.1. Perform the startup procedure.

3.1.1. Push the fluorescence filter into the optical path of the microscope and camera. Start the program and follow the instructions on the screen for automated implementation.

3.1.2. Select the correct cell number (Z158_C1149_Fluor) in the “Cell Check” tab (cell definition) for fluorescence measurement. Select the reference position for the optics to make sure that laser and microscope are in a common focus (laser and microscope move to this position automatically).

3.1.3. Flush the cell channel with a syringe filled with 10 mL of distilled water. Ensure that the measurement cell is free of air bubbles and do not inject air bubbles into the system.

3.1.4. Prepare a calibration suspension containing uniform 200 nm sized fluorescence-labelled polystyrene particles that have carboxylate groups on their surface. Dilute 10 µL of the particles with 990 µL distilled water. Then dilute 10 µL of this particle solution in a 15 mL tube with 10 mL of distilled water to obtain the required concentration.

3.1.5. Inject 2.5 mL of the diluted particle solution in the cell channel and click “Optimize focus” to adjust the camera.

3.2. Measure the sample.

3.2.1. Flush the cell channel multiple times with a syringe filled with 10 mL of distilled water prior to each sample measurement. Inject the stained EV suspension (prepared in step 2) into the cell channel.

3.2.2. Adjust the following main camera parameters in the “Cell Check” tab in the software as needed (**Table 2**). Use the reference position or position 0.41193 to adjust the parameters.

3.2.2.1. For sensitivity, find the optimal sensitivity range by clicking on the button “Number of Particles *vs.* Sensitivity” to display a curve of measured particles per screen for different sensitivity levels.

Note: A higher sensitivity level allows visualization of smaller particles but also increases artifacts related to background noise.

3.2.2.2. For shutter, adjust the period of time that the camera allows light to pass for a determined interval.

3.2.2.3. For post-acquisition parameters, choose a minimum brightness of 20, a minimum size of 20 nm, and a maximum size of 500 nm for measurement.

3.2.3. Note the number of detected particles counted in the field of vision from the display. The scattering bar must be in the green to orange range (50-300 particles). If the scattering bar is red, the scatter will fuse individual particles and they are counted as one single particle, thus leading to false results. In such a case, further dilute the sample to avoid overlapping of the particles or lower the sensitivity.

3.2.4. Click on “Check particle drift at 0 V” in the “Cell Check” tab before starting the measurement. If the drift is higher than 5 µm/s, wait until the sample stops flowing to continue the measurement.

Note: If the drift is too high at the beginning of the measurement, the repeated measurements can differ and sophisticate the results. Due to the underlying principles of NTA, a relevant drift may have an impact in the determined particle size, as calculated by the software.

3.2.5. Click on “Run Video Acquisition” in the “Measurement” tab. Select the number of experiments (3-5) and the time delay between them (0 min).

3.2.6. Define the number of (individual subvolume-) positions (11) and the number of (measurement) cycles (10) at each measurement position, where particles should be analyzed

3.2.7. Select a folder, create a new file name, and click “OK” to start the measurement.

4. Interpretation of the Results

4.1. View the results and parameters on the “Analysis” tab after the measurement. Check the following parameters after the analysis before cleaning the cell channel: average number of particles per position, total number of traced particles and particle concentration, the distribution width of the particles (x10, x50, and x90 values), value of mean and standard deviation. Repeat the measurement of the sample if required.

Note: The results are also saved as a .pdf or .txt file.

4.2. Click on “Analysis” tab to view the graph calculated after the measurement, which shows the distribution of the detected particles by size. Click on “Display” in the “Analysis” tab and use the icons to adjust and change the graph settings for particular requirements.

5. Validation *via* EV Detection by Western Blotting

5.1. Dissolve the EV pellet (prepared in step 1) in RIPA lysis and extraction buffer, pipette thoroughly and incubate on ice for 30 min. Centrifuge the sample at 8,000 x g for 10 min at 4 °C to clarify the lysate and transfer the supernatant to a new tube.

5.2. Measure the total protein by a Lowry protein assay kit. Dilute 1 µL of the isolated EV suspension with 49 µL of RIPA buffer and use 5 µL in triplicates for analysis. Dilute the EV suspension with 2x Laemmli loading buffer to a final concentration of 2 µg/µL and heat for 10 min at 95 °C.

5.3. Load 20 µg of protein per well. Separate and transfer the proteins by polyacrylamide gel electrophoresis and tank blotting according to standard protocols.

5.4. Block the membrane (0.2 µm polyvinylidene difluoride) with bovine milk powder (5%) for 1 h at RT and incubate the membrane with specific primary antibodies (CD9, CD63, and vimentin) overnight at 4 °C.

Note: The primary antibodies are used at a 1:1,000 dilution.

5.5. Wash the membrane with TBST 3x 5 min and incubate the membrane with horseradish peroxidase (HRP)-conjugated secondary antibody (1:20,000 in TBST) for 60 min at RT.

5.6. Wash the membrane with TBST 3x 5 min and detect the proteins by chemiluminescence with a high sensitivity substrate solution on an imaging system.

Note: Because CD63 antigen is extensively and variably glycosylated, the molecular weight can vary, and bands can appear between 40-65 kDa.

REPRESENTATIVE RESULTS:

EVs were isolated from whole blood and characterized by nanoparticle tracking analysis with fluorescing reagents. The optimal sensitivity for measurement of unstained particles was identified to range at 70% during our experiments. The fluorescent beads used for adjustment and calibration of the measurement showed an optimum setting at a sensitivity of 85% (**Figure 2A**). Between a sensitivity of 70% and 90%, the number of detected particles increased rapidly, while further increasing the sensitivity can lead to a deterioration of the particle size distribution where the number of particles is re-dropping. The settings of the camera displayed a sharp picture (**Figure 2B**) and repeated measurements showed low standard deviation (**Figure 2C**). Insofar, the protocol for processing the samples of EVs was adjusted so that all measurements could be conducted with the same settings (**Table 2**). The distribution width is defined by three values on the x-axis, the x10, x50, and x90. The x50, or median particle size is the diameter at which half of the population lies below this value. Similarly, the x10 and x90 indicate the diameter at which 10% and 90% of the detected particles are under the reported size. Staining with PKH67 cell linker kit, including a fluorescent cell linker that incorporates a green fluorescent dye with long aliphatic tails into lipid regions of the cell membrane, demonstrated a strong correlation between the sensitivity and the number of particles measured **(Figure 3A)**. PKH67 is often used for proliferation monitoring but has also proven useful for monitoring exosome or liposome uptake as well as for *in vivo* cell trafficking. Due to the non-specific labeling of PKH67, a wide variety of EVs can be labeled and detected. The distribution of the particles was in a range between 266 nm (x10) and 1946 nm (x90) with a peak maximum at 857 nm (x50) and with a low standard deviation between the measurements (28.1 nm). LAMP-1, also known as lysosome-associated membrane glycoprotein 1, and CD107a reside primarily across lysosomal membranes. After staining with an Alexa Fluor 488 labeled specific antibody against LAMP-1, the distribution of the particles ranges from 220 nm (x10) to 1145 nm (x90) with a peak maximum at 541 nm (x50) and a standard deviation of 11.7 nm **(Figure 3B)**. For characterization of EVs, we used Alexa Fluor 488 labeled antibodies against common exosomal markers and confirmed our findings by Western blotting. After staining with Alexa Fluor 488-labeled CD9-antibody, the distribution of the particles ranges from 251 nm (x10) to 1139 nm (x90) with a peak maximum at 548 nm and a second minor peak at approximately 25 nm **(Figure 4A).** Staining with Alexa Fluor 488 labeled CD63 (**Figure 4B**) and vimentin (**Figure 4C**) yielded similar results. Western blotting analysis substantiated our positive result for antibodies used here. Repeated measurements showed reproducible results for all antibodies used in this report. As controls, we stained vesicle-free water with respective antibodies (**Figure 5A**), where PKH67 and LAMP1 antibodies virtually detected no EV up to a sensitivity close to 100%. Using the example of vimentin, high sensitivity increased the number of emerging artefacts, even when the sample is essentially free of particles. If measurement is started when the drift is yet too high (> 5 µm/s), the individual repetitions distinctly deviate among themselves **(Figure 5B).** As represented with three different antibodies, it is crucial that the drift is as minimal as possible before starting the measurement. According to our experience, using fluorescein isothiocyanate (FITC) as fluorochrome results in measurements that are not accurate and reproducible because FITC is prone to rapid photo bleaching (**Figure 5C**). Therefore, we recommend using exclusively Alexa Fluor 488 labeled antibodies for EV characterization. In this protocol, EVs were isolated by a polymer-based exosome precipitation solution containing polyethylene glycol. To ensure that our results are not falsified by the applied isolation method, we characterized EVs after isolation with ultracentrifugation. As represented with PKH67 and two different antibodies (CD63 and LAMP-1), the results of our applied isolation with exosome precipitation solution (**Figure 6A**) are comparable with EVs isolated *via* ultracentrifugation (**Figure 6B**). Unfortunately, because of the poor yield of EVs after ultracentrifugation, the initial set of serum for isolation must be distinctly higher compared to isolation with exosome precipitation solution.

