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Size Determination and Phenotypic Analysis of Urinary Extracellular Vesicles Using Flow Cytometry

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May 26th, 2020

Benjamin Werth
Sr. Science Editor – Chemistry | Biochemistry
JoVE

Dear Dr. Werth

Attached you will find the first revision of the manuscript entitled “Size determination and phenotypic analysis of urine extracellular vesicles using Flow Cytometry” as a peer-reviewed video methods for JoVE. This manuscript has not been submitted for publication elsewhere.

In this protocol, we describe a method for the isolation of urine extracellular vesicles (uEVs) from human healthy-donors and their phenotypic characterization by size and surface marker expression using BD Accuri C6 flow cytometer.

All the authors named in the submitted version of the protocol have read and agree with the content of the manuscript. We hope you find our manuscript interesting enough to be considered by JoVE.

We hope to hear from you soon.

Sincerely:

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TITLE:

Size Determination and Phenotypic Analysis of Urinary Extracellular Vesicles Using Flow Cytometry

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KEYWORDS:

urinary extracellular vesicle, exosome, flow cytometry, quantification, size, biomarker

SUMMARY:

This protocol describes a method for the isolation of urinary extracellular vesicles, uEVs, from healthy human donors and their phenotypic characterization by the size and surface marker expression using flow cytometry.

ABSTRACT:

Extracellular vesicles, EVs, are a heterogeneous complex of lipidic membranes, secreted by any cell type, in any fluid such as urine. EVs can be of different sizes ranging from 40-100 nm in diameter such as in exosomes to 100-1000 nm in microvesicles. They can also contain different molecules that can be used as biomarkers for the prognosis and diagnosis of many diseases. Many techniques have been developed to characterize these vesicles. One of these is flow cytometry. However, there are no existing reports to show how to quantify the concentration of EVs and differentiate them by size, along with biomarker detection. This work aims to describe a procedure for the isolation, quantification, and phenotypification of urinary extracellular vesicles, uEVs, using a conventional cytometer for the analysis without any modification to its configuration. The method's limitations include staining a maximum of four different biomarkers per sample. The method is also limited by the amount of EVs available in the sample. Despite these limitations, with this protocol and its subsequent analysis, we can

obtain more information on the enrichment of EVs markers and the abundance of these vesicles present in urine samples, in diseases involving kidney and brain damage.

INTRODUCTION:

In mammals, blood is filtered by passing through the kidneys 250 - 300 times; during this time, urine is formed. Production of this biofluid is the result of a series of processes, including glomerular filtration, tubular reabsorption, and secretion. Metabolic waste products and electrolytes are the main components of urine. Also, other byproducts such as peptides, functional proteins, and extracellular vesicles (EVs) are excreted[1-6]. Initially, urinary extracellular vesicles (uEVs) were identified in urine samples from patients suffering from water-balance disorders. These patients showed the presence of molecules such as aquaporin-2 (AQP2), which was then used as a biomarker for this disease[7]. Several subsequent studies focused on identifying the cellular origin of uEVs, describing that these structures can be secreted by kidney cells (glomerulus, podocytes, etc.) and other cell types of endothelial or leukocytic lineages. Moreover, the number and molecule-enrichment in uEVs can correlate with the status of many diseases and disorders[8-14].

Altogether, EVs make up a highly heterogeneous family of particles enclosed by lipid bilayers and released by cells through passive or active mechanisms into different fluids. Depending on their origin, EVs can be classified as endosome originated exosomes or plasma membrane-derived microvesicles/microparticles. However, this classification criterion can only be applied when the biogenesis of the particles is directly observed. Therefore, other non-trivial criteria, including physical, biochemical, and cellular origin, have been endorsed by several researchers in the field[15-17]. Depending on the nature of the isolate analyzed, different analytical techniques were suggested for EVs characterization. For example, based on the enrichment of big (≥100 nm) or small (≤100 nm) EVs, quantification via flow cytometry or nanoparticle tracking is suggested, respectively[18].

Nowadays, the use of EVs as biomarkers for many diseases has become relevant, so the search for different sources are been investigated. One of the most promising sources is the urine as it can be obtained in an easy and non-invasive manner. Therefore, this protocol describes a procedure for the isolation of uEVs by differential centrifugation, processing with fluorochrome-conjugated antibodies, and downstream analysis using a conventional 2-lasers/4-colors cytometer.

PROTOCOL:

The human urine samples were obtained from healthy volunteers who had signed donor-informed consent. These procedures were also approved by the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán Research Ethics Committee.

1. Isolation of urinary extracellular vesicles

NOTE: The isolation protocol of uEVs is modified from ref.[19]. **Figure 1** depicts the representation of the protocol to isolate uEVs.

[Place **Figure 1** here]

1.1. Use the first-morning urine (15 mL) from healthy volunteers. Centrifuge the urine at 3,000 x *g* for 10 min at 4 °C, to remove all the cells and debris.

NOTE: Preferentially use fresh urine; if not available, use urine stored for a maximum period of 3 months at -20 °C, or stored for a maximum period of 6 months at -70 °C. Thaw the urine sample on ice and vortex it vigorously. Perform all the procedures on ice or at 4 °C. An optional step for western blot analysis is to make a duplicate tube and aliquot 200 µL of the first-morning urine in a separate tube. Store the sample at -20 °C until use, labeling it as "whole urine", to check for extracellular vesicle markers.

1.2. Transfer the supernatant obtained in step 1.1 to a new 15 mL conical centrifuge tube, then centrifuge at 10,000 x *g* for 45 min at 4 °C. Transfer the supernatant to an 8 mL polycarbonate ultracentrifugation tube and preserve this on ice.

NOTE: Optionally, aliquot 200 µL of the supernatant obtained in step 1.1 in a separate tube labeled as "urine without cells" for western blot analysis.

1.2.1. Removal of Tamm-Horsfall protein, THP

NOTE: THP is present in urine and is enriched when an individual has renal disease. It has been reported that THP diminishes the yield of uEVs because it can bind to uEVs. To remove this protein, use of a reducing agent is neccesary[3,6].

1.2.1.1. Prepare the isolation solution by mixing 250 mM sucrose with 10 mM triethanolamine. Adjust the pH to 7.6.

1.2.1.2. Mix the pellet obtained in step 1.2 with 500 µL of isolation solution, and then add 5 µL of β-mercaptoethanol.

1.2.1.3. Incubate the pellet-mix at 37 °C for 10 min, vortexing every 2 min. Add 500 µL of isolation solution and then centrifuge at 17,000 x *g* for 10 min at 37 °C. Collect the obtained supernatant (containing reduced-non aggregated THP plus uEVs).

1.3. Mix both the reserved supernatants (from steps 1.2 and 1.2.1.3) in the same 8 mL polycarbonate ultracentrifugation tube, and then centrifuge at 160,000 x *g* for 70 min at 4 °C, using an ultracentrifuge fixed-angle rotor.

NOTE: Optionally, for western blot analysis, store 200 µL of the supernatant obtained above labeling it as "supernatant without EVs". This sample will serve as a negative control when searching for extracellular vesicle markers.

1.4. Discard the supernatant. Add ice-cold 1x PBS to the 8 mL polycarbonate tube and centrifuge at 160,000 x *g* for 70 min at 4 °C.

NOTE: Be careful not to discard the pellet, as it contains the uEVs. The 1x PBS needs to be sterilized and filtered with at least 0.22 µm pore syringe-filter.

1.5. Discard the supernatant. Add the rest of the supernatant to the 8 mL polycarbonate tube and centrifuge at 160,000 x *g* for 70 min at 4 °C.

1.6. Discard the supernatant and add 8 mL of ice-cold PBS to the pellet to wash the uEVs.

1.7. Discard the supernatant. Let the pellet dry and then resuspend it with 1 mL of ice-cold PBS. Store at -70 °C until use.

NOTE: If performing western blot analysis, resuspend the duplicate tube's pellet with 50 µL of RIPA buffer plus protease inhibitors. Store at -20 °C until use.

2. Staining of uEVs

NOTE: Before staining and analysis of uEVs, it is essential to perform at least one methodology recommended by MISEV2018[18] to verify proper isolation of uEVs; here, western blot analysis is depicted. **Figure 2** shows a representative protocol to uEVs stain.

[Place **Figure 2** here]

2.1. Calculate the relative number of uEVs by quantifying the protein content using any conventional colorimetric protein assay, as mentioned in the **Table of Materials**. Perform a 1:5 dilution of the uEVs and follow the instructions of the datasheet provided in the assay protein kit.

2.2. Based on a previously reported formula[20], consider 1 µg/mL of uEVs protein to be equal to 800,000 uEVs/ µL. Ensure 500,000 uEVs are present in 20 µL of ice-cold PBS.

2.3. Label the tubes, as indicated in **Table 1**.

[Place **Table 1** here]

NOTE: The number of tubes used will depend on the number of antibodies employed, the limitation for 2-lasers/4 colors cytometer is a maximum of 4 antibodies per tube that could be used; therefore, only FL1, FL2, FL3, and FL4 in the cytometer could be read, although compensation is needed. For these procedures, use 1.5 mL microcentrifuge tubes or 5 mL round-bottom tubes for flow cytometry. Needed tubes are depicted in **Table 1**. Tubes 4 and 5 consist of a cocktail of all the antibodies to be used in this protocol. Two problem tubes (8 and 9) are given as an example; therefore, this set of controls must have the combination to be

used in each tube.

2.4. Add 20 µL of PBS containing 500,000 uEVs to the labeled tubes.

2.5. Add the antibodies as indicated in **Table 1**, previously titrated. Incubate overnight at 4 °C.

NOTE: Before staining, it is recommended to centrifuge the antibodies at 4 °C at full speed for at least 5 min, to prevent the aggregates. The antibodies used here are an example of proteins present in the uEVs and belong to an independent manuscript currently in preparation.

2.6. Add 0.4 µL of carboxyfluorescein succinimidyl ester (CFSE) [5 nM] to tube number 3. Incubate for 10 min at 37 °C.

NOTE: CFSE is a dye used to stain all the EVs present in a sample and discriminate between the background noise when a flow cytometer analysis is performed. For more information, see the **Discussion** section.

2.7. Add 400 µL of ice-cold 1x PBS to all the tubes.

2.8. Keep all the tubes at 4 °C.

3. Acquisition of uEVs using a conventional cytometer

NOTE: Instructions for the use of the flow cytometer (see **Table of Materials**) are described here.

3.1. Perform the quality calibration of the cytometer, using the 6- and 8-peak beads.

NOTE: The %CV of the last peak must be a value under 6; the cytometer technician will take care of this.

3.2. Open the flow cytometer software.

NOTE: Once the software is open, a new experiment will open, showing a screening with a template for 96 samples, the parameters for running, and an "empty" section to create dot plots or histograms.

3.3. Adjust the parameters on the screen of the software that is running: 100 µL of sample to capture, slow running, set the threshold at 10,000 on FSC-H, and 2,000 on SSC-H.

NOTE: The threshold recommended here is an example; it is required to set the threshold based on the samples analyzed.

3.4. Load tube number 1 (Megamix tube), as indicated in **Table 1**. Capture the beads. Create

two dot plots in the "empty" part of the software screen by clicking on the **Dot Plot** option. Create as panel A and B of the **Figure 3**.

[Place **Figure 3** here]

NOTE: The Megamix fluorescent beads (beads used to delimit the sizes of 0.1, 0.3, 0.5, and 0.9 µm and create the template for sizes), can be analyzed using the FL1 (FITC) detector. All the dot plots and histograms need to be displayed in height values. Record as many events as possible; any modification in the worksheet does not alter the data.

3.5. In the "empty" section of the software screen create a dot plot and histogram for each fluorochrome used; use the algorithm in **Supplementary Figure 1** as an example. To do this, click on the **Dot Plot** or **Histogram** option for each fluorochrome.

NOTE: Background noise will always be present, as shown in **Figure 3A**. All the dots between 0 to 1,000 of FL1 (x-axis) are outside the created gate. Therefore, it is essential to use all the control tubes to obtain adequate results. Between every tube loading, backflush the sheath fluid and perform an unclog cycle. Gently shake every tube before loading. After loading five consecutive tubes, run 100 µL of PBS 1x.

3.6. Load tubes number 2 and 5, to set the cut-off values (negative). To do this, select the **Rectangle Gate** icon (located under the dot plot created), or **Line Gate** icon (located under the histogram created); and place it where there is no signal, in order to obtain dot plots and histograms like the ones shown in **Supplementary Figure 1**.

NOTE: All these tubes require the placement of the positive region not further than 0.70% because there will not be "fluorescence" more than 10^3 (1,500). If there is any fluorescence further than this region, dilute the reagents used. Therefore, it is important to titer the reagents before final measurements.

3.7. Load tube number 6 (autofluorescence tube), to set the negative regions for the sample. To do this, select the **Rectangle Gate** icon (located under the dot plot created), or **Line Gate** icon (located under the histogram created); place it where there is no signal.

3.8. Load the next tubes (tubes 7 to 9, depicted in **Table 1**).

3.9. Save the experiment.

NOTE: For the cytometer software used here, the command **Save Experiment**, refers to save the data generated. In other words, all the tubes that are acquired by the flow cytometer, and the data generated for each tube, need to be saved. To do this, it is necessary to click on the **Save Experiment** button.

3.10. Export data as FSC files.

4. Analysis of the data with a flow cytometer software.

NOTE: Instructions for using the flow cytometer software depicted in the table of materials, are described in this section. **Figure 4** shows the workspace with the steps to create the size gates.

[Place **Figure 4** here]

4.1. Open the flow cytometer software. Add the samples, by clicking on the **Add Samples** button, and select the exported FCS files.

4.2. Click on the **Megamix tube** to open a dot plot SSC-H (y-axis) VS FSC-H (x-axis). Adjust both axes to view 0 to 100,000 by clicking on the **T button** (Transform Data Display), click on **Customize Axis**, click on **SSC-H** or **FSSC-H** and change the scale value to min 0 and max 100,000 (see **Figure 4A-F**).

4.3. Set the gates for 0.1, 0.3, 0.5, and 0.9 µm, in the dot plot generated in step 4.2. Select the **Rectangle Gate** button to create a gate as depicted in **Figure 4G-I**.

NOTE: **Figure 5** shows the workspace with the steps to create the positive regions, and to obtain the Mean Intensity for the fluorochrome.

[Place **Figure 5** here]

4.4. Apply the generated gates (0.1, 0.3, 0.5, and 0.9 µm) to all the samples. Select the **Gates**, drag and drop in the **All Samples** option, as depicted in **Figure 5A**.

4.5. Click on the **Autofluorescence tube**. Set the positive regions for each fluorochrome, for each size. Open the dot plot SSC-H VS FSC-H with the size gates, click on **Single Gate Size**. In the new window, click on the **y-axis** to select the histogram option. In the **x-axis**, select FL1-H, then select the **Range** icon to create the positive region. Repeat the operation for FL2-H and FL4-H (see **Figure 5B**).

4.6. Apply the gates to all the samples. Select **Gates**, drag and drop in the **All Samples** option, as depicted in **Figure 5C**.

4.7. Open the Layout Editor.

4.8. Drag and drop each sample in the editor. Select the size of the “autofluorescence” tube, drag and drop, then select the same size of the stained tube, drag, and drop.

4.9. Click the right bottom on the histogram. Open the **Properties** menu. Click on **Legend**. Add the mean intensity for the fluorochrome.

NOTE: In **Properties**, there are other available tools to modify the appearance of the histogram.

4.10. Repeat the same procedure for the remaining fluorochromes and sizes.

5. Analysis to obtain the number of uEVs per sample.

NOTE: **Figure 6** shows the workspace with the steps to obtain the number of uEVs per sample.

[Place **Figure 6** here]

5.1. Click on **Autofluorescence tube**. In the dot plot SSC-H VS FSC-H with the size gates, create a region including all the sizes. Click on **New Region**. Set the positive region (see **Figure 6A,B**).