FIGURE AND TABLE LEGENDS:

Figure 1: **Schematic setup of the nanoparticle tracking analysis**. The microscope/video axis and laser beam are orientated orthogonally to each other, crossing at the cell channel cross section. A fluorescence filter is placed between the cell channel and the microscope. Light scattered by the particles is displayed in the “live-view” window of the software. After acquisition, the results are displayed as a size distribution curve coordinate system.

Figure 2: Calibration with fluorescence labeled beads. (**A**) The number of particles *vs.* sensitivity curve displays the particles in one position at one point in time during an automatic sensitivity scan. (**B**) Visualization of particles on the live view screen. (**C**) Particle size distribution after repeated measurements.

Figure 3: Representative results after staining with PKH67 and LAMP-1. Number of particles *vs.* sensitivity curve (1), visualization of particles on the live view screen (2), and particle size distribution (3) after staining with PKH67 (**A**) and LAMP-1 (**B**). A similar size distribution of particles is observed, while changes in the sensitivity setting lead to a similar but yet not entirely identical change of detected particles.

Figure 4: Representative results after staining with Alexa Fluor 488 labeled CD9, CD63, and vimentin. Number of particles *vs.* sensitivity curve (1), visualization of particles on the live view screen (2), particle size distribution (3), and representative Western blots of two different EV suspensions (4) for CD9 (24-27 kDa, **A**), CD63 (26 kDa, **B**), and vimentin (54 kDa, **C**). Late occurrence of particle signals along the increasing sensitivity (x-axis) correlates with lower signal intensity in the Western blot analysis, confirming the lower amount of the respective particle surface marker (*e.g.,* vimentin *vs.* CD63). Note the almost identical curves of representative measurements for all analyzed markers indicating high reproducibility (3).

Figure 5: Representative results for used controls and possible error sources. (**A**) Vesicle-free water stained with PKH67 (1), LAMP-1 (2), and vimentin (3) as controls. (**B**) Representative particle size distributions after staining with LAMP-1 (1), CD63 (2), and CD9 (3), and measurements performed when suspension drift is yet too high. (**C**) Number of particles *vs.* sensitivity curve (1) and particle size distribution (2) after staining with FITC-labelled CD63 antibody. A clear bleaching effect is observed after each measurement, resulting in progressively lower particle numbers.

Figure 6: Comparison of different isolation methods for EV*s.* Particle size distribution of EVs isolated with exosome precipitation solution (**A**) and ultracentrifugation (**B**) after staining with PKH67 (1), LAMP-1 (2), and CD63 (3).

Table 1: List of used antibodies and dilution range of samples.

Table 2: Acquisition parameters for nanoparticle tracking analysis.

DISCUSSION:

We demonstrate a detailed protocol for isolation of EVs from whole blood and fast characterization of specific surface markers with fluorescence-based nanoparticles tracking analysis. In the conducted experiments, we exclusively used EVs isolated from serum samples, but this method is also suitable for ethylenediaminetetraacetic acid (EDTA) and citrated plasma and can also be expanded to other bodily fluids such as urine, breast milk, saliva, cerebrospinal fluid, and semen. Moreover, this protocol can be adjusted for characterization of EVs from cell culture supernatants. In this protocol, the EV suspension was generated from 100 µL of serum using an exosome precipitation reagent, which contains a proprietary polymer that gently precipitates exosomes and EVs according to a corpuscular size ranging from 30 nm to 200 nm, whereby 10-20 µL of EVs were appointed for characterization of each surface marker. Unfortunately, the isolation step is inevitable, because the high amount of protein in serum samples (*e.g.,* albumin and globulin) interferes with the antibody staining procedure and results in high level of background and sophisticated findings. Furthermore, based on the biological availability of the exosomes in the samples, the amount of the employed EV suspension as well as the dilution before processing must be adjusted for other source materials. To compare multiple samples, a standardized approach for the dilution of the samples as well as consistent acquisition parameters (sensitivity, shutter, *etc.*) is necessary. Another important point is that the measurements are not started until the drift is low (in our hands, < 5 µm/s). If the drift was too high, repeated measurements of the sample yielded high standard deviation among themselves, but with a low drift, the resulting data were highly consistent and confirmed a high level of reproducibility. It is important that the selected antibodies have an appropriate fluorochrome. Antibodies must be conjugated with Alexa Fluor 488, because FITC has a high rate of photo-bleaching. Possibly more stable fluophores will certainly lead to increased assay stability in the future. Normally, many researchers use PBS as a diluent for EV*s.* For this protocol, it is crucial to use distilled water as a diluent for the EV suspensions. When EVs are labeled with fluorescing dyes, the high osmolality and ion concentration of other diluents, such as PBS, can interfere with the measurement and lead to altered results.

While methods for analysis of EVs have been considerably improved over the last decade, there is still no standardized method for isolation and characterization of EV*s.* The major disadvantage of flow cytometry, where EVs are often bound to beads to provide a larger surface, is that many EVs dock onto the surface to provide a strong and detectable signal[10]. SEM and TEM have the disadvantage that the preparation of samples is time-consuming and EVs can only be distinguished by their size and morphology[11]. Up to date, the best-established and commonly used method for qualitative (*i.e.,* biochemical) EV characterization is Western blotting, where proteins can be analyzed with specific antibodies[12,13]. However, the disadvantages of all these methods lie in the inability to analyze single EVs for specific surface markers. Furthermore, the long processing times and long washing/isolation procedures used by many of the current protocols involve labor-intensive steps, making them not suitable for high sample throughput and characterization of single EV*s.* Our protocol provides a complete workflow for quick isolation and characterization of single EVs with specific surface markers such as CD63, CD9, vimentin, and CD107a, and can be expanded for a broad spectrum of other surface markers to determine the origin of released EV*s.* Because of a permanent technical advancement of the NTA device, we confirmed our findings in cooperation with the manufacturer with the newest analyzer. Irrespective of the future progress of research on EV biology, particularly concerning exosomes, the protocol that is presented here will provide a rapid and reliable method for characterization of single EVs with specific markers. Because aggregation of EVs during the isolation and staining procedure is so far unavoidable, future research should focus on developing methods to prevent EV aggregation and enable an accurate size determination of fluorescent-labeled EV*s.*

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES

1 Ibrahim, A. & Marban, E. Exosomes: Fundamental Biology and Roles in Cardiovascular Physiology. *Annual Review of Physiology.* **78**, 67-83, doi:10.1146/annurev-physiol-021115-104929, (2016).

2 Su, S. A. *et al.* Emerging role of exosome-mediated intercellular communication in vascular remodeling. *Oncotarget.* **8** (15), 25700-25712, doi:10.18632/oncotarget.14878, (2017).

3 Lasser, C. *et al.* Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. *Journal of Translational Medicine.* **9**, 9, doi:10.1186/1479-5876-9-9, (2011).

4 Li, M. *et al.* Analysis of the RNA content of the exosomes derived from blood serum and urine and its potential as biomarkers. *Philosophical Transactions of The Royal Society B Biological Sciences.* **369** (1652), doi:10.1098/rstb.2013.0502, (2014).

5 Lin, J. *et al.* Exosomes: novel biomarkers for clinical diagnosis. *The Scientific World Journal.* **2015**, 657086, doi:10.1155/2015/657086, (2015).

6 Properzi, F., Logozzi, M. & Fais, S. Exosomes: the future of biomarkers in medicine. *Biomarkers in Medicine.* **7** (5), 769-778, doi:10.2217/bmm.13.63, (2013).

7 Thery, C., Zitvogel, L. & Amigorena, S. Exosomes: composition, biogenesis and function. *Nature Reviews Immunology.* **2** (8), 569-579, doi:10.1038/nri855, (2002).

8 De Toro, J., Herschlik, L., Waldner, C. & Mongini, C. Emerging roles of exosomes in normal and pathological conditions: new insights for diagnosis and therapeutic applications. *Frontiers in Immunology.* **6**, 203, doi:10.3389/fimmu.2015.00203, (2015).

9 Thery, C., Amigorena, S., Raposo, G. & Clayton, A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Current Protocols in Cell Biology.* **Chapter 3** Unit 3 22, doi:10.1002/0471143030.cb0322s30, (2006).

10 Inglis, H., Norris, P. & Danesh, A. Techniques for the analysis of extracellular vesicles using flow cytometry. *Journal of Visualized Experiments.* (97), doi:10.3791/52484, (2015).

11 Wu, Y., Deng, W. & Klinke, D. J., 2nd. Exosomes: improved methods to characterize their morphology, RNA content, and surface protein biomarkers. *Analyst.* **140** (19), 6631-6642, doi:10.1039/c5an00688k, (2015).

12 Bianco, N. R., Kim, S. H., Morelli, A. E. & Robbins, P. D. Modulation of the immune response using dendritic cell-derived exosomes. *Methods in Molecular Biology.* **380**, 443-455, doi:10.1007/978-1-59745-395-0_28, (2007).

13 Conde-Vancells, J. *et al.* Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. *Journal of Proteome Research.* **7** (12), 5157-5166 (2008).

14 Mehdiani, A. *et al.* An innovative method for exosome quantification and size measurement. *Journal of Visualized Experiments.* (95), 50974, doi:10.3791/50974, (2015).