5.2. Apply the gates to all the samples. Select the gates, drag, and drop in the **All Samples** option.

5.3. Click on **CFSE + uEVs tube**. Open the new region and verify the positive region for CFSE (FL1-H), as depicted in **Figure 6C**.

5.4. At the workspace in **Figure 6D**, copy the # cells data for the tube. This number is the #uEVs obtained by flow cytometry (#uEVs FC).

5.5. Apply the following formula to calculate the number of uEVs per microliter:

$$\#uEVs/\mu L = \frac{\#uEVs\ FC \times Dilution\ Factor}{100}$$

5.6. To have the #uEVs per microliter for each fluorochrome (#uEVs/µL FLX), at the workspace, copy the statistics for the FLX-H subset, this number is the percentage (%FLX subset).

5.7. Apply the following formula to calculate the number of uEVs per microliter for each fluorochrome.

$$\#uEVs/\mu L\ FLX = \frac{(\%FLX\ subset) \times (\#uEVs\ \mu L)}{100}$$

NOTE: Depending on whether the selected gate will be for one size or for all the uEVs, verify the selected data.

REPRESENTATIVE RESULTS:

There are several checkpoints through the protocol, and before the staining of uEVs. Therefore, it is essential to first verify the amount of protein present in the extract of uEVs. All the research groups that work with extracellular vesicles quantify the protein, as indicated in step 2.1. **Supplementary Figure 2** shows a representative 96 well plate containing uEVs fraction in wells 4E, 5E, and 6E. Wells 1A, 2A, and 3A consist of blanks, but if there are no uEVs purified, the wells will take similar color.

After this step, there is a need to verify the presence of uEVs. **Supplementary Figure 3** shows a representative result of a polyacrylamide gel, stained with Coomassie blue, to show the amount of proteins present in all the collected fractions, and to perform comparison with other methodologies to isolate uEVs. Among the two different reducing agents, dithiothreitol (DTT) and β-mercaptoethanol, the second one showed better protein yield.

Another important thing is to validate the presence of uEVs using any of the methodologies recommended by the MISEV2018. **Supplementary Figure 4** shows a representative result of the enrichment of several proteins such as CD63 and CD9 in the uEVs and the collected fractions used as negative controls. In the uEVs fraction, no visible bands correspond to these proteins, indicating that there is no uEVs isolation.

Supplementary Figure 5 shows a representative result of the uEVs quantification in 12 healthy individuals without any significant or manifested disease, thereby making this method an excellent choice to isolate EVs in homeostatic conditions.

Once isolation of EVs is confirmed, the next step is to prove that the flow cytometer can differentiate between different sizes. **Figure 7** shows an example of graphs obtained with the Megamix FSC beads and other commercial beads with different sizes. As shown, r^2 value is very close to 1.0, indicating the cytometer's sensitivity to differentiate between 0.1, 0.3, 0.5, and 0.9 µm bead sizes. If the r^2 value is less than 0.7, do not use that cytometer for the protocol presented here.

[Place **Figure 7** here]

It is then essential to verify that all the negative controls are set in the correct position; also, consider avoiding switching staining panels without readjusting cytometer settings since results obtained will be different when antibodies with different fluorochromes are used. **Supplementary Figures 6** and **Supplementary Figure 7** show two tubes containing the same sample but stained in a different tube using different antibodies; therefore, it is important to verify these details before applying statistics to the results or to perform any calculations. **Supplementary Figure 6** is an example of an incorrect analysis, using only the PBS tube and one fluorochrome detector to set the negative and positive gates. On the contrary, **Supplementary Figure 7** shows a correct analysis, considering all the negative controls based on the different antibodies with different fluorochromes. These figures endorse the importance of all the controls mentioned here.

The next critical step is to obtain #uEVs/µL (see **Figure 8**). It is essential to verify that the statistic number will be the same as the generated dot plot; if not, there is a mistake, and the resulting calculations will be wrong.

[Place **Figure 8** here]

Once the #uEVs/µL is obtained, , one can obtain the number of uEVs/µL for the sizes defined by the Megamix beads by following the procedure shown in **Figure 9**. It is important to verify the correct statistic number for the generated gate.

[Place **Figure 9** here]

Figure 10 is an example of how the #uEVs/µL can be obtained for FL1 that corresponds to CD9. Do the same for all the antibodies and tubes.

[Place **Figure 10** here]

An example of the results obtained using this technique is presented in **Figure 11**.

[Place **Figure 11** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the uEVs isolation for flow cytometry analysis. In this protocol, first centrifuge the first urine of the day to remove the cells and debris. Then centrifuge to remove the large vesicles with treatment to remove the THP protein and finally perform ultracentrifugation to enrich and obtain the uEVs with a single wash. Steps to keep urine fractions for the WB validation are marked.

Figure 2: Overview of the uEVs staining and capture in the cytometer. (**A**) Representation of the uEV staining. For 500,000 uEVs, the antibody was mixed and incubated at 4 °C for 12 h. Then CFSE was added and incubated at 37 °C for 10 min. The uEVs had the CFSE inside, and the antibody will bind to the surface of the antigen. 400 µL of cold PBS was used to resuspend and to capture 100 µL of the sample in the flow cytometer at a slow velocity. (**B**) Analysis strategy. The first dot plot (SSC-H VS FL-X) depicts the negative control for uEVs, followed by the dot plot showing uEVs staining with CFSE, and finally, a histogram with the antibody staining of uEVs (black line), the negative control is shown in the grayline.

Table 1: Tubes labeling. Example showing how to label the tubes. The first tubes are all the controls needed. The tubes with the antibodies-fluorochromes will depend on the staining.

Figure 3: Megamix FSC beads dot plots. The dot plots showed were generated using the flow cytometer software; in the flow cytometer, the image will be very similar. (**A**) The first dot plot generated to select the beads avoiding the background noise. (SSC-H VS FL1-H). (**B**) The dot plot generated by the selection of the previous gate, showing the different sizes of the beads. (SSC-H VS FSC-H).

Supplementary Figure 1: Dot plots and histograms for each fluorochrome used in the example. For this example, three different fluorochromes were used. On the left side, the histogram is shown, and on the right side, the corresponding dot plot is shown. The gates were

selected using the autofluorescence tube to obtain the positive gate. (**A**) Histogram and dot plot for the FL1-H. (**B**) Histogram and dot plot for the FL2-H. (**C**) Histogram and dot plot for the FL4-H.

Figure 4: Workspace with all the steps to begin the analysis of the data. All the images were generated by screen printing of the workspace. (**A**) Workspace generated with the sample data added (left), dot plot generated by the selection of the tube 1, megamix FSC beads, (right). (**B,C**) show the modification of the axis, to have SSC-H and FSC-H. (**D-F**) show step-by-step customization of both axes. (G-I) show the selection and generation of the different bead sizes.

Figure 5: Workspace to analyze the data obtained. All the images were generated by the screen printing of the workspace. (**A**) Workspace generated with the size gate applied to all the samples. (**B**) Autofluorescence tube selected, dot plot showing the size gate, and the histogram for one selected size (0.1 μm), use this histogram to obtain the positive gate for each fluorochrome and size. (**C**) Workspace generated with the positive gates for each fluorochrome and size. (**D**) Workspace (left) and Layout Editor (right) generated for the samples. In the Layout Editor is shown the histogram for autofluorescence tube and positive tube for FL1-H, and how to obtain the properties panel to modify them. (**E**) The image shows how to obtain the mean intensity fluorescence value. (**F**) Histograms generated for three different fluorochromes, showing all the changes that the software allows to do with the statistic information.

Figure 6: Workspace to analyze the CFSE tube. All the images were generated by screen printing of the workspace. (**A**) Workspace generated by the selection of all the sizes region, uEVs total (left), dot plot showing the gate selected (right). (**B**) Dot plot SSC-H VS FLI-H for CFSE negative region in the autofluorescence tube. (**C**) Dot plot SSC-H VS FLI-H for CFSE in the staining tube. (**D**) Image of the table obtained with the statistics of the CFSE staining, showing the number of uEVs in the sample.

Supplementary Figure 2: uEVs protein quantification. The image shows a 96-well plate after the incubation with the reagents; each condition is a triplicate. A1 – A3 is the blank. Wells 1 -3 from B to H is the standard solution of bovine serum albumin at different concentrations. B1 – B3: 2 μg/mL. C1 – C3: 1.5 μg/mL. D1 – D3: 1.0 μg/mL. E1 – E3: 0.75 μg/mL. F1 – F3: 0.5 μg/mL. G1 – G3: 0.25 μg/mL. H1 – H3: 0.125 μg/mL. A4 – A6: whole urine. B4 – B6: urine without cells. C4 – C6: supernatant without uEVs. D4 – D6: Urine cells. E4 – E6: EVs diluted 1:10.

Supplementary Figure 3: Polyacrylamide gel of urine fractions and uEVs isolated by two different methodologies. The image shows a 15% polyacrylamide gel of urine fractions and uEVs isolated with polyethylene glycol (PEG) 8000 or ultracentrifugation. Line 1: protein marker. Line 2: whole urine. Line 3: urine cells. Line 4: Supernatant without uEVs. Line 5 – 7 uEVS isolated with PEG 8000 in PBS. Line 5 without any reducing agent. Line 6 with DTT. Line 7 with β-mercaptoethanol. Line 8 – 10 uEVS isolated with ultracentrifugation. Line 8 without any reducing agent. Line 9 with DTT. Line 10 with β-mercaptoethanol.

Supplementary Figure 4: Characterization of uEVs. The image shows a western blot of different

markers of uEVs. Line 1: Whole urine. Line 2: Urine without cells. Line 3: Supernatant without uEVs. Line 4: uVEs. The upper panel shows the tetraspanin CD63 40 kDa. The down panel shows CD9 22 kDa.

Supplementary Figure 5: uEVs protein quantification in healthy individuals. The graph is a representative example of uEVs protein quantification, n = 12 healthy individuals.

Figure 7: Validation of flow cytometer to discriminate 0.1 – 0.9 µm. The graphs show representative results. (**A**) Graph of Mean FSC-H VS size of the Megamix beads (black line) and FITC+ beads (red line) the r^2 of both beads are close to 1.000. (**B**) Graph of Mean SSC-H VS size of the Megamix beads (black line) and FITC+ beads (red line) the r^2 of both beads are close to 1,000.

Supplementary Figure 6: Dot plots with an incorrect setting of negative regions. Representative dot plots of different conditions, the negative regions were set using only the PBS tube. (**A**) to (**D**) dot plots SSC-H VS FL2-H. (**A**) PBS tube. (**B**) PBS + CD37 FITC, CD53 PE, ADAM10 APC tube. (**C**) autofluorescence tube (**D**) uEVs + CD37, CD53, ADAM10. (**E**) to (**H**) dot plots SSC-H VS FL1-H. (**E**) PBS tube. (**F**) PBS + CD9 FITC, TSPAN33 AF647 tube. (**G**) autofluorescence tube. (**H**) uEVs + CD9 FITC, TSPAN33 AF647.

Supplementary Figure 7: Dot plots with a correct setting of negative regions. Representative dot plots of different conditions, the negative regions were set using all the tube controls. (**A**) to (**D**) dot plots SSC-H VS FL2-H. (**A**) PBS tube. (**B**) PBS + CD37 FITC, CD53 PE, ADAM10 APC tube. (**C**) autofluorescence tube (**D**) uEVs + CD37, CD53, ADAM10. (**E**) to (**H**) dot plots SSC-H VS FL1-H. (**E**) PBS tube. (**F**) PBS + CD9 FITC, TSPAN33 AF647 tube. (**G**) autofluorescence tube. (**H**) uEVs + CD9 FITC, TSPAN33 AF647.

Figure 8: Analysis strategy to obtain the number of uEVs per microliter. The image shows a representative workflow to obtain the number of uEVs per microliter. (**A**) Dot plot SSC-H VS FL1-H showing the negative region considering all the tube controls. (**B**) Dot plot SSC-H VS FL1-H of the CFSE staining tube, highlighted the percentage of staining. (**C**) The table obtained in the software highlights the percentage and number of uEVs. (**D**) Calculus to obtain the real number of uEVs present in the sample.

Figure 9: Analysis strategy to obtain the number of uEVs per microliter by size. The image shows a representative workflow to obtain the number of uEVs per microliter by size, 0.1 µm. (**A**) Dot plot SSC-H VS FL1-H shows the negative region considering all the tube controls. (**B**) Dot plot SSC-H VS FSC-H of the CFSE staining tube, highlights the percentage of staining in the 0.1 µm gate. (**C**) The table obtained in the software highlights the percentage of 0.1 µm uEVs. (**D**) Calculus to obtain the real number of 0.1 µm uEVs present in the sample.

Figure 10: Analysis strategy to obtain the number of uEVs per microliter with a marker. The image shows a representative workflow to obtain the number of uEVs per microliter with the marker, CD9+. (**A**) Dot plot SSC-H VS FL1-H shows the negative region considering all the tube

controls. (**B**) Dot plot SSC-H VS FL1-H of the CD9+ staining tube. (**C**) The table obtained in the software highlights the percentage of CD9+ uEVs. (**D**) Calculus to obtain the real number of CD9+ uEVs present in the sample.

Figure 11: Example results obtained by the strategies analysis. Representative graphs of the results obtained from 12 healthy individuals. (**A**) The number of uEVs per microliter. (**B**) The number of CD37+ uEVs per microliter. (**C**) The number of CD53+ uEVs per microliter. (**D**) The number of CD9+ uEVs per microliter. (**E**) The number of TSPAN33+ uEVs per microliter. (**F**) The number of ADAM10+ uEVs per microliter.

DISCUSSION:

Nowadays, the use of extracellular vesicles as biomarkers for several diseases has augmented, especially for those that can be isolated from non-invasive sources such as urine[5,21-24]. It has been proved that the isolation of uEVs is a vital resource to know the status of a healthy individual, and the diagnosis/prognosis of patients suffering several diseases[6,13,16,25-27]. This protocol shows how to obtain uEVs to perform their examination by flow cytometry and analytic strategy to obtain the absolute number of uEVs per microliter, segregating them by size and by any biomarker of interest.

Before the flow cytometry staining, the first thing to do is to corroborate and choose the adequate method to isolate uEVs. Shown here is an ultracentrifugation method plus a reducing agent (β-mercaptoethanol) to isolate the uEVs. The reducing agent is added to eliminate Tamm-Horsfall protein's presence since this affects the uEVs isolation[28]. First, in **Supplementary Figure 2**, there is the image of the result obtained when a protein quantification was made; it is essential to mention that the uEVs fraction was diluted 1:10 to have enough sample to perform the Western blot validation. **Supplementary Figure 3** shows an example of two different methodologies reported by literature[19,29-33], showing a better yield of proteins of the uEVs fraction using ultracentrifugation plus β-mercaptoethanol; and then with these samples, a successful western blot for detecting the EVs. The Western blot obtained with other methodologies is not shown because they render no signal, so they represent a non-viable methodology for this purpose.

Once we have the western blot validation of uEVs, the next step is to validate whether the cytometer can discriminate between uEVs and background noise, so the fair use of all the controls mentioned in this protocol is needed[34]. In **Figure 3**, we show a dot plot depicting the Megamix beads with 0.1-0.9 µm and the separation by size, with previously adjusted threshold and zoom. **Figure 7** shows graphs displaying the mean FSC-H and SSC-H VS size of Megamix beads and another type of beads not suitable for this purpose; in both cases, the r^2 is very close to 1.0, indicating good discrimination from the cytometer, despite other researcher groups mentioning that this cytometer cannot perform this function[35]. Contrariwise, other groups use this cytometer to perform extracellular vesicle analysis[36].

Another critical step is the uEVs staining, with a reagent that should stain almost all the extracellular vesicles present in the sample, as reported before[37-39]. CFSE was then selected for

this purpose. It is important to mention that the evaluation of other dyes was done, but the staining ratio was extremely deficient (Data not shown).