Figure 1

Figure 1

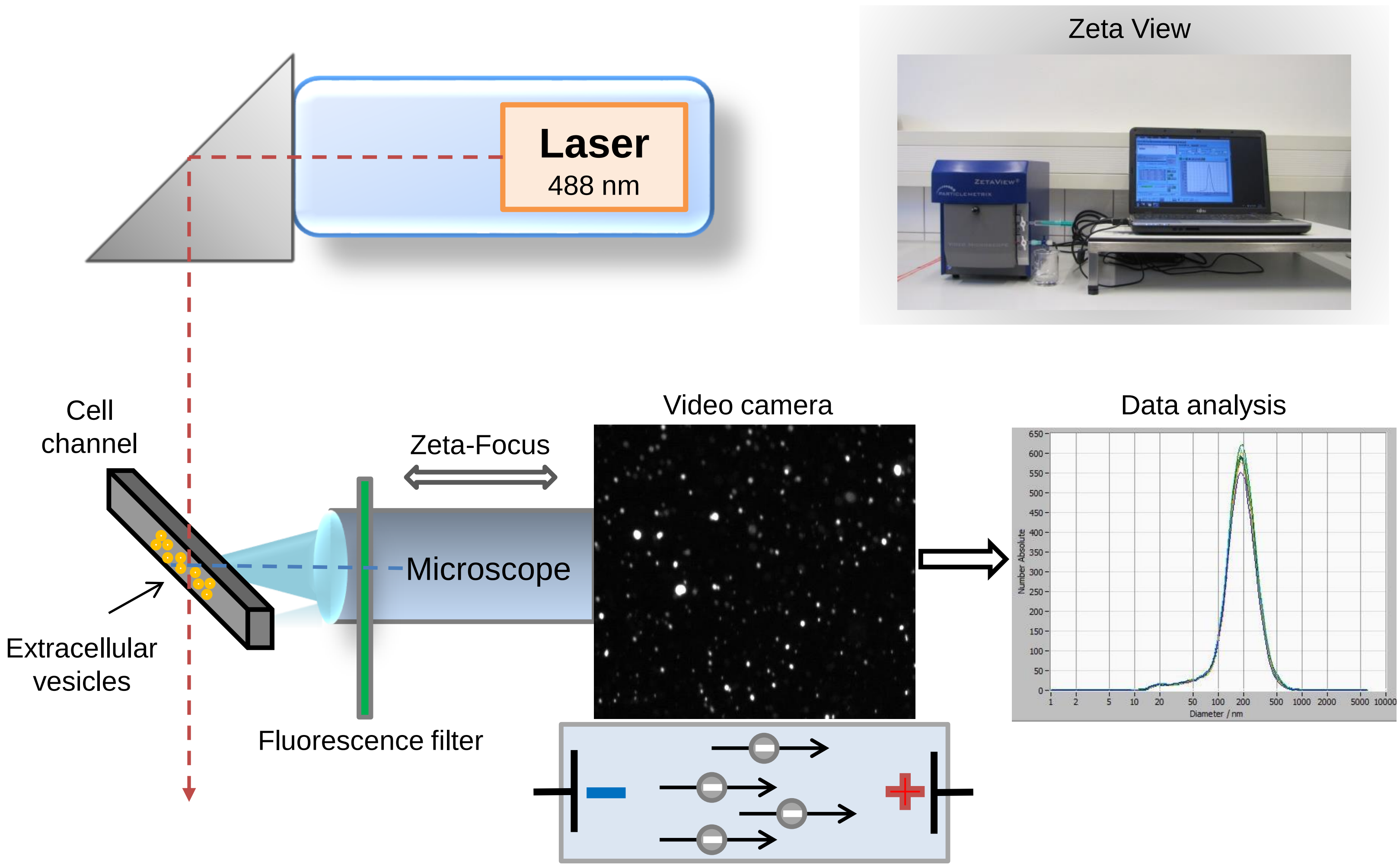


Figure 2

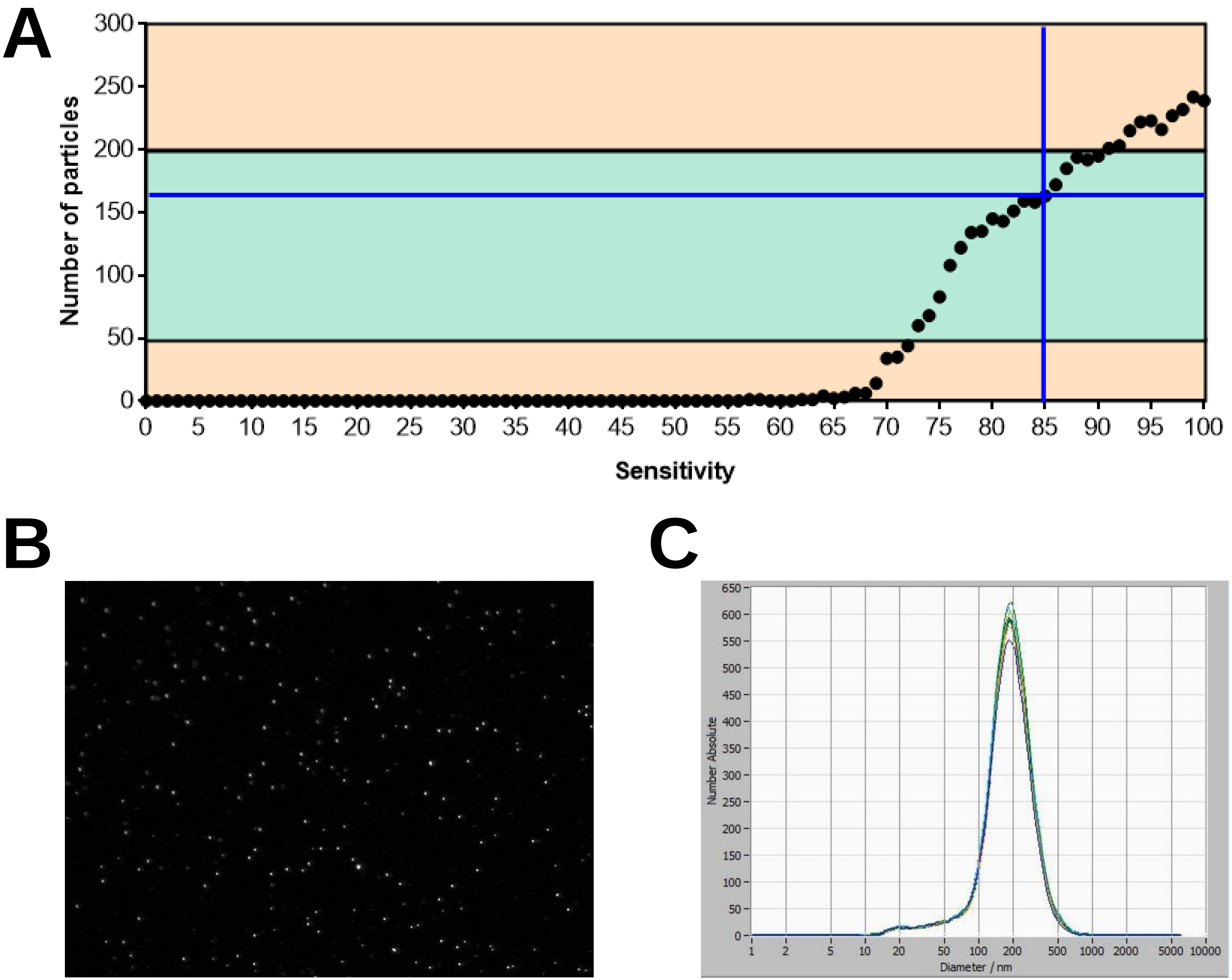
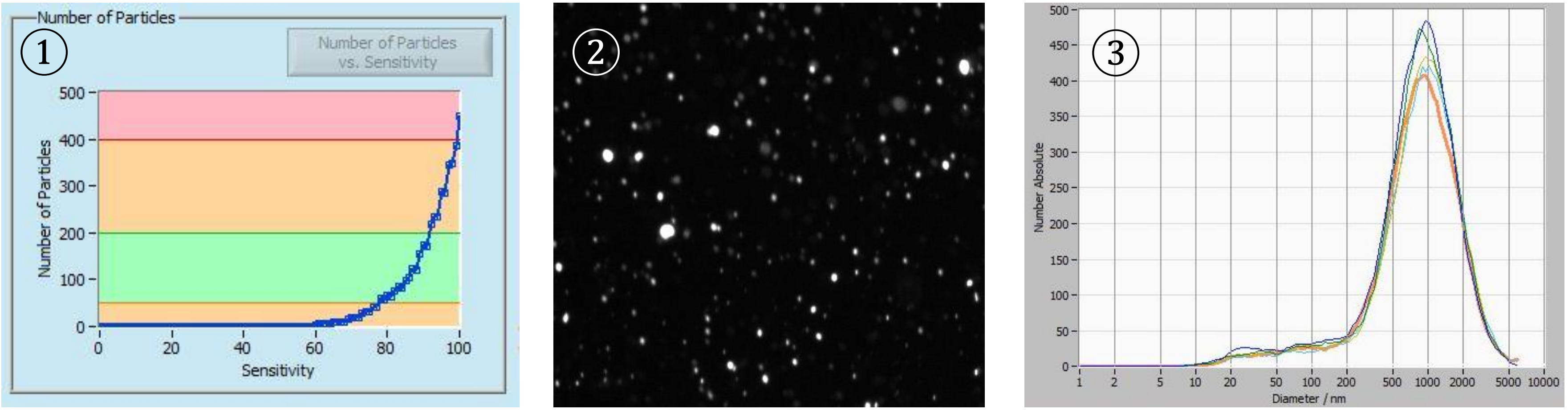

A
Number of particles
Sensitivity
B
C
Number Absolute
Diameter / nm

Figure 3

A

PKH67



B

LAMP-1

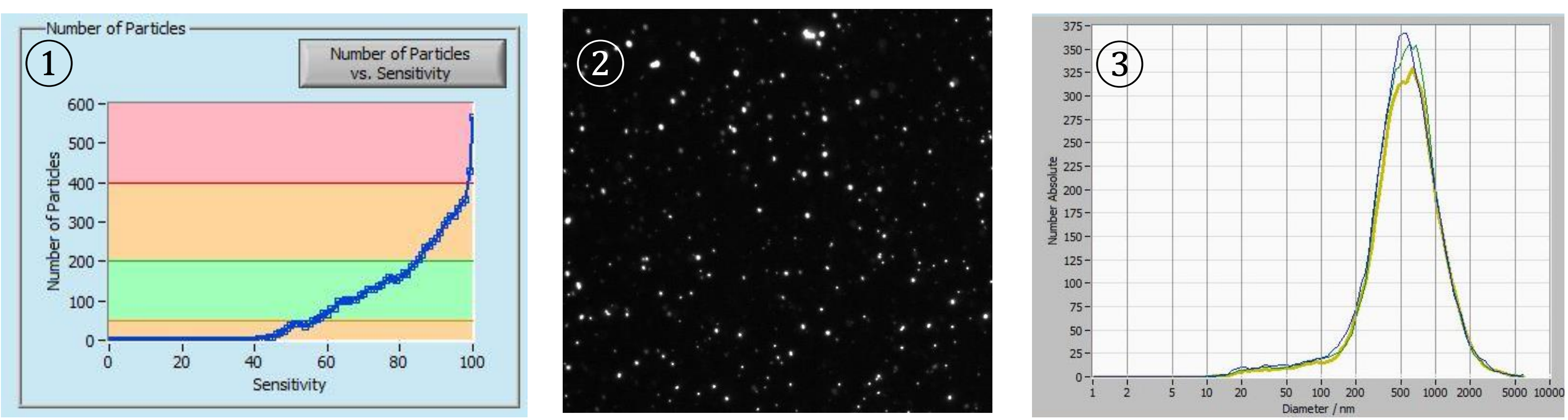


Figure 4

Figure 4

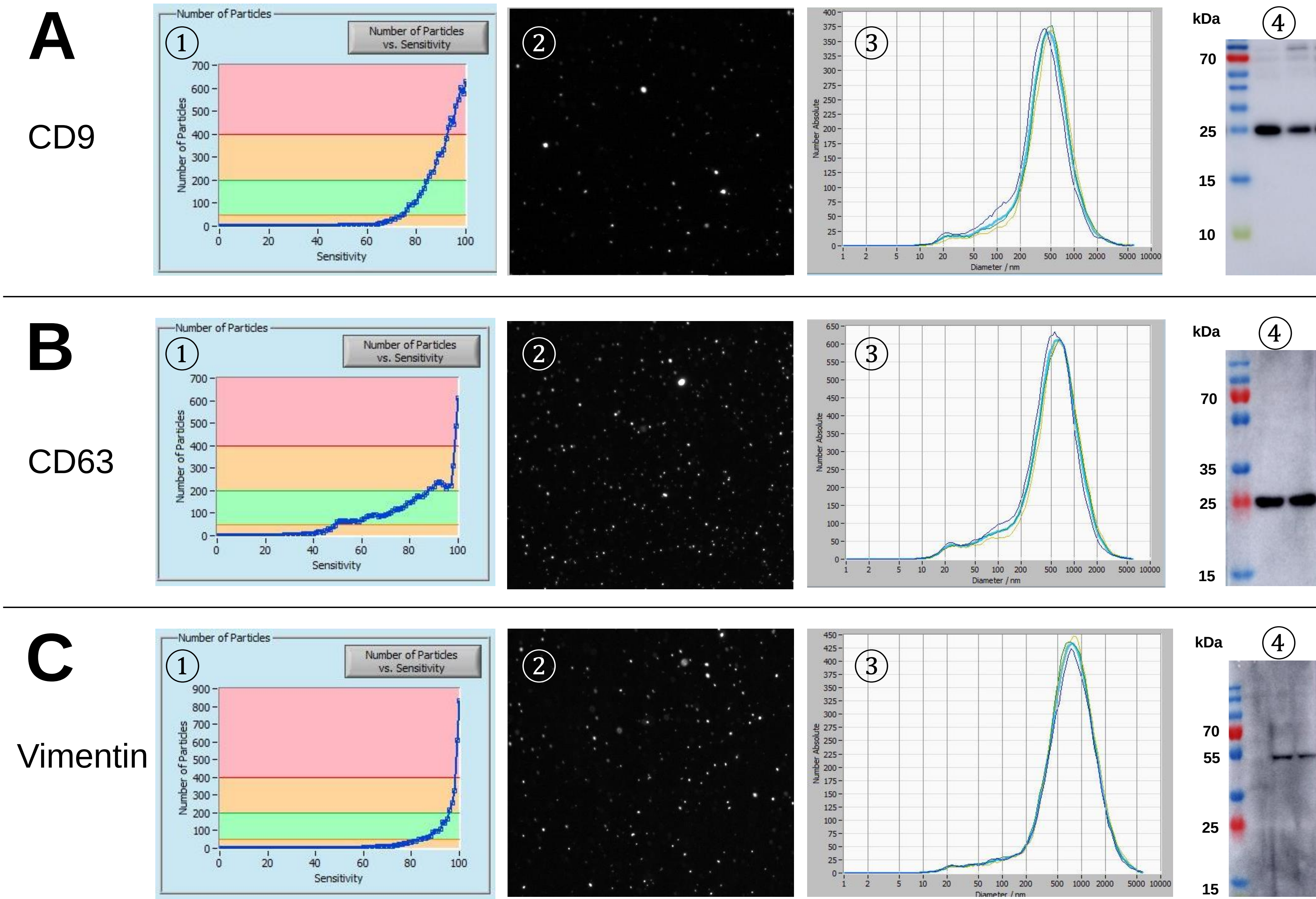


Figure 5

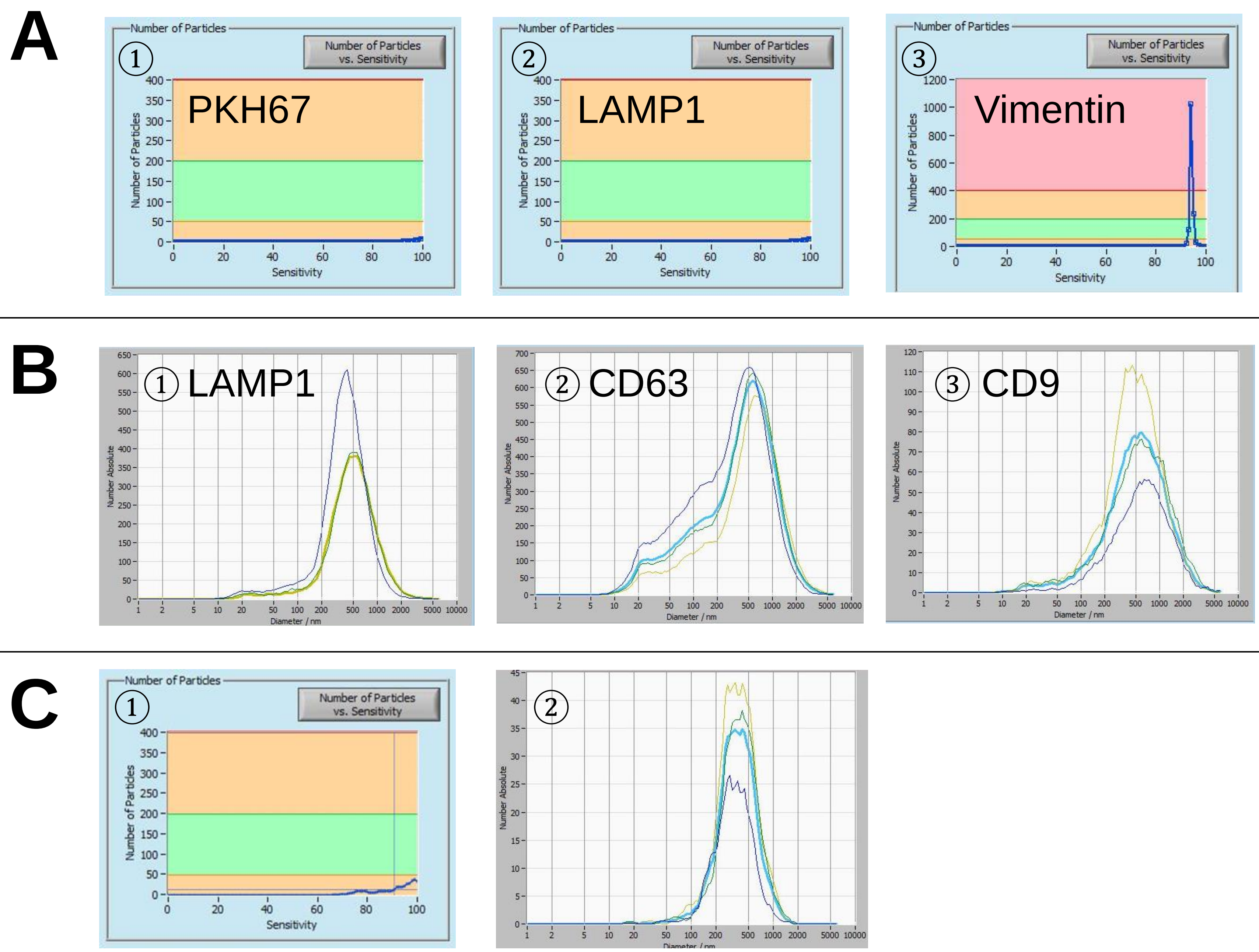

A
Number of Particles
Number of Particles vs. Sensitivity
① PKH67
② LAMP1
③ Vimentin
Number of Particles
Sensitivity
B
① LAMP1
② CD63
③ CD9
Number Absolute
Diameter / nm
C
Number of Particles
Number of Particles vs. Sensitivity
①
Number of Particles
Sensitivity
②
Number Absolute
Diameter / nm