The choice of biomarkers shown here was based on an independent project that we are working on, so representative results of the healthy individuals are shown in this protocol. Since the literature indicates that there has not been any step-by-step analysis strategy reported for absolute numbers of uEVs by size and by defined/specific biomarkers, we developed the protocol shown in **Figure 8**, **Figure 9**, and **Figure 10**. As mentioned, it is crucial to have all the controls and to verify that the statistics are correct for each data set.

Using this analytic strategy, we can obtain more information about our samples, and perform better analysis and correlations with clinical data. It is essential to mention that using other methodologies to measure biomarkers is highly recommended to validate the data obtained by flow cytometry. A significant limitation of the particular and simple cytometer used here is that the maximum number of biomarkers per tube is 4; so, the use of several tubes and the use of more sample is required; despite this, the use of this method is a good option if no other devices are available.

Finally, a key attribute of this cytometer is that instead of having a pressurized system for the fluidics system, it possesses a peristaltic pump, so the acquisition of the whole sample volume is possible without any loss, than facilitating to obtain results per volume analyzed.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare that the research was conducted in the absence of any financial or commercial relationship that could be construed as a potential conflict of interest.

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Figure 1

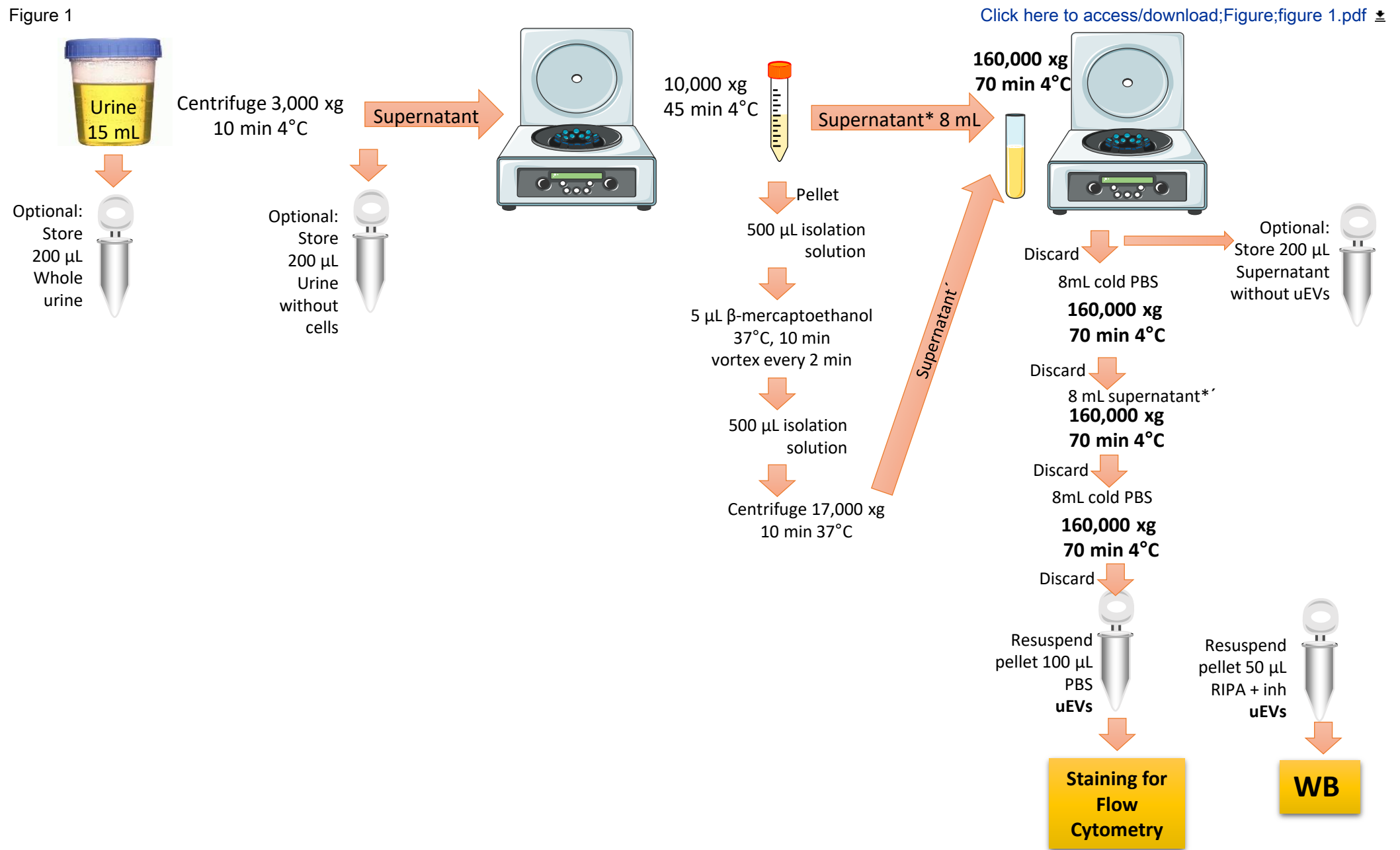

Figure 2

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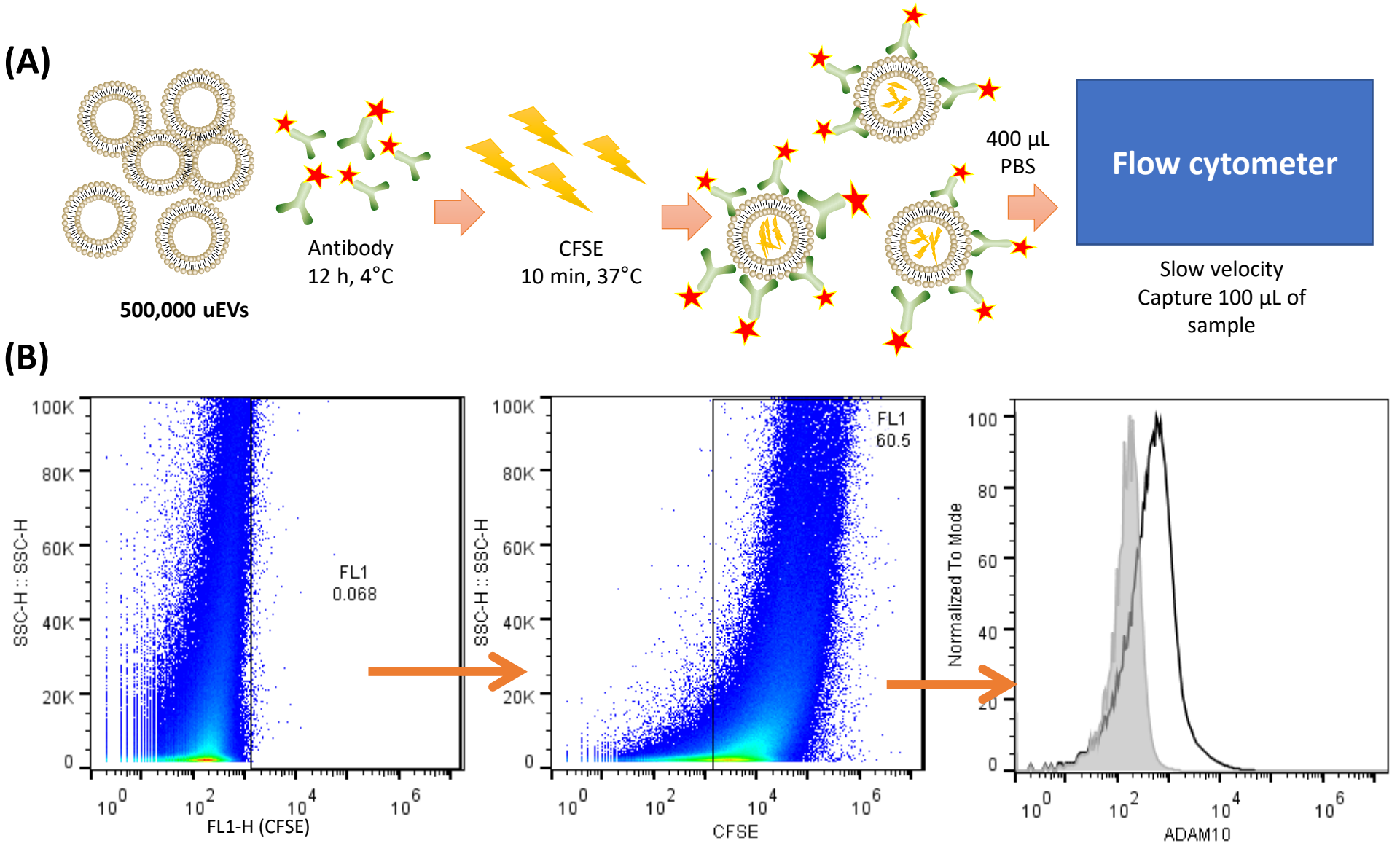

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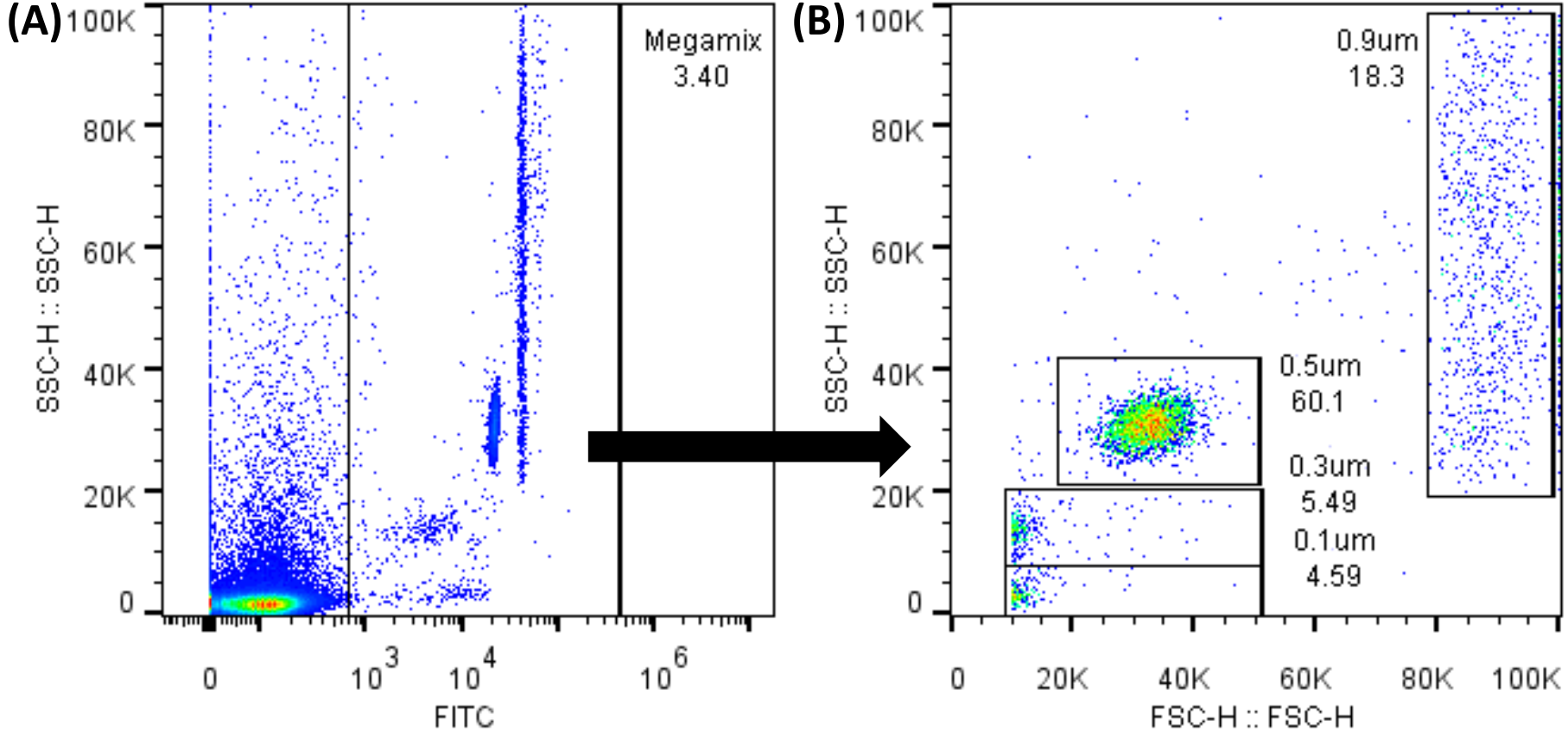

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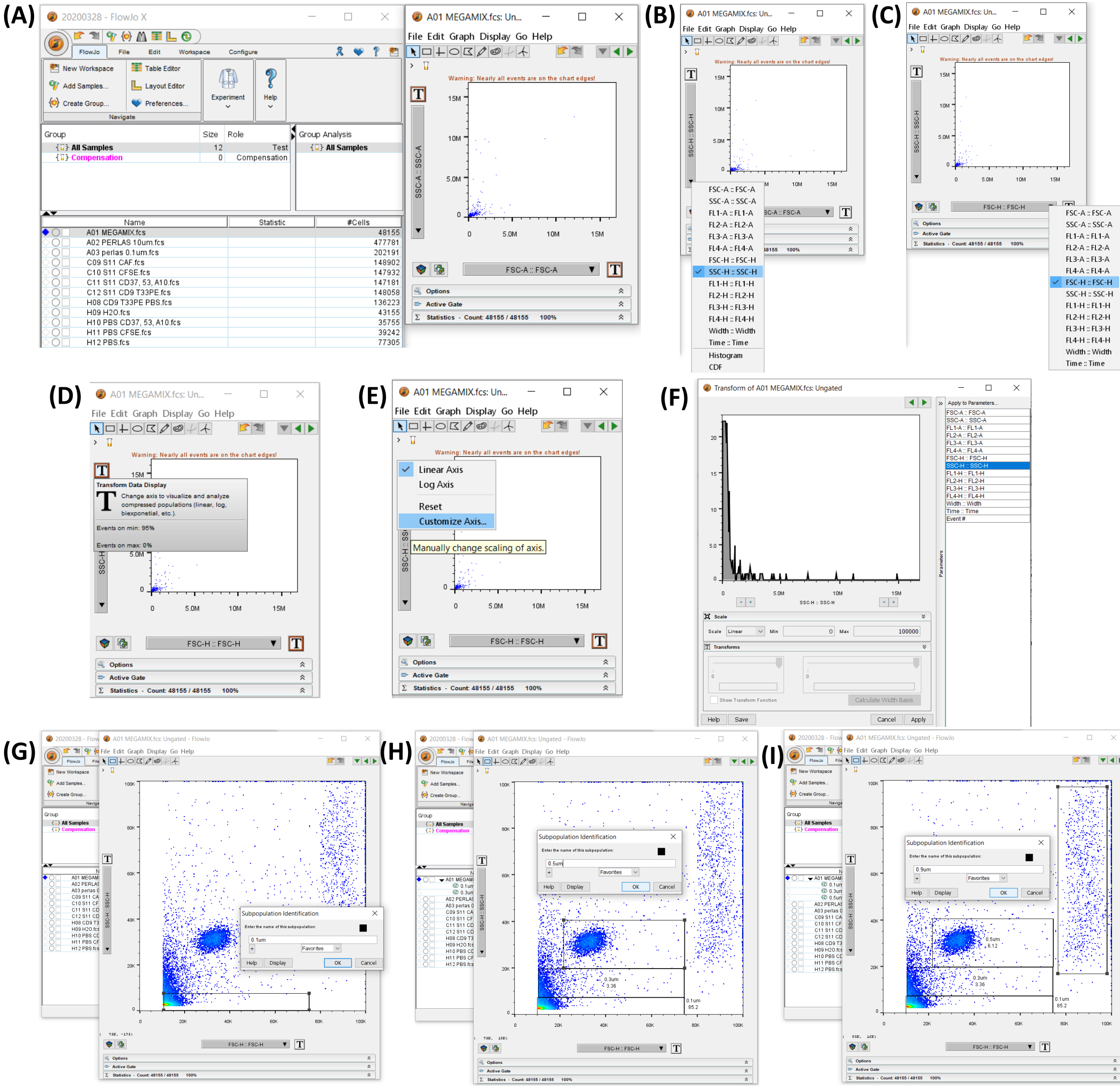