Figure 6

Figure 6

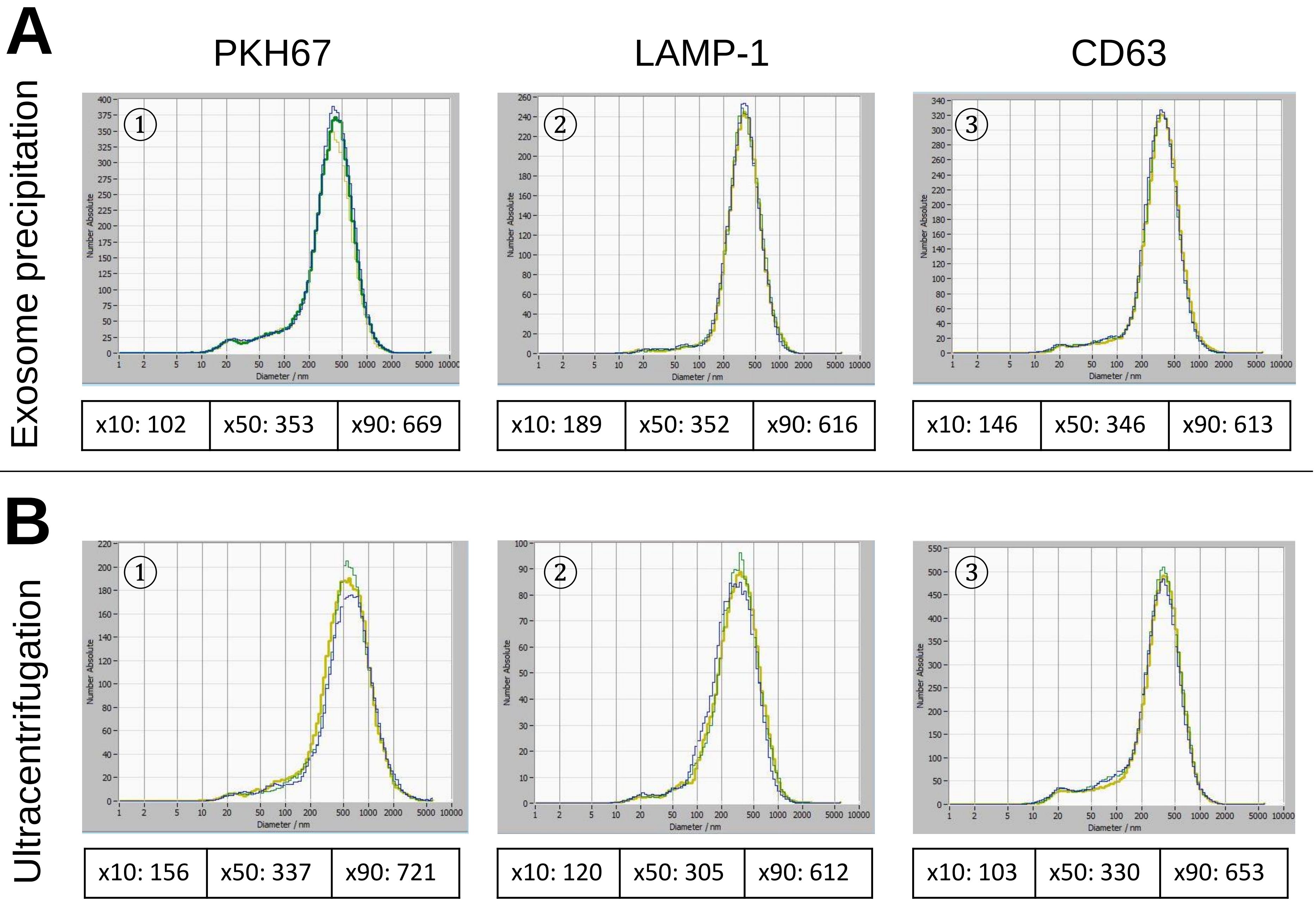


Table 1 Click here to access/download;Table;Table1.xlsx

Table 1

	LAMP-1	CD9	CD63	Vimentin
Antibody (µL)	5	2.5-5	2.5-5	5
EV suspension (µL)	10	20	10	10
H_2O (µL) for staining	50	50	50	50
Final volume (mL)	5-10	2.5-5	5	10

Table 2

Acquisition parameters	
Sensitivity (%)	85
Shutter	70
Min. Brightness	20
Max. Size (nm)	500
Min. Size (nm)	20
Polarity	Negative
Voltage	Off
Particle drift at 0 V (µm/s)	< 5
Positions	11
Cycles	10
Multiple acquisitions	3-5
Time delay (min)	0

Name of Material/ Equipment	Company
Serum-separation tube	BD
Ampuwa water	Fresenius Kabi
Dulbecco's phosphate-buffered saline	Sigma
Falcon tube, 15 mL	Greiner Bio One
Falcon tube, 50 mL	Greiner Bio One
Microcentrifuge tube, 1.5 mL	Eppendorf
Tube with Snap-On Cap 1.5 mL	Beckman Coulter
Polybeads Microspheres 0.2 µm	Polysciences, Inc.
Fluoresbrite YG Carboxylate Microspheres beads 0.2 µm	Polysciences, Inc.
Syringe, 2 mL	Braun
Syringe, 10 mL	Braun
Exoquick	SBI
Laemmli Sample Buffer (2x)	BioRad
DC Protein Assay Kit II	BioRad
PKH67 Green Fluorescent Cell Linker Kit	Sigma
Alexa Fluor 488 anti-human CD107a Antibody	BioLegend
Human CD9-Alexa Fluor 488	R&D Systems
Anti-CD9 Antibody	SBI
CD63-Alexa Fluor 488	ThermoFisher
FITC anti-human CD63 Antibody	BioLegend
CD63. Antibody, polyclonal	SantaCruz
Alexa Fluor 488 anti-vimentin Antibody	BioLegend
Anti-Vimentin Antibody	SBI
Goat anti mouse IgG + IgM	Jackson Immuno
Goat anti rabbit IgG	Dianova
SuperSignal West Femto Maximum Sensitivity Substrate	ThermoFisher
ZetaView	Particle Metrix
Centrifuge	Eppendorf
Ultracentrifuge	Beckman Coulter
Chemiluminescence Imager	GE Healthcare

Catalog Number	Comments/Description
366882	BD Vacutainer
10060	
56064C	
188271	
227270	
30120086	Speciality tubes for ultra centrifugation
357448	
7304	Alignment Solution
09834-10	Alignment Solution
4606027V	
4606728V	
EXOQ20A-1	EV precipitation solution
1610737	
5000112	Lowry prtein assay
PKH67GL-1KT	For general membrane labelling
328609	Lysosomal-associated membrane protein-1 (LAMP-1)
FAB1880G	
EXOAB-CD9A-1	
MA5-18149	
353005	
Sc-15363	
677809	
EXOAB-VMTN-1	
315-035-048	
111-035-003	
34094	
PMX 100, Type	
5804R	
Optima MAX-XP	
Amersham Imager 600	

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Author(s):	Andreas Weber, Julia Christin Wehmeyer, Artur Lichtenberg, Payam Akhyari

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The authors are grateful for the opportunity to revise the manuscript. In particular, we are thankful for the comprehensive review and the valuable suggestions of the reviewers. In the following, we would like to respond to the comments and questions of the reviewers in a point-by-point manner.

Editorial comments:

Changes to be made by the Author(s):

***Comment 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.*

Answer: We thank the editor for this hint and carefully proofread the manuscript.

***Comment 2**: Please revise lines 57-59, 63-67, 84-87, 89-90, and 238-240 to avoid previously published text.*

Answer: We are thankful to the editor for this important annotation and revised the stated lines.

***Comment 3**: Please revise the title to be more concise.*

Answer: We revised the title.

***Comment 4**: Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.*

Answer: We carefully reviewed the complete manuscript and revised the units, in particularly all units including the unit micro. Please state, if used abbreviations continued to be not conform to the International system of Units.

***Comment 5**: JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Exoquick, Snap-On, Fluoresbrite®, SuperSignal, etc.*

Answer: We thank the editor for this remark. We revised the complete manuscript and removed all commercial language.

Comment 6*: Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.*

Answer: We adjusted the numbering of the protocol and removed the bullets.

Comment 7*: Please revise the protocol (lines 206-214, etc.) to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.*

Answer: We thank the editor for this note. In the revised version now only imperative tense is used. The required safety procedures are included in the manuscript.

Comment 8*: Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.*

Answer: Following your recommendations we have added more details to the protocol steps in the revised version. Because the processing of the sample is already published extensively in Mehdiani et al., we will not go into detail, but focus on the differences and specific steps. This fact was already stated in protocol step 3. For further alterations, please state more explicitly, which steps should be described in more detail.

Comment 9*: Line 107: Please specify the blood source. What volume of blood is collected?*

Answer: Blood source and obtained volume are indicated now in the revised manuscript.

Comment 10*: Line 109: Transfer 1 mL of serum or plasma? Please specify.*

Answer: We adapted the whole protocol for the use of serum to avoid ambiguities.

***Comment 11**: Line 224: Please specify incubation conditions.*

Answer: We specified the incubation conditions in the revised manuscript.

***Comment 12**: Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.*

Answer: As already stated in our response to comment 8, detailed information about the complete measurement process are already published in our previous JoVE publication. Please specify more explicitly, which steps should be more explicitly explained.

***Comment 13**: Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.*

Answer: We combined the shorter protocol steps as far as possible. Please note that some steps are critical and combining those steps could compromise the conduction of this protocol.

***Comment 14**: Please include single-line spaces between all paragraphs, headings, steps, etc.*

Answer: We included single line spaces between all paragraph, headings and steps.

***Comment 15**: After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings 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.*

Answer: We highlighted approximately 2.75 pages of manuscript.

***Comment 16**: Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.*

Answer: We thank the editor for these recommendations and highlighted complete sentences in imperative tenses only. Please state if there are any ambiguities left.

Comment 17*: Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.*

Answer: We considered these recommendations at highlighting. The highlighted steps should include all relevant details to perform the step. Please state if there are any ambiguities left.

Comment 18*: Figure 4: Please explain the right panels (4) in the figure legend.*

Answer: We thank the editor for this note. In the revised version of the manuscript, panel 4 is explained in the figure legend.

Comment 19*: References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.*

Answer: We revised the references and wrote out the journal titles. Volume and issue numbers are included as far as they are provided by the National Center for Biotechnology Information.

Reviewer #1:

Manuscript Summary:

Overall a good description of EV analysis by combination of nanoparticle analysis and labelling of EV. Simple to follow protocols that would be accessible to most laboratories starting work on EVs.

> Answer: We thank the reviewer for acknowledging the original character and the topic of our work. We also appreciate for bringing these points to our attention, all of which have been now addressed in the revised version of the manuscript:

Major Concerns:

No major concerns

Minor Concerns:

***Comment 1**: Lines 69 onwards in text - the authors use FACS as a term when I think they would be better to use flow cytometry. Reason - imaging flow cytometry instruments are now available for EV, and sorting is also an option, but analysis without sorting is probably used by most groups, therefore I think it would be better to go through the text and use flow cytometry as an overall term. They can expand on the various types of flow cytomerty - imaging, sorting, analysis etc in an additional sentence if they wish.*

> Answer: We thank the reviewer for this suggestion. We revised the complete manuscript and replaced the term FACS by flow cytometry.

***Comment 2**: Line 122 - description of ultracentrifugation verges on dangerous! Many standard 1.5 ml tubes would not survive 110,000 x g and most rotors would not support these types of tubes anyway. Authors are using a bench top ultra - many other labs would use bigger floor standing ultras, so they need to be more specific to prevent accidental mis-use by individuals not well versed in use of ultra. I think some more explanation of tube types and machine compatibility would help here, and a recommendation that supervision should be sought in the use of ultracentrifuges.*

> Answer: We thank the reviewer for this hint. We revised the complete paragraph and added further detailed information about the used materials and handling of the ultracentrifuge.

We revised protocol step 1.2 as follows (lines 137 – 154):

1. Take the pre-cooled rotor (TLA-55 fixed-angled) from the fridge and cool-down the ultracentrifuge before use.
2. Transfer 1.25 mL PPP (prepared in section 1.1) to a suitable 1.5 mL ultracentrifugation tube with cap and centrifuge the sample at 110,000 x g for 90 min at 4°C. Make sure the rotor load is balanced before starting.
3. Decant the supernatant and place tube turned upside-down on a paper towel for 2 min. Re-suspend the pellet in 500 µL PBS and centrifuge the sample at 110,000 x g for 90 min at 4°C.
4. Aspirate the supernatant and re-suspend the pellet in 50 µL PBS.

Note: Use only rotors and accessories designed for the ultracentrifuge you are operating. Pretest the tubes in the rotor by using water because the strength of the tubes can vary between lots.

Comment 3*: line 139. Justify the use of water as a dilution reagent in this section and later sections. Does the change is osmolality between an EV being in serum versus water alter the results? I see no fixation step, so this is a concern. Insert reference to published data on serum free media or PBS versus water as evidence base for use in this protocol. Is there a limit on storage of EV preps in water?*

Answer: We thank the reviewer for this important question. Unfortunately, when we use PBS as dilution reagent, the detection of fluorescence labeled particles is quite difficult. Based on the high concentrations of ions in the diluent we suppose that the high osmolality affects the detection method. When analyzing an unstained suspension of particles, we generally used carefully selected water, which contains near to zero particles. The use of water as diluent did not affect the number of particles in the samples, but after a couple of hours, the particles begin to swell, however, we did not observe any particle burst. When diluting the samples for measurement, we recommend processing the sample as soon as possible to avoid variation in the detected size. We added an appropriate note in the protocol section as well as in the discussion.

We have added to protocol (lines 169 – 170):
"Note: It is crucial to use water as diluent for the EV suspension, because other diluents (e.g. PBS) can impair the measurement. "

We have also added to the discussion (lines 430 – 433):

"Normally, many researchers use PBS as diluent for EVs. For this method, it is crucial to use distilled water as diluent for the EV suspensions. When EVs are labeled with fluorescing dyes, the high osmolality and ion concentration of other diluents, such as PBS can interfere with the measurement and lead to altered results."

Comment 4: *Figure 4 a-c - why two lanes in the blots? Are they different loading or the same loaded twice? Please indicate in figure legend. Also CD63 blots are notorious for smearing from 20- 60 kDa range. It would be helpful to indicate that this might occurs in some samples.*

Answer: We thank the reviewer for this comment. All conducted experiments were performed with blood samples from two different donors, and therefore we used both samples for Western blot analysis. We added an appropriate annotation in the figure legend. We also added a note in the protocol that CD63 can be extensively and variably glycosylated, and that the molecular weight can vary and bands can appear between 40 - 65 kDa.