Figure 5

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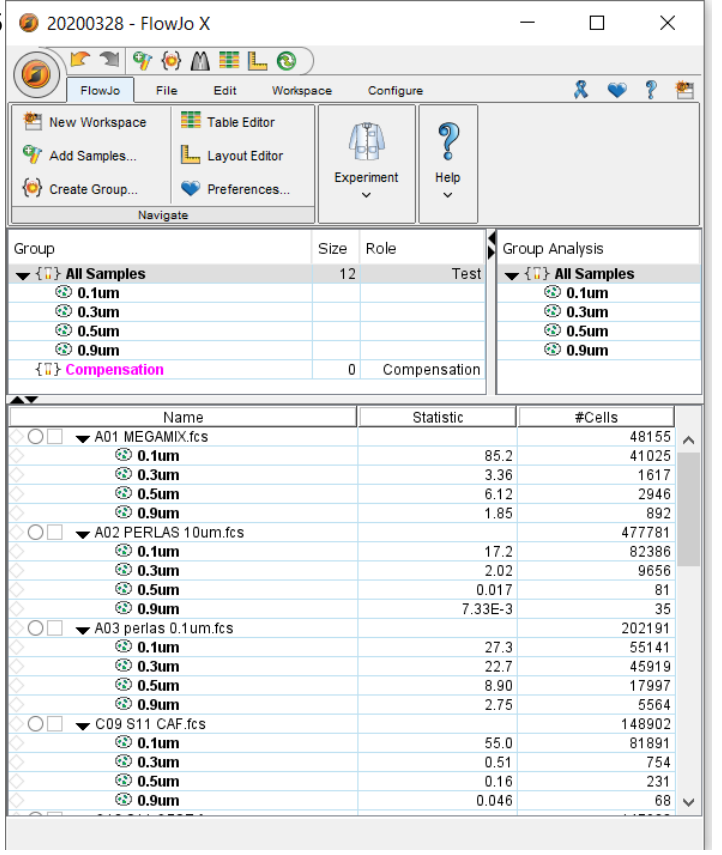

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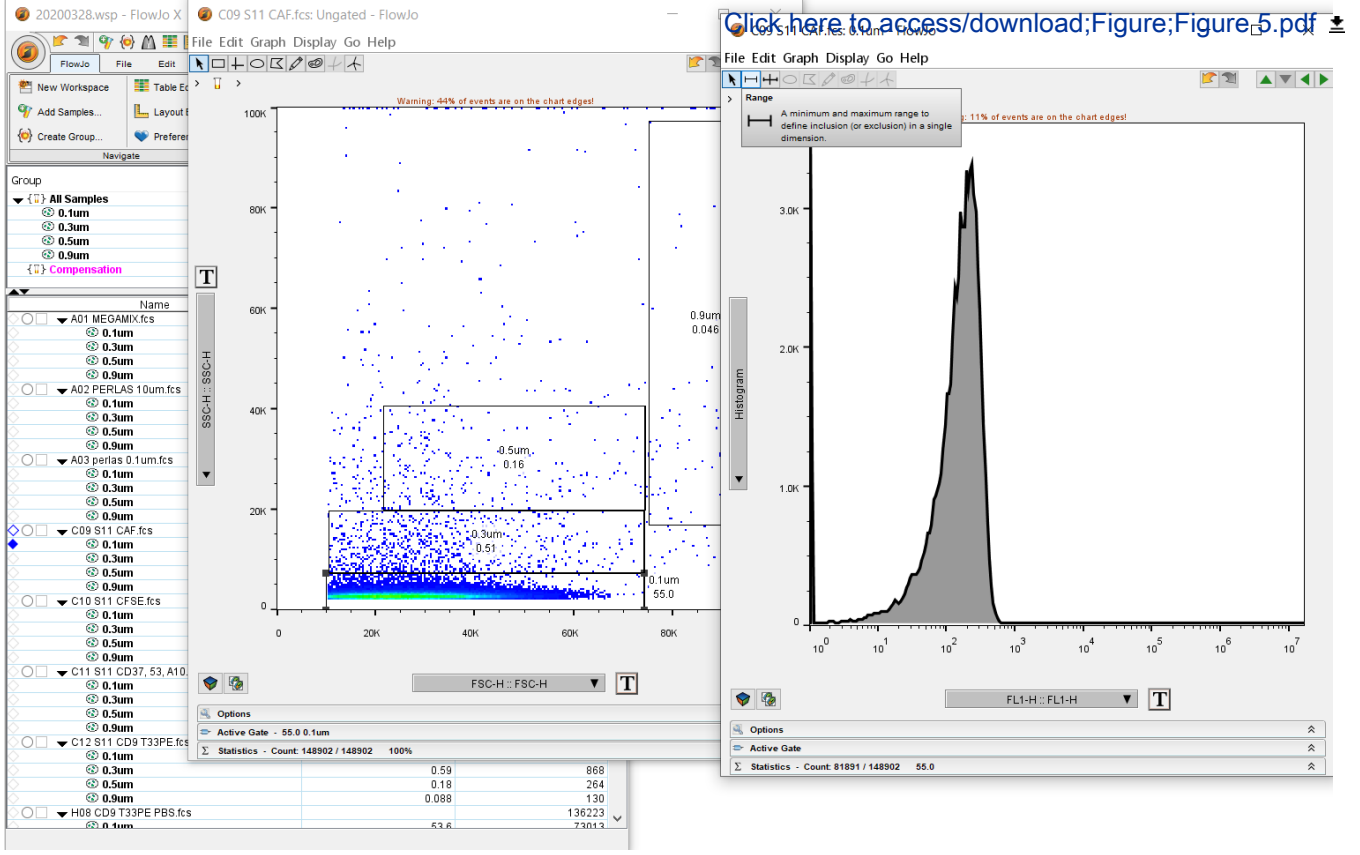

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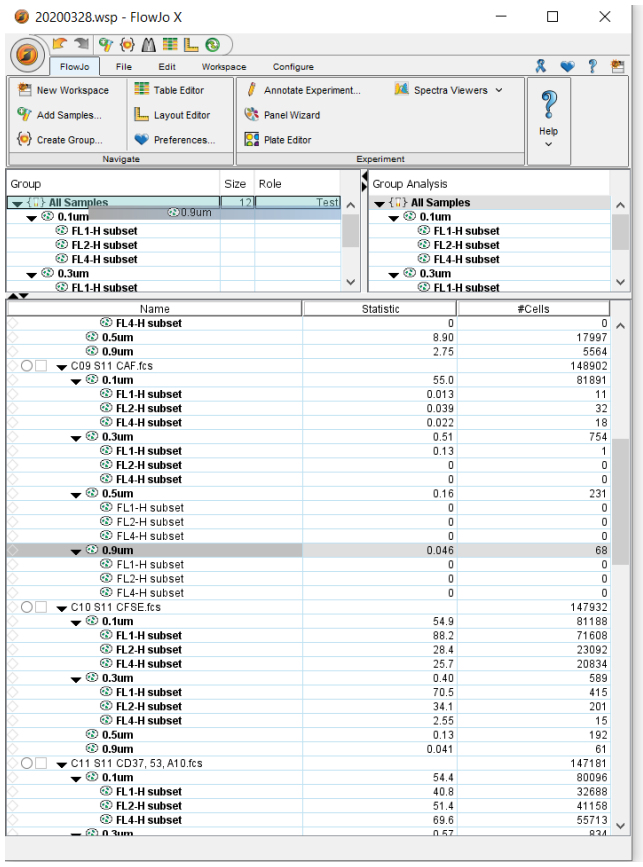
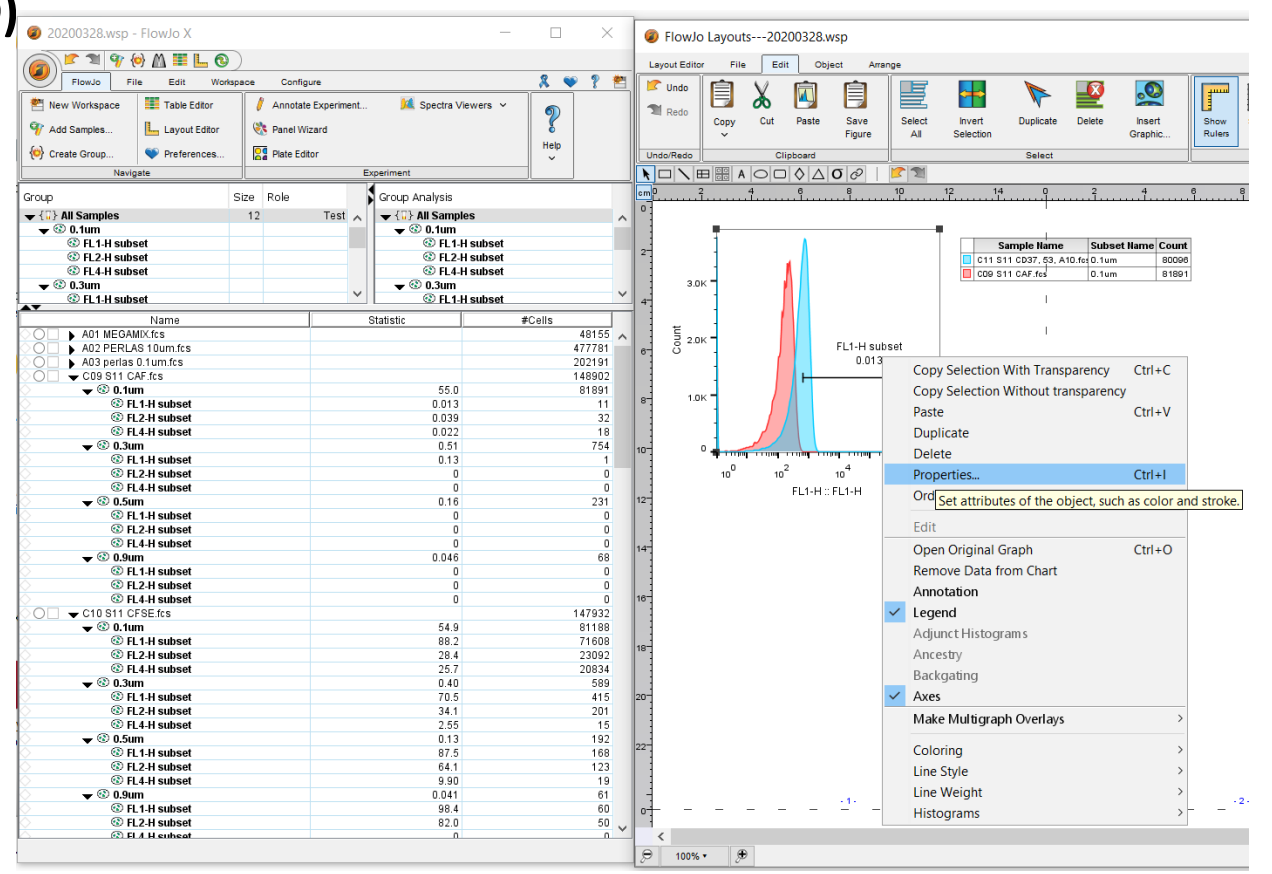


(D)



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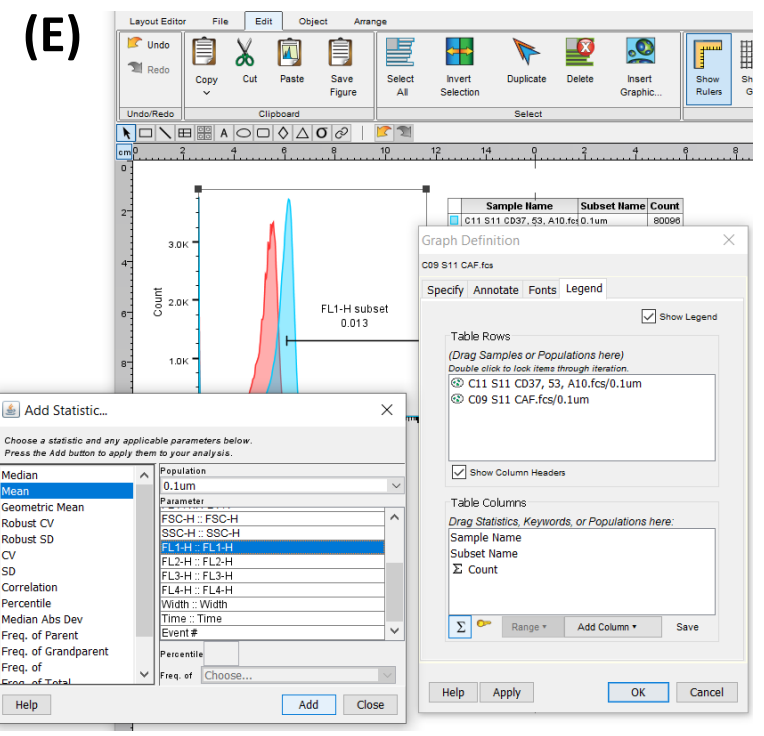

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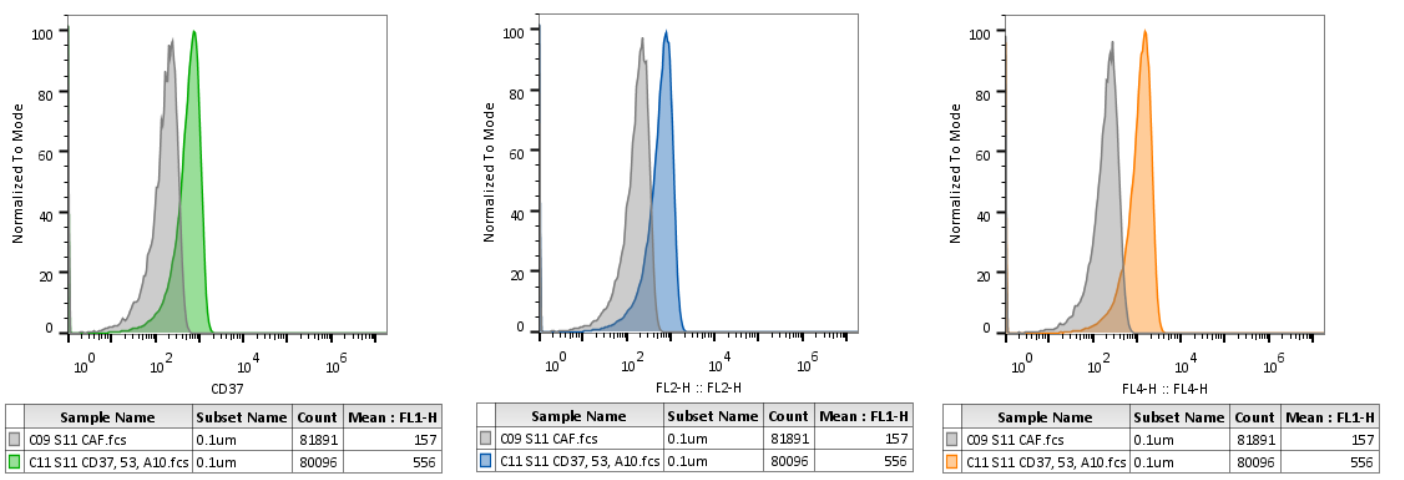

Figure 6

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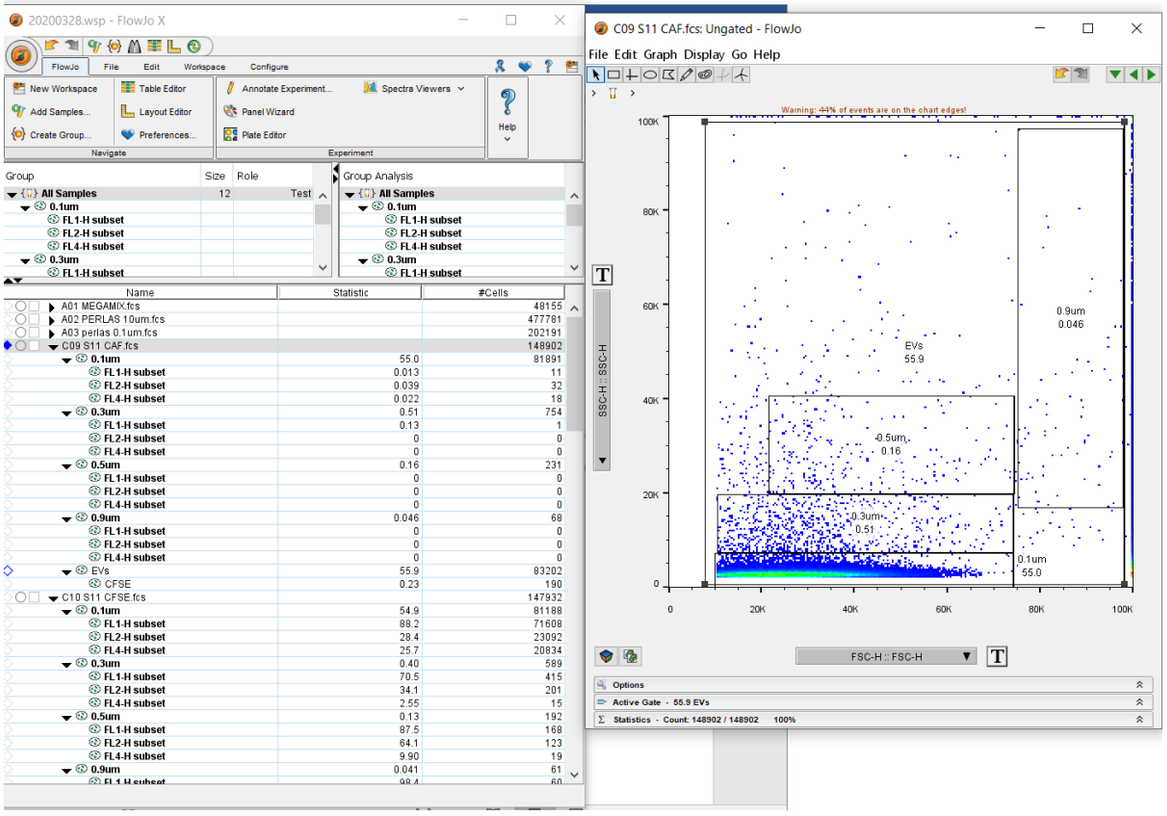

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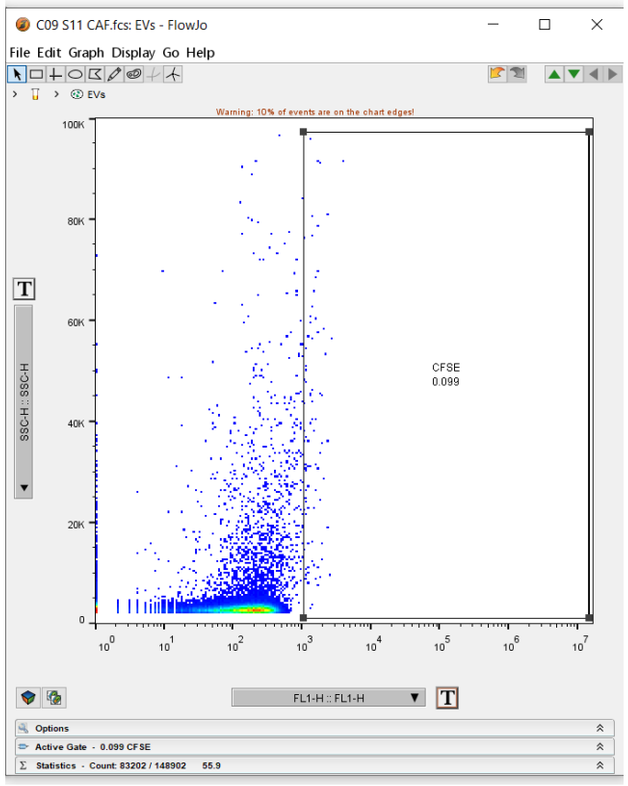

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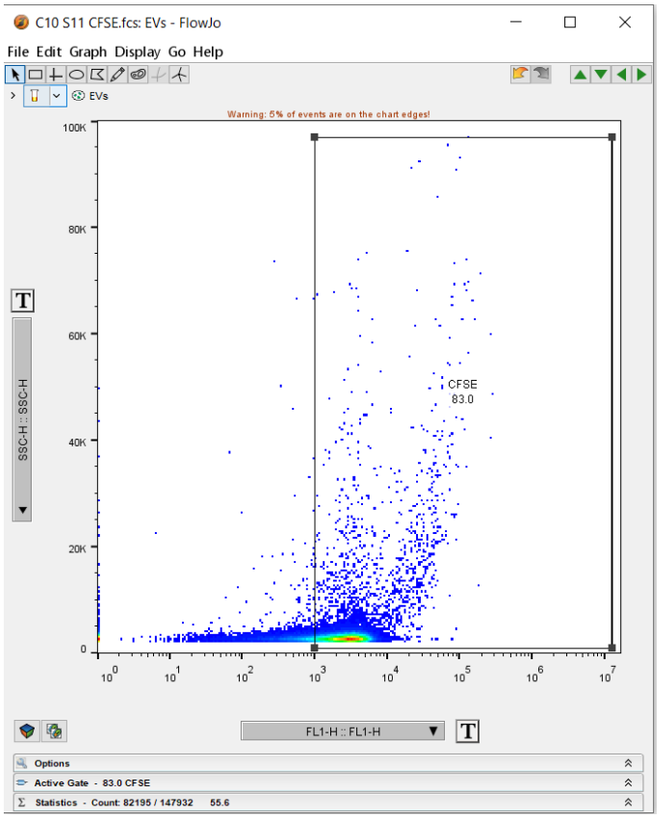

(D)

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C10 S11 CFSE.fcs		147932
0.1um	54.9	81188
0.3um	0.40	589
0.5um	0.13	192
0.9um	0.041	61
EVs	55.6	82195
CFSE	83.0	68227

Figure 7

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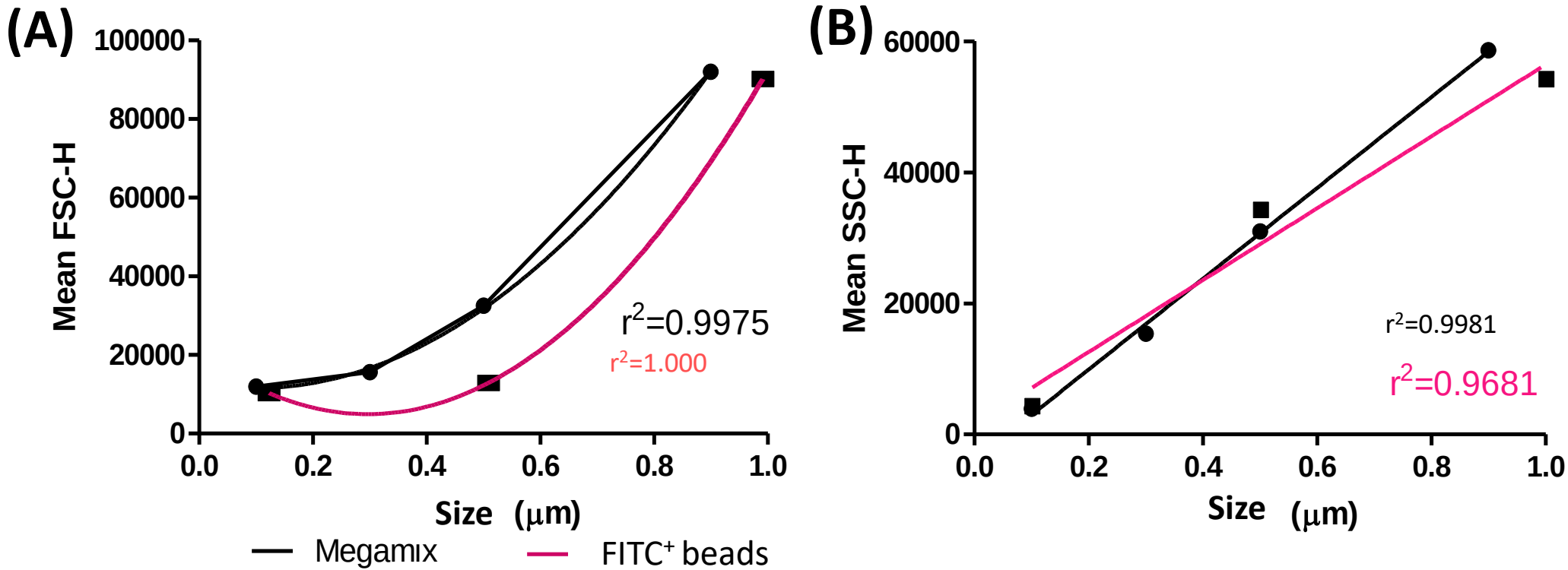
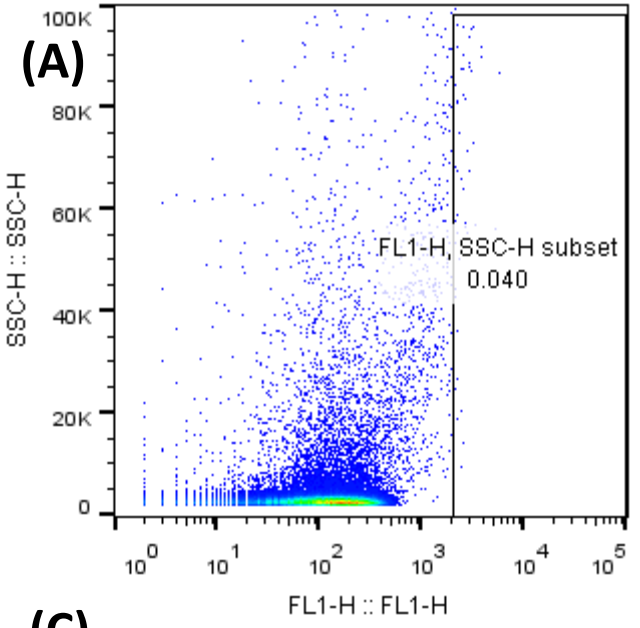
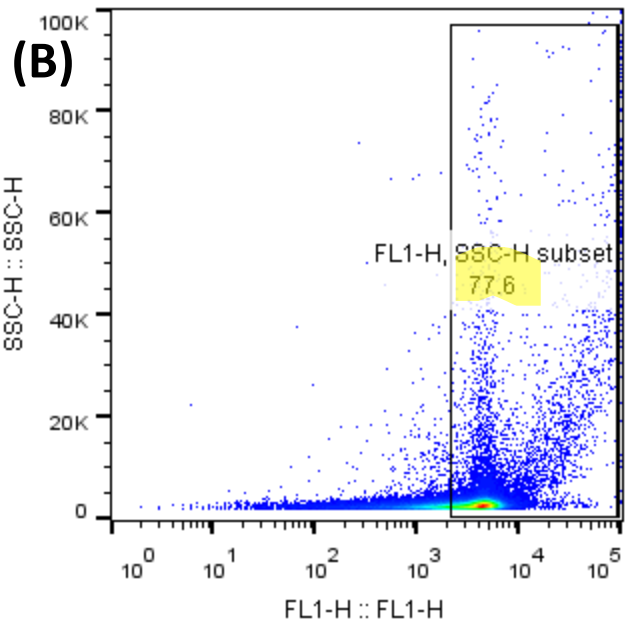


Figure 8





(C)

Name	Statistic	#Cells
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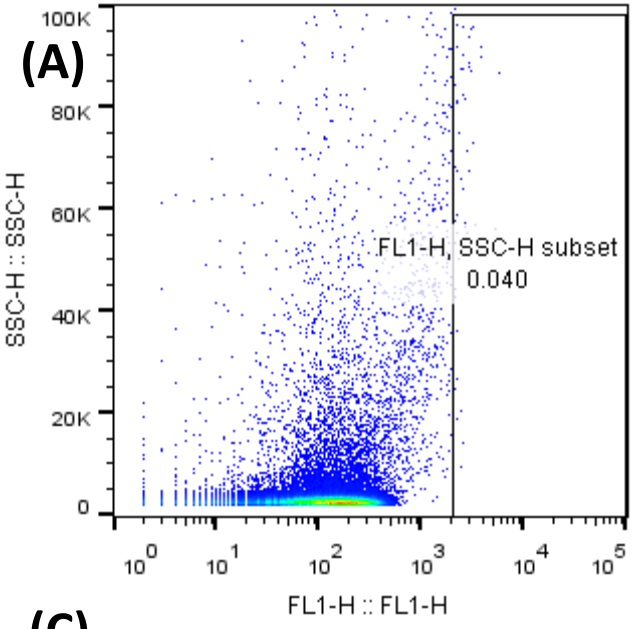
A02 PERLAS 10um.fcs		477781
A03 perlas 0.1um.fcs		202191
C09 S11 CAF.fcs		148902
C10 S11 CFSE.fcs		147932
0.1um	54.9	81188
0.3um	0.40	589
0.5um	0.13	192
0.9um	0.041	61
EVs	55.6	82195
CFSE	83.0	68227
FL1-H, SSC-H subset	77.6	114755

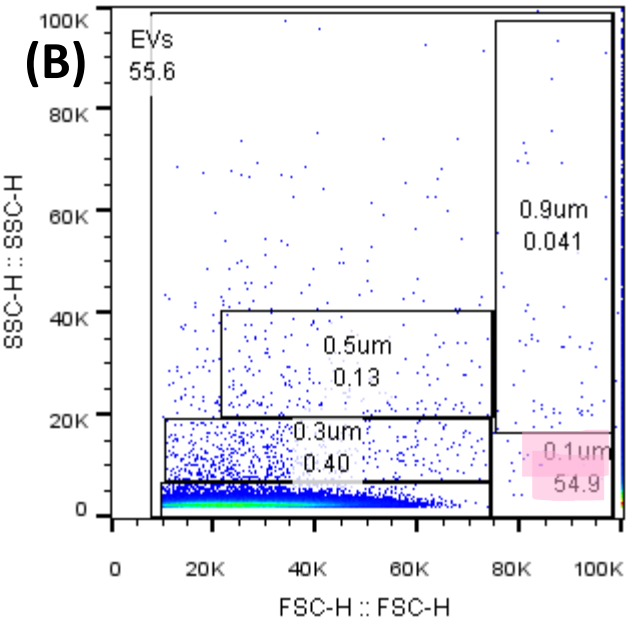
(D)

$$\#\text{uEVs}/\mu\text{L} = \frac{\#uEVs\ FC \times Dilution\ Factor}{100}$$

$$\#\text{uEVs}/\mu\text{L} = \frac{\#114755 \times 20}{100} \Rightarrow \#\text{uEVs}/\mu\text{L} = 22{,}951$$

Figure 9





(C)

Name	Statistic	#Cells
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A02 PERLAS 10um.fcs		477781
A03 perlas 0.1um.fcs		202191
C09 S11 CAF.fcs		148902
C10 S11 CFSE.fcs		147932
0.1um	54.9	81188
0.3um	0.40	589
0.5um	0.13	192
0.9um	0.041	61
EVs	55.6	82195
CFSE	83.0	68227
FL1-H, SSC-H subset	77.6	114755