We added to the legend of figure 4 (lines 383 – 384):
"…representative Western blots of two different EV suspensions (4) for…"

We have also added to protocol (lines 309 – 310):
"Note: Because CD63 antigen is extensively and variably glycosylated, the molecular weight can vary and bands can appear between 40 - 65 kDa."

Reviewer #2:

<u>Manuscript Summary:</u>

Extracellular vesicles (EVs), including exosomes are nano-sized vesicles found in bodily fluids, which are released, from many cell types to play roles in regulating cell-cell communication in diverse range of biological processes. EVs studies are highlighted as potential biomarkers for early diagnosis of several diseases and/or prediction of disease progression. Exosomes are often described by the presence of molecules that they are specifically associated with regardless of the cell types they are driven from. Many methods are used for EVs purification methods to describe the characterization, however most of methods have disadvantages, such as time-consuming, difficult to analyze specific markers of interest due to the lack of discrete populations. There is still no standardized method for characterization of single EVs. Authors developed a semi-automated method for characterization of single EVs by fluorescence-based nanoparticle-tracking analysis. Their new advanced methods are based on their former method of nanoparticle-tracking analysis. Their protocol presents rapid isolation of EVs and characterization with both PKH67, a general cell membrane linker, as well as with specific surface markers such as CD63, CD9, vimentin and LAMP-1. They used human serum samples for EVs isolation, but their method is suitable for EDTA and citrated plasma and from cell culture supernatants. Their method resulted a high level of reproducibility, which is confirmed by Western blot. This manuscript has an interesting topic, innovated results, and showing potential significance. Reviewer suggests addressing few issues and data to revise their manuscript.

> <u>Answer:</u> We would like to thank the reviewer for careful and thorough reading of this manuscript and for the thoughtful comments and constructive suggestions, which help to improve the quality of this manuscript. In the following we would like to respond to the comments and questions of the reviewers.

<u>Major Concerns:</u>

Comment 1*: In 1.1, please describe more detail about the methods used. For example, after whole blood collection, did you incubate at RT for how long before the centrifugation? The description is not user friendly.*

> <u>Answer</u>: We thank the reviewer for this suggestion. We revised the complete paragraph and added more details about the isolation process.

The paragraph was revised as follows (lines 110 – 132):

1. Collect 2 mL human whole blood in serum-separating tubes (SST) via venipuncture and incubate the tube for 15 min at room temperature (RT) until coagulation is finished.
2. Centrifuge the SST at 1,700 x g for 10 min at RT to separate cells from serum and transfer 1 mL of the serum to a 1.5 mL reaction tube. Centrifuge the platelet-rich plasma (PRP) at 3,000 x g for 15 min at 4°C to remove platelets and transfer 100 µL of platelet-poor plasma (PPP) to a new 1.5 mL reaction tube.
3. Add 25 µL exosome precipitation solution (4 parts PPP, 1 part exosome precipitation solution) and vortex thoroughly. Incubate the sample for 30 min on ice. Keep the tube upright and do not rotate or mix the tube during the incubation period.
4. Centrifuge the sample at 1,500 x g for 30 min at 4°C to pellet the EVs. After centrifugation, the EVs appear as a beige or white pellet at the bottom of the vessel. Aspirate the supernatant and centrifuge the sample at 1,500 x g for 5 min at 4°C.
5. Remove all traces of fluid and re-suspend the pellet in 100 µL phosphate-buffered saline (PBS) by frequently pipetting up and down. Store the EV suspension at -80 °C when analysis is not be performed immediately.

Note: When isolating EVs from plasma, fibrinogen and fibrin can impede efficient recovery and re-suspension is heavier and takes more time.

Comment 2: *Spell PBS in full, at least for the first time such as phosphate-buffered saline (PBS).*

Answer: We thank the reviewer for this hint. We reviewed and revised all the used abbreviations in this manuscript.

Comment 3*: Authors used PKH67 to staining the EVs. This dye is non-specific membrane dye and now exosomes specific dyes are available in commercially. Reviewer suggests using such dyes to in their method.*

Answer: We thank the reviewer for this suggestion. We also tested an Exo-Glow staining kit but unfortunately, these fluorescing dyes are not stable enough for the applied method. Of course, we will consider the use of novel dyes for refining our method for future applications

Comment 4*: Lowry protein assay needs to be described.*

Answer: We added a more precise explanation how to handle and prepare the samples for Lowry protein assay.

We have added to protocol (lines 291 – 297):

2. Measure the total protein by Lowry protein assay kit. Dilute 1 µL of the isolated EV suspension with 49 µL RIPA buffer and use 5 µL in triplicates for analysis. Dilute the EV suspension with 2x Laemmli loading buffer to a final concentration of 2 µg/ µL and heat for 10 min at 95°C.
3. Load 20 µg of protein per well. Separate and transfer the proteins by polyacrylamide gel electrophoresis and tank blotting according to standard protocols.

Comment 5: *How did you store the purified exosomes?*

Answer: Normally, we store the purified exosomes at -80°C for long-term storage. But all analyses for this manuscript were performed immediately after isolation. We added a respective comment in 1.1.

We have added to protocol (lines 131– 132):

"Store the EV suspension at -80 °C when analysis is not performed immediately."

Minor Concerns:

Comment 1: *Add detailed information for the reagents you used, such as Exoquick, PKH67, etc.*

Answer: We thank the reviewer for this hint. We added more details for the used reagents in the results and in the discussion as well as in the materials table.

We have added to representative results (lines 328– 330 and 354 - 355):

"PKH67 is often used for proliferation monitoring but has also proven useful for monitoring exosome or liposome uptake as well as for in vivo cell trafficking. Due to the non-specific labeling of PKH67, a wide variety of EVs can be labeled and detected".

"EVs were isolated by a polymer-based exosome precipitation solution containing polyethylene glycol"

We also have added to the discussion (lines 411 – 414)

"In this protocol, the EV suspension was generated from 100 µL serum using an exosome precipitation reagent, which contains a proprietary polymer that gently precipitates exosomes and EVs according to a corpuscular size ranging from 30 nm to 200 nm"

Comment 2*: Please correct the reference for LOTAN etc. This format needs to be revised to match other references.*

Answer: Because there is no reference for "LOTAN" we edited all references according to the journal guidelines

Comment 3*: The last paragraph of Introduction can be revised to strengthen the discovery.*

Answer: We revised the last paragraph of the introduction and added further useful information for practical application.

We revised in the introduction (lines 97– 102):
"In this protocol, we describe the complete workflow for rapid isolation of EVs from human whole blood and fast characterization of specific markers by fluorescence-based nanoparticles-tracking analysis. EVs can be detected by staining with PKH67, a general cell membrane linker, as well as with specific exosomal markers, e.g. CD63, CD9 and vimentin. Our protocol is also suitable for EDTA and citrated plasma as well as other body fluids and cell culture supernatants."

Comment 4*: Revising Abstract is suggested in the last few sentences to highlight the discovery.*

Answer: We revised the abstract in the last few sentences.

We revised in the abstract (lines 49– 55):
"In the conducted experiments, we exclusively used EVs isolated from human serum samples, but this method is also suitable for plasma or other body fluids and can be adjusted for characterization of EVs from cell culture supernatants. Irrespective of the future progress of research on EV biology, the protocol that is presented here provides a rapid and reliable method for rapid characterization of single EVs with specific markers."

Again we would like to thank the reviewers for their critical review and suggestions. Following their recommendations we feel that the manuscript has gained in clearness and validity, and we hope that the revised form of our work will find the approval of the reviewers as well as the editors of JoVE.