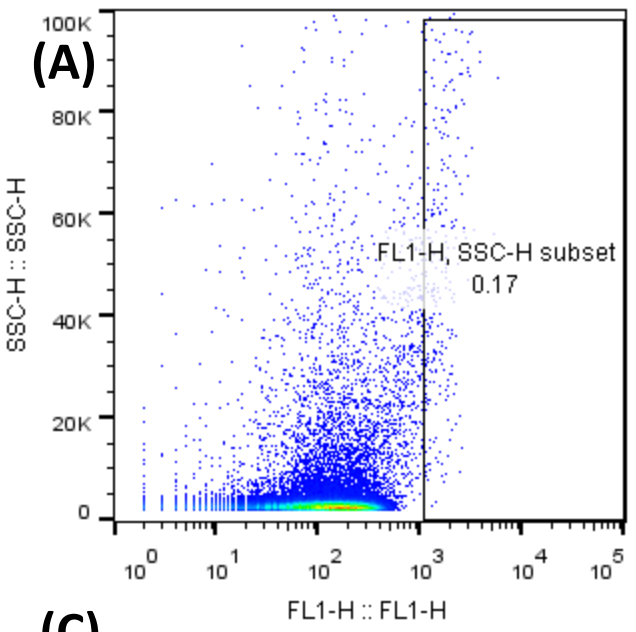
(D)

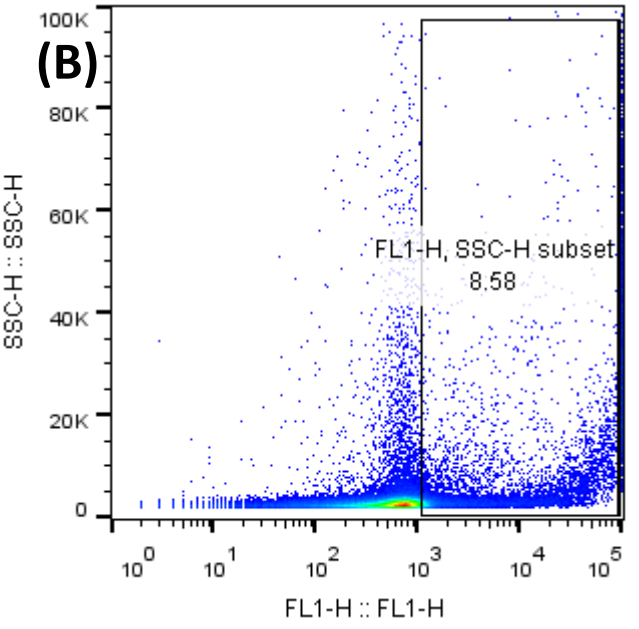
$$\#\text{uEVs/µL FLX} = \frac{(\%FLX\ subset) \times (\#uEVs\ \mu L)}{100}$$

$$\#\text{uEVs/µL FLX} = \frac{(54.9) \times (22951)}{100}$$

#uEVs/µL de 0.1µm =12,600

Figure 10





(C)

Name	Statistic	#Cells
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A02 PERLAS 10um.fcs		477781
A03 perlas 0.1um.fcs		202191
C09 S11 CAF.fcs		148902
C10 S11 CFSE.fcs		147932
C11 S11 CD37, 53, A10.fcs		147181
C12 S11 CD9 T33PE.fcs		148058
0.1um	54.7	80988
0.3um	0.59	868
0.5um	0.18	264
0.9um	0.088	130
EVs	55.8	82579
FL1-H, SSC-H subset	8.58	12699

(D)

$$\#\text{uEVs/µL FLX} = \frac{(\%FLX\ subset) \times (\#uEVs\ \mu L)}{100}$$

$$\#\text{uEVs/µL FL1} = \frac{(8.58) \times (22951)}{100} \Rightarrow \#\text{uEVs/µL CD9}^{+} = 1969$$

Figure 11

Click here to access/download;Figure;Figure 11.pdf

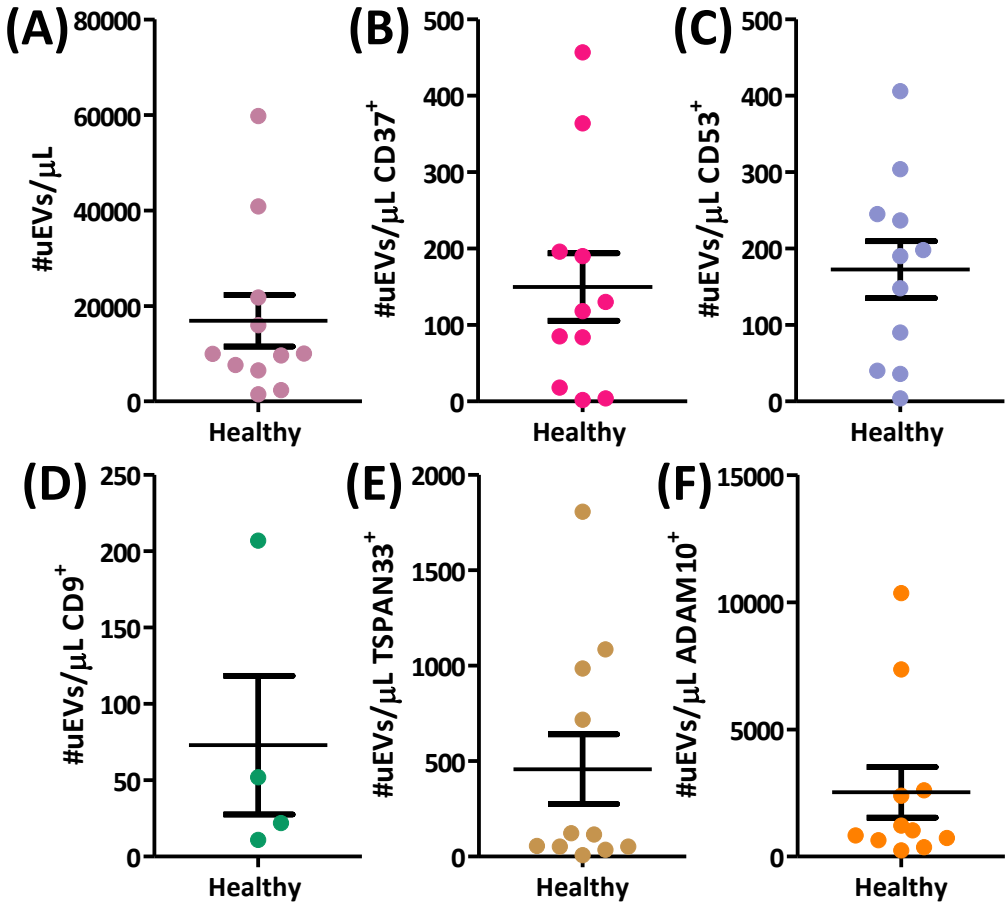


Table 1 Click here to access/download;Table;Table 1.xlsx

Tube 1.	Megamix FSC beads	
Tube 2.	PBS	
Tube 3.	PBS with CFSE	
Tube 4.	PBS with all antibodies of problem 1	
Tube 5.	PBS with all antibodies of problem 2	
Tube 6.	Autofluorescence control	uEVs without any reagent, only in PBS.
Tube 7.	#uEVs	uEVs with CFSE
Tube 8.	Problem 1	uEVs with CD37 FITC, CD53 PE, ADAM10 APC
Tube 9.	Problem 2	uEVs with CD9 FITC, TSPAN33 APC

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
APC anti human CD156c (ADAM10) antibody	BioLegend	352706	Add 5 µL to the 20 µL of uEVs in PBS
APC anti human TSPAN33 (BAAM) antibody	BioLegend	395406	Add 5 µL to the 20 µL of uEVs in PBS
Avanti centrifuge with JA-25.5O fixed angle rotor	Beckamn Coulter	J-26S XPI	
BD Accuri C6 Flow Cytometer	BD Biosciences		
β-mercaptoethanol	SIGMA-Aldrich	M3148	
Benchtop centrifuge with A-4-44 rotor	Eppendorf	5804	
BLUEstain 2 protein ladder	GOLDBIO	P008	
CD9 (C-4) mouse monoclonal antibody	Santa Cruz Biotechnology	sc-13118	
CD63 (MX-49.129.5) mouse monoclonal antibody	Santa Cruz Biotechnology	sc-5275	
Cell Trace CFSE cell proliferation kit for flow cytometry	Thermo Scientific	C34554	
Chemidoc XRS+ system	BIORAD	5837	
FITC anti human CD9 antibody	BioLegend	312104	Add 5 µL to the 20 µL of uEVs in PBS
FITC anti human CD37 antibody	BioLegend	356304	Add 5 µL to the 20 µL of uEVs in PBS
Fluorescent yellow particles	Spherotech	FP-0252-2	
Fluorescent yellow particles	Spherotech	FP-0552-2	
Fluorescent yellow particles	Spherotech	FP-1552-2	
FlowJo Software	Becton, Dickinson and Company		
Goat anti-mouse immunoglobulins/HRP	Dako	P0447	

Halt protease inhibitor cocktail	Thermo Scientific	78429	
Immun-Blot PVDF membrane 0.22µm	BIORAD	1620177	
Megamix-Plus FSC beads	COSMO BIO CO.LTD	7802	
NuPAGE LDS sample buffer 4X	Thermo Scientific	NP0007	
Optima ultracentrifuge with rotor 90Ti fixed angle 355530	Beckamn Coulter	XPN100	
Page Blue protein staining solution	Thermo Scientific	24620	
PE anti human CD53 antibody	BioLegend	325406	Add 5 µL to the 20 µL of uEVs in PBS
Pierce BCA Protein assay kit	Thermo Scientific	23227	
Pierce RIPA buffer	Thermo Scientific	89900	
Polycarbonate thick wall centrifuge tubes	Beckamn Coulter	355630	
Spherotech 8-Peak validation beads (FL1-FL3)	BD Accuri	653144	
Spherotech 6-Peak validation beads (FL4)	BD Accuri	653145	
Sucrose	SIGMA-Aldrich	59378	
Triethanolamine	SIGMA-Aldrich	90279	

Abstract comments:

Line 35. Please include e.g. as done for size.
There was a misunderstanding about that line, despite the efforts to have specific molecules in order to classify if an extracellular vesicle is a microvesicle, or exosome, there is no report about this, yet. So, this line was rewrite, as following "

Line 41 and 42. So you are differentiating on the basis of size or protein expression? Please bring out clarity. or different protein expression is associated with different size? In the protocol the main is to differentiate on the basis of size; the protein expression is an example of how we can characterize more these extracellular vesicles.

Introduction comments:

Line 59 and 60: Please reword for clarity. Done
"Moreover, the enrichment of several molecules in the uEVs and number of these can reflect the development and/or correlate with the status of various diseases and disorders"

Line 74 and 76: Please reword for clarity. Done
"Nowadays the use of extracellular vesicles as biomarkers for many diseases has become relevant, so that the searching for different sources has been investigated, one of the most promising sources is the urine, especially for being non-invading, and easier to obtain."
Protocol comments:

Please write steps in the order. Please be crisp with respect to the action being performed. Done

Line 99: Is this done before or after centrifugation in step 1.1? Please place the step accordingly.
"Transfer the supernatant obtained in step 1.1, to a new 15 mL conical centrifuge tube, then centrifuge at 10,000 *x g* for 45 min at 4 °C."

Line 100: What temp ? Store at -20°C, until use.

Line 106: This is introduced for the first time, Please include what is the significance of this protein and why it is necessary to remove this in short under this step as a NOTE. Please include relevant citation as well.
NOTE. The THP is a protein present in urine, enriched when an individual has a renal disease, it has been reported that the THP diminished the yield of uEVs, because of their binding capacity with uEVs. To remove it there is the need to use a reducing agent [3,6].

3 Pisitkun, T., Johnstone, R. & Knepper, M. A. Discovery of urinary biomarkers. *Molecular & Cellular Proteomics.* **5** (10), 1760-1771, doi:10.1074/mcp.R600004-MCP200, (2006).

6 Merchant, M. L., Rood, I. M., Deegens, J. K. J. & Klein, J. B. Isolation and characterization of urinary extracellular vesicles: implications for biomarker discovery. *International Urology and Nephrology.* **13** (12), 731-749, doi:10.1038/nrneph.2017.148, (2017).

Line 121: Please reword to bring out clarity. Supernatant from which step? "NOTE. For western blot analysis, store 200 µL of the supernatant obtained in step 1.3. labeling as "supernatant

without EVs", that will serve as negative control when searching for extracellular vesicle markers."

Line 143: Does this mean analysis of uEVs is important before staining? What kind od analysis is performed in your case? To guarantee that we have uEVs before staining, is important to do at least one methodology, like here we do Western blot analysis, or can do electronic microscopy, or nano tracking analysis, any of these.

Line 149: So you do not check for contamination in this case? Total protein content cannot be directly used for uEVs. Please expand this part to bring out clarity. All the research groups that work with extracellular vesicles quantify the amount of protein in the extracellular vesicle fraction, and use the same protein assay kit used here.

Line 151: Which datasheet? The datasheet for the protein assay kit mentioned in the table of materials.

Line 165: What are problem tubes? Please reword for clarity.
"The tubes depicted in table 1, tubes 4 and 5 are PBS with cocktail of all the antibodies to be used, in this protocol two problem tubes (tube 8 and 9) are given as an example, therefore this set of controls must have the combination to be used in each tube".

Line 170: Which antibodies are used in this case, please include it here with citation? Also please include the rationale for using these antibodies somewhere either in introduction or result section. Please ensure that the dilution is included in the table of materials.
"Add the antibodies establish in the table 1, previously titrated. Incubate overnight at 4°C.
NOTE. Before staining it is recommended to centrifuge the antibodies at 4 °C at full speed for at least 5 min, to prevent the aggregates. The antibodies used here are an example of proteins present in the uEVs and belong to other manuscript in preparation."

Line 177: Please include the reason for adding CFSE here with citation.
"NOTE: CFSE is a dye used to stain all the EVs present in a sample and to discriminate between the background noise when a flow cytometer analysis is performed. For more information go to discussion section."
"Another critical step is the uEVs staining, with a reagent that should stain almost all the extracellular vesicles present in the sample, as reported before[37-39]. CFSE was then selected for this purpose. It is important to mention that the evaluation of other dyes was done, but the staining ratio was extremely deficient (Data not shown). "

37 Puzar Dominkus, P. *et al.* PKH26 labeling of extracellular vesicles: Characterization and cellular internalization of contaminating PKH26 nanoparticles. *Biochimica et Biophysica Acta (BBA) - Biomembranes.* **1860** (6), 1350-1361, doi:10.1016/j.bbamem.2018.03.013, (2018).

39 de Rond, L. *et al.* Comparison of Generic Fluorescent Markers for Detection of Extracellular Vesicles by Flow Cytometry. *Clinical Chemistry.* **64** (4), 680-689, doi:10.1373/clinchem.2017.278978, (2018).

In 3. Acquisition of uEVs using a conventional cytometer: For all the steps in this protocol please include button clicks wherever applicable. e.g., Click on the "Analyze" button to analyze the results.

Line 196: What is being seen here. Please use scientific terms to describe what is to be observed. "Load tube number 1 (Megamix tube), depicted in table 1. Capture the beads. Create two dot plots as panel A and B of the **Figure 3**".

Line 201: Please include the details. This is introduced here for the first time.
"The Megamix fluorescent beads (beads used to delimit the sizes of 0.1, 0.3, 0.5 and 0.9 µm and create the template for sizes), can be analyzed using the FL1 (FITC) detector".

Line 205: Figure 3 A or B? Both, the panel A of figure 3 is to localize the beads, using a dot plot of the SSC-H VS FL1 (FITC fluorescence of the bead), then once the gate was selected in this region, create a new dot plot (panel B) SSC-H VS FSC-H to appreciate the different sizes of the beads. The objective is that the person who use this protocol can replicate these images shown here.

Line 208: What is the background noise in figure 3? The background noise is depicted in panel A of figure 3, all the dots between 0 to 1000 of FL1 (*y* axis), note that there are outside of the created gate.

Line 213: How is this done? "Load the tubes number 2 and 5, to set the cut-off values (negative). To do this select the rectangle gate icon (localize under the dot plot created), or line gate icon (localize under the histogram created); and put it on where there is no signal. In order to obtain dot plots and histograms like in the **Supplementary figure 1**".

Line 219: Which regions? How is this done? "Load the tube number 6 (autofluorescence tube), to set the negative regions for the sample. To do this select the rectangle gate icon (localize under the dot plot created), or line gate icon (localize under the histogram created); and put it on where there is no signal".

Line 221: What are next tubes in your case? As depicted in table 1, there will be the next tubes (tubes 7 to 9).

Line 223: Save the results? For the cytometer software use here, the command "save experiment", refers to save the data generated. In other words, all the tubes that we charge to the flow cytometer, and the data generate for each tube, are needed to save it, and for do it is necessary to click in the "save experiment" button.

Step 4. Analysis of the data with a flow cytometer software: For this step please include all the button clicks in the software.

Line 235: Is this the file name here? Please bring out clarity. As depicted in figure 4, the name of the tube, is Megamix tube, because here we charge the tube with the Megamix beads, depicted in

table 1. Is important to first stablish all the parameters to see the data generated, in other words do zoom in the data, using this tube named Megamix tube.

Line 238: What different sizes are being looked at? As depicted in figure 3 panel B so as figure 4 panel g, h and i, there are shown the different sizes (0.1, 0.3, 0.5 and 0.9 µm) that we can analyze with this protocol, so we can recreate this dot plot for all the analyzed tubes.

Line 247: What are the next steps. What is exactly being done. Please be clear and concise.
Once generated all the gates to analyze the extracellular vesicles, the next steps are analyze each tube with the samples plus antibodies, to obtain the data of percentage of extracellular vesicles, by size and by the protein expression, plus the mean fluorescence intensity, as depicted in figure 5, in all the panels.

Step 5 Analysis to have number of uEVs per sample: How is this done ? Following this sentence, there is the explanation of how to do it.

Line 266 Again please be specific with respect to the what are the next steps. The next steps are in order to obtain the number of uEVs per sample, as the step 5 is titled.

Representative results comments:

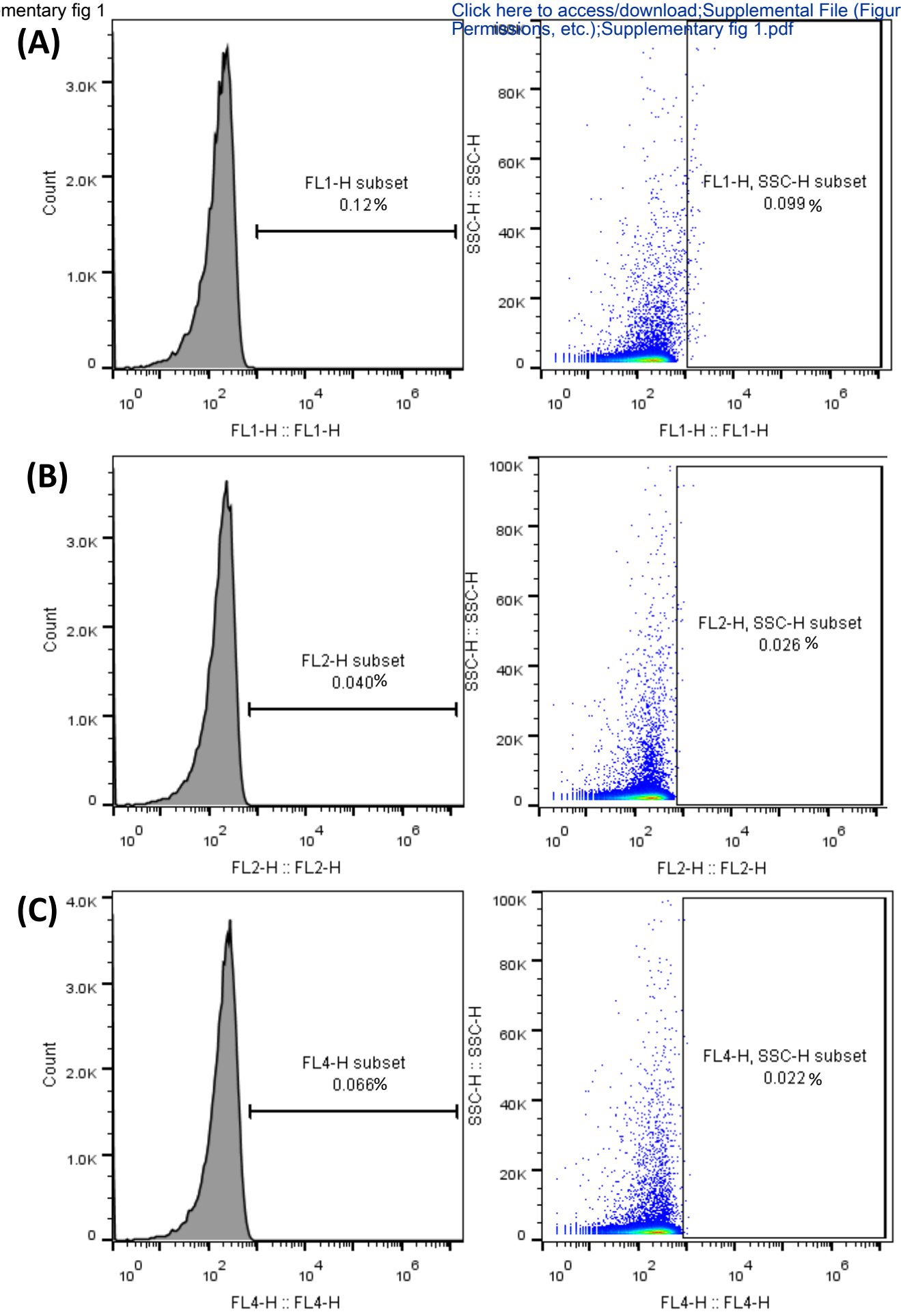
Please write this section as if you are describing the representative result for your own experiment performed using the technique presented above.
e.g., We performed uEV isolation from 5 heathy control. CD63 expression was observed in all the cases. This is a marker for uEVs (included citation). Since it is difficult to work with urine samples (include the reasonings) total protein concentration was used as loading control. As expected, increased level of CD63 protein was observed in the uEV fractions. …etc.

Line 299: ???. As depicted in the sentence, if there is no isolation of uEVs, there will be no signal in the protein quantification, that is the reason we write “be careful to obtain uEVs”.

Line 302: Is this blotted? What is being seen here? How do you ensure equal loading?
The supplemental figure 3 is a poly-acrylamide gel, stained with blue Coomasie, of the proteins present in all the collected fractions, depicted in the figure legend “Line 1: protein marker. Line 2: whole urine. Line 3: urine cells. Line 4: Supernatant without uEVs. Line 5 – 7 uEVS isolated with PEG 8000 in PBS. Line 5 without any reducing agent. Line 6 with DTT. Line 7 with β-mercaptoethanol. Line 8 – 10 uEVS isolated with ultracentrifugation. Line 8 without any reducing agent. Line 9 with DTT. Line 10 with β-mercaptoethanol”. We quantify the protein present in all the fractions with the assay protein mention in the table of materials, and this gel is only to show why we use the ultracentrifugation method with β-mercaptoethanol, instead of PEG with DTT to isolate the uEVs.
Line 309 – 311: Need clarity. What is the loading control in this case? Without control the data is of no value. For the study of extracellular vesicles the use of western blot is only to show the presence of markers of extracellular vesicles, like CD9, CD63, tsg101, etc., there are no loading control, because there are no cells, there are extracellular vesicles, and we can not control how many protein is going to be packed in the extracellular vesicle. Also, the isolation of the extracellular vesicles is for healthy urine, so to guarantee the presence of a protein in urine, is difficult.

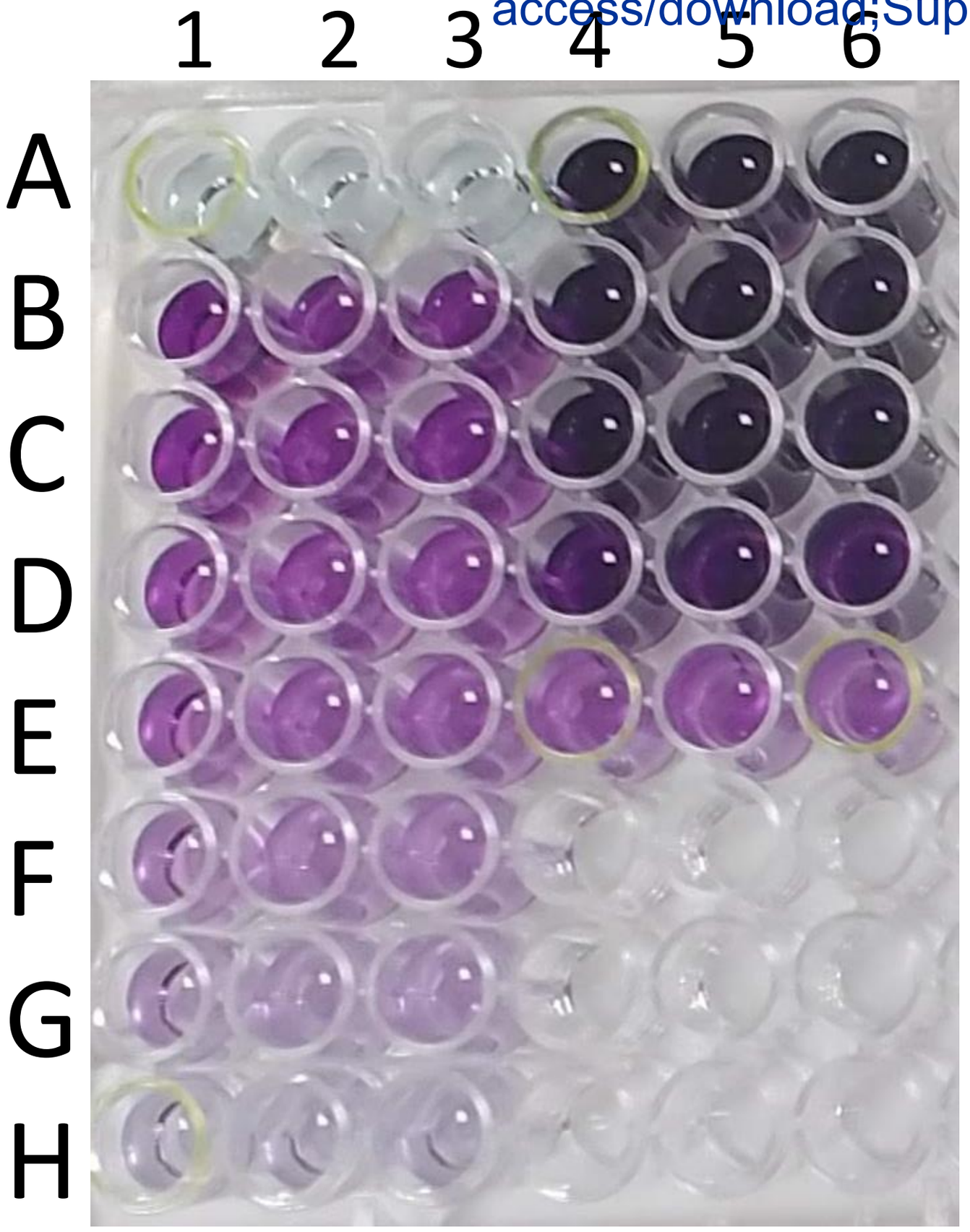
Line 313: How is the quantification performed? As depicted in table of materials we use an assay protein kit, and we follow the instructions of the datasheet.

Supplementary fig 1

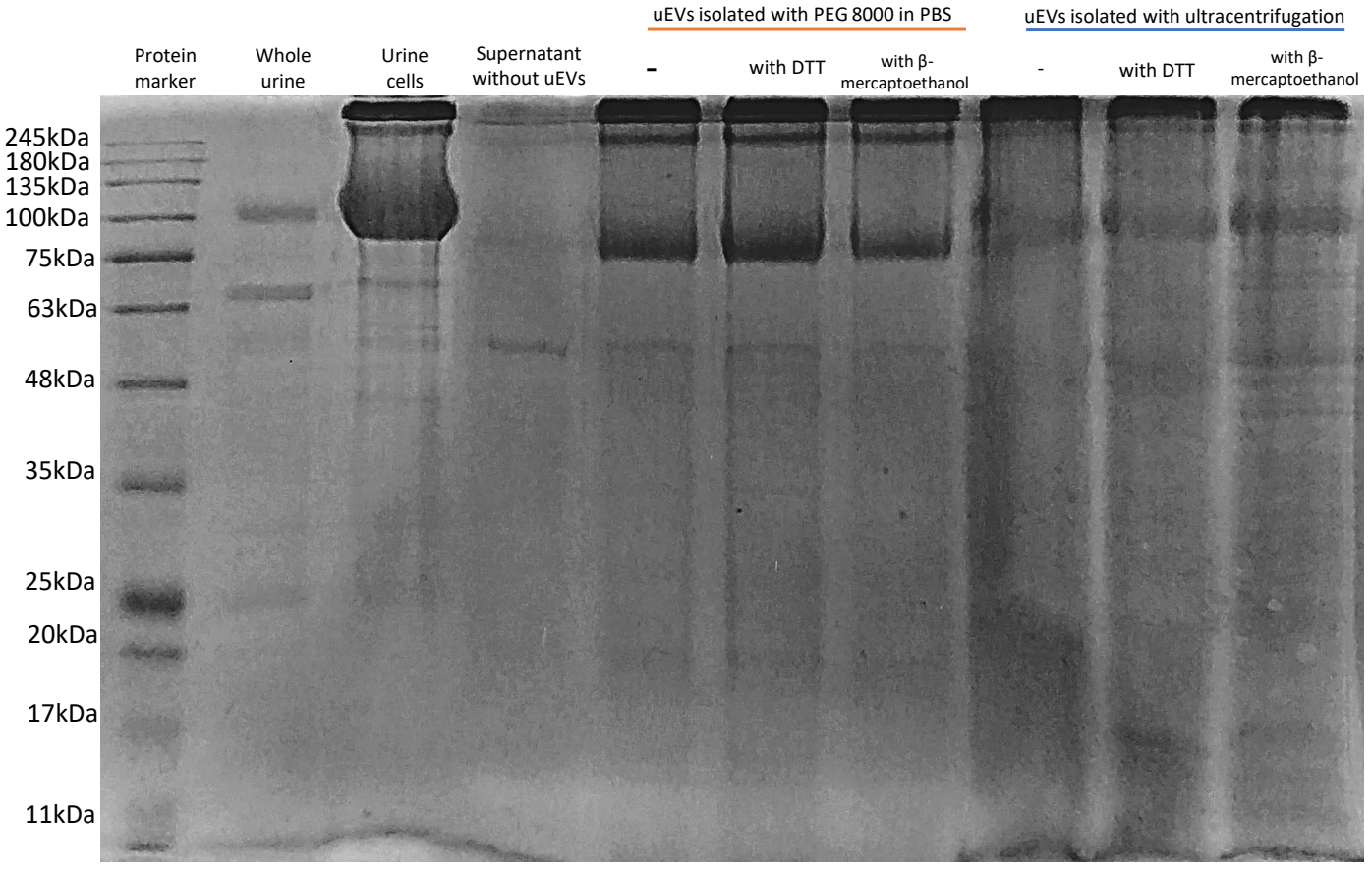


Supplementary fig 2

Click here to access/download;Supplem

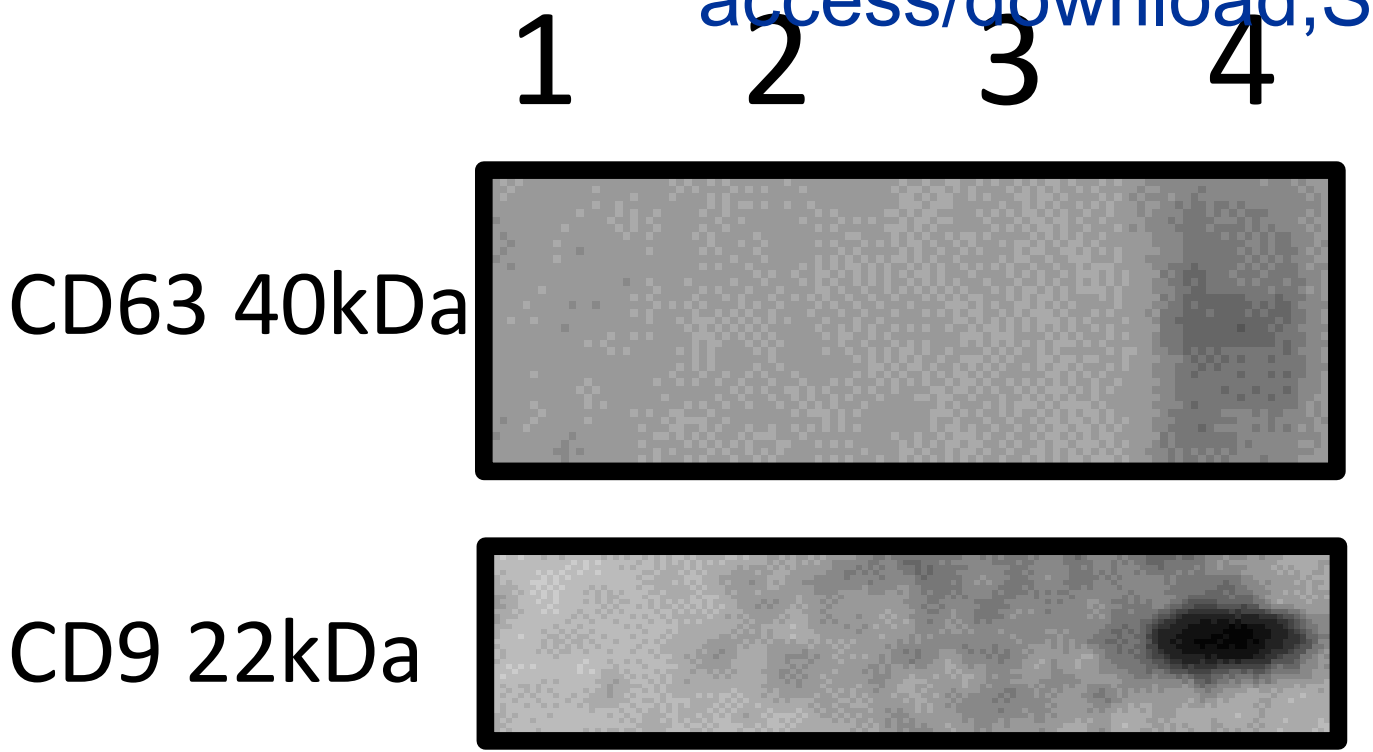


Supplementary fig 3



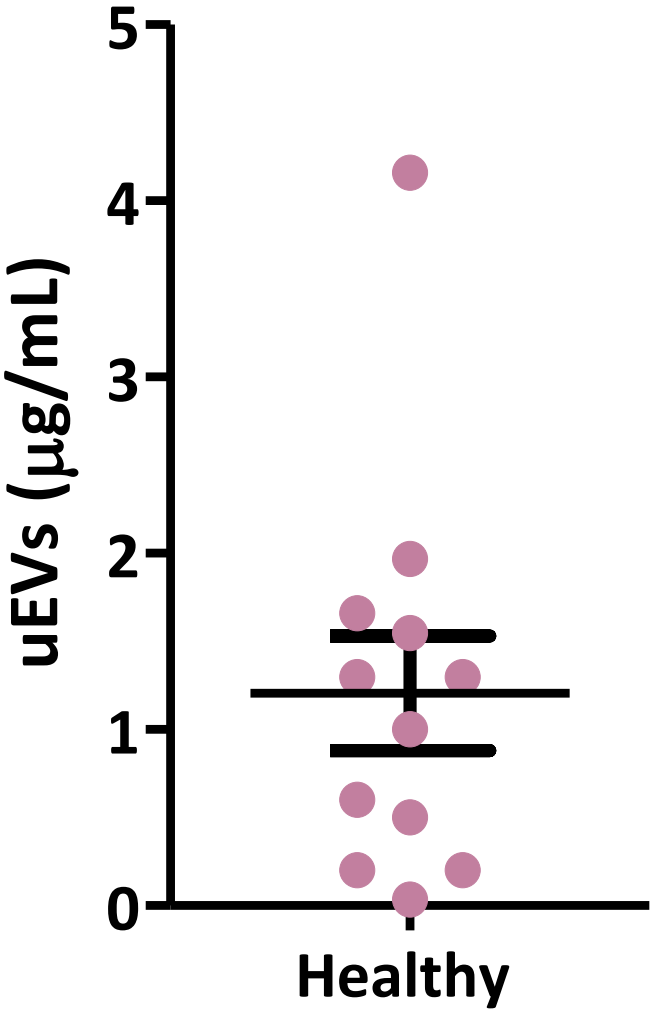
Supplementary fig 4

Click here to access/download;Suppl



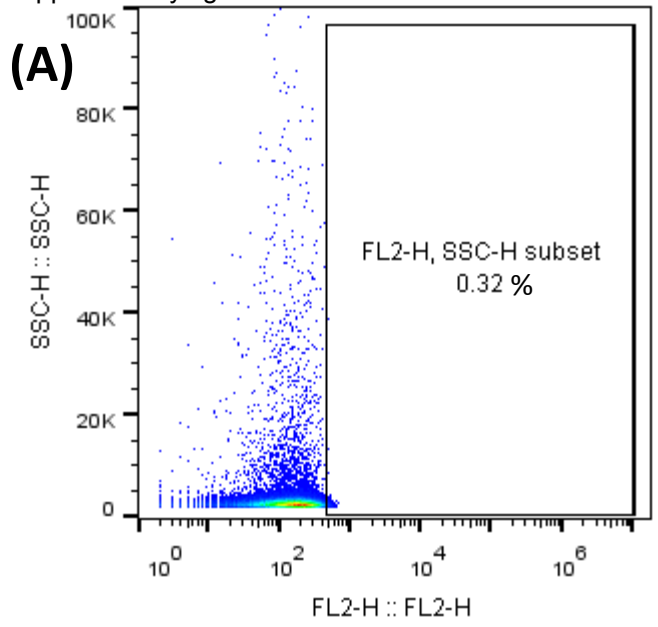
Supplementary fig 5

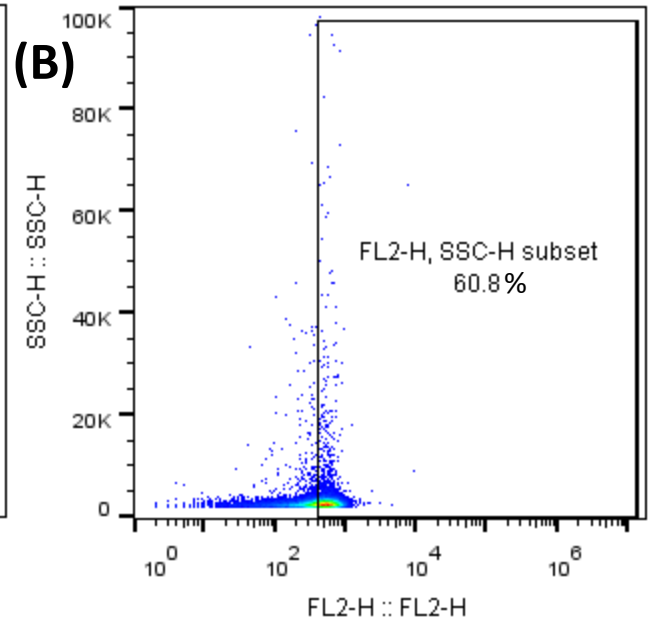
Click here to

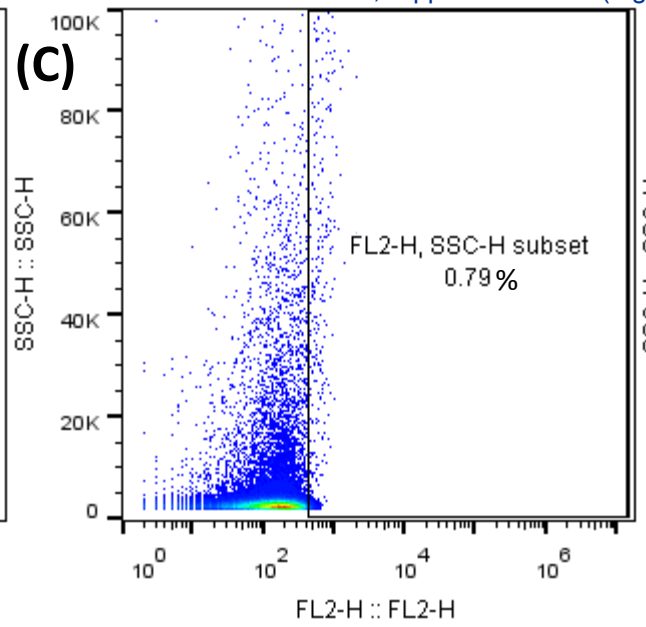


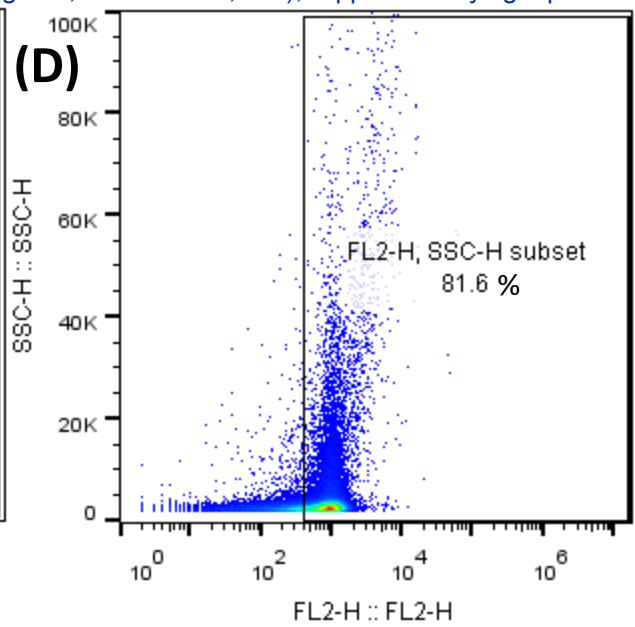
Supplementary fig 6

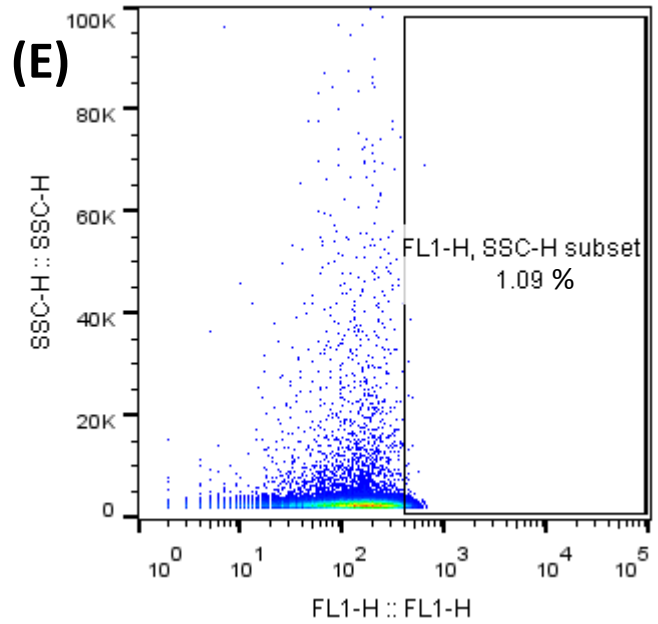
Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary fig 6.pdf

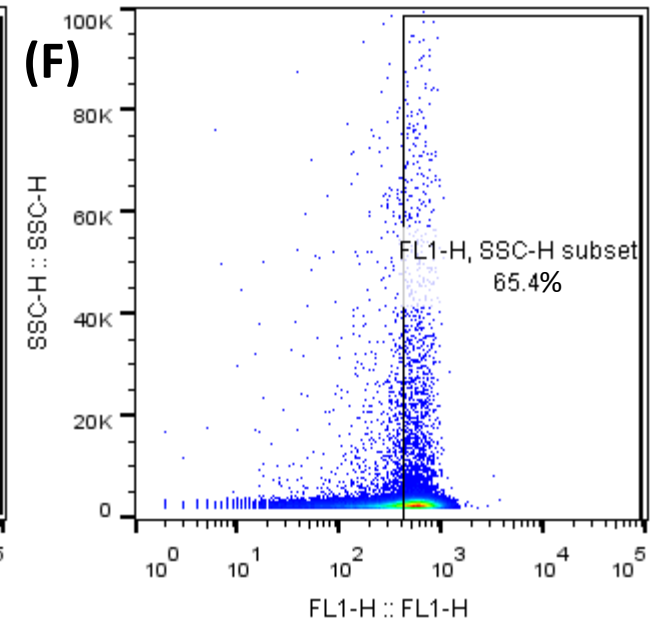


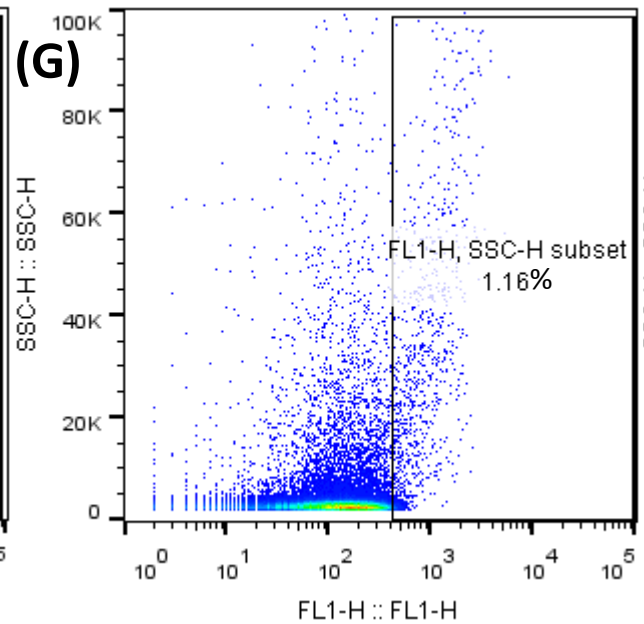


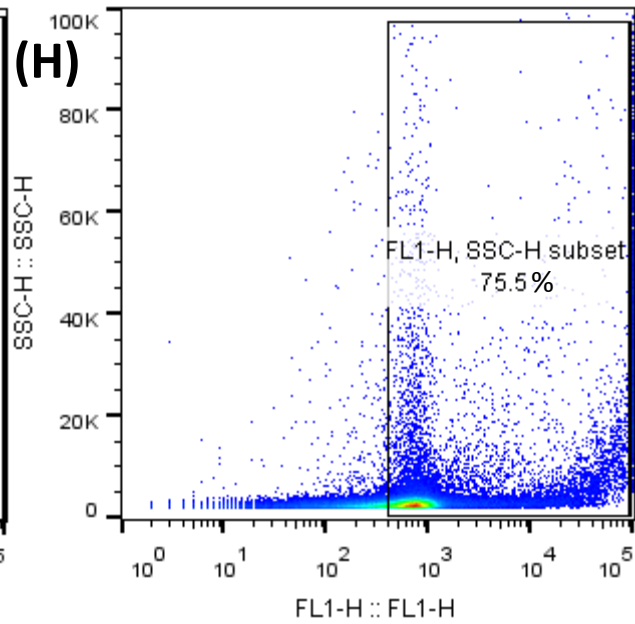












Supplementary fig 7

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary fig 7.pdf