Supporting Information

Direct Isolation of Circulating Extracellular Vesicles from Blood for Vascular Risk Profiling in Type 2 Diabetes Mellitus

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Materials and Methods

Microfluidic Device Fabrication

The 2-inlet, 4-outlet ExoDFF device, shown in Figure 1, was fabricated with poly-dimethylsiloxane (PDMS; Dow Corning, Midland, MI, USA) using standard soft lithography methods. PDMS prepolymer was mixed well with the curing agent in a ratio of 10:1 (w/w) and poured over a silicon wafer patterned with ExoDFF design. The mixture was de-gassed before curing in an oven at 80 °C for 2 h. The cured PDMS slab was carefully peeled from the wafer, and inlets and outlet holes were punched out with a 1.5 mm biopsy puncher. The PDMS slab was cleaned thoroughly with isopropanol and bonded to a 1 mm thick glass slide (70 mm × 50 mm) using an air plasma machine (PDC-002, Harrick Plasma Inc, Ithaca, NY, USA).

Computational Fluid Dynamics (CFD) Simulation

Computation fluid dynamics simulation was conducted using ANSYS FLUENT (Canonsburg, PA, USA). Steady state pressure-based laminar flow was analyzed using SIMPLE scheme. Fluid is modelled as water to mimic PBS. A complete hexahedral discretization mesh was built using ANSYS ICEM CFD (Canonsburg, PA, USA) with maximum element dimension of 2 μm along height and width directions, 25 μm along length direction. Determinant, maximum orthogonality and maximum warpage of element was set to minimum value of 0.3. Only upper half of the channel was modelled due to symmetry in height direction. Boundary conditions were set to velocity inlet, zero gauge pressure outlet, and no slip at channel walls. 1000 fluid streamlines were initiated from sample inlet using discrete phase model (DPM) without interaction with the continuous phase. Coordinates of streamlines were then exported and processed with MATLAB (MathWorks, Natick, MA, USA). Total flow rate was set to 480 μL/min with different sample to sheath flow rate ratio ranging from 1:3 to 1:40.

Western Blot

Equal volume (15 μL) of ExoDFF outlets, UC microvesicles (UC MV) and UC exosomes (UC Exo) and a reduced volume (0.5 μL) of plasma were lysed in Radioimmunoprecipitation assay (RIPA) buffer (Pierce, Thermo Scientific) containing protease inhibitor cocktail (Thermo Scientific) with the samples chilled on ice. The samples were mixed with loading buffer containing β-mercaptoethanol (Merck, Darmstadt, Germany), heated to 70 °C for 10 min, loaded on 15% SDS-PAGE gels and electrophoresed to detect exosome markers CD9, Flotillin-1 and TSG101. Proteins were transferred to 0.45 μm nitrocellulose membranes (Bio-rad, Feldkirchen, Germany) and stained with REVERT total protein stain (Li-COR Biosciences, Lincoln, NE, USA) for normalisation. Membranes were blocked for 1 h with Odyssey blocking buffer TBS (Li-COR Biosciences, Lincoln, NE, USA) at room temperature, and incubated with 1:1000 of the following antibodies - Rabbit monoclonal [EPR2949] anti-CD9 (Abcam ab92726); mouse monoclonal anti-Flotillin-1 (C2) (Santa Cruz Biotechnology, sc-74566, Dallas, TX, USA) and mouse monoclonal [4A10] anti-TSG101 (Abcam, ab83, Cambridge, MA, USA) overnight at 4 °C. The membranes were next washed using 1X Tris Buffered Saline (TBS) 0.1% Tween 20 (TBS-T), and incubated with 1:15,000 each of IRDye 800CW anti-mouse IgG secondary antibody (Li-COR Biosciences, Lincoln, NE, USA) and IRDye 680LT anti-rabbit IgG secondary antibody (Li-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature. Membranes were washed again with 1X TBS-T and

scanned with an Odyssey CLx imaging system (Li-COR Biosciences) using 700- and 800-nm channels and visualized using ImageStudio software version 5.2 (LI-COR Biosciences).

RNA Isolation and Measurement

Total RNA was extracted from extracellular vesicles using miRNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. The purity and concentration of the isolated RNA were measured using NanoDrop One Microvolume UV-Vis Spectrophotometer (ND-ONE-W, Thermo Scientific) and RNA size distribution was analysed using 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA) with the RNA 6000 Pico Kit according to the manufacturer's protocol.

Cell Culture

Human umbilical vein endothelial cells (HUVEC; Lonza) were cultured in endothelial cell growth medium (EGM-2; Lonza) supplemented with 1% penicillin-streptomycin (P/S; Gibco, Grand Island, NY, USA) in a T75 flask. The cells were maintained at 37 °C in a humidified 5% CO_2 incubator and medium was changed thrice weekly. For EVs uptake assay, confluent HUVEC was dissociated using 0.25% trypsin with 1 mM EDTA (Gibco) and seeded in a 24-well plate at 8×10^4 cells per well with 400 μL EGM-2 and grown to confluency prior use.

Platelet Count and Platelet-derived Microvesicles

To examine the platelet shearing effects of centrifugation, whole blood was first centrifuged at 1,000 g for 5 min to obtain platelet rich plasma (PRP). The PRP was then either subjected to high speed centrifugation at 20,000 x g for 45 min or perfused into ExoDFF device for sorting. Pre-processed PRP sample, pellet from high speed centrifugation and ExoDFF fractions (O2 for platelet-derived microvesicles, O3 – O4 for platelets) were then stained with FITC-conjugated anti-human CD41a antibody to detect and compare intact residual platelets and CD41a+ microvesicles using flow cytometry.

Transmission Electron Microscopy (TEM)

EVs isolated from ExoDFF were subjected to SEC to remove plasma proteins before TEM analysis. For TEM analysis, a carbon-coated grid (CF300-CU, Electron Microscopy Sciences, Hatfield, PA, USA) was glow-discharged for 1 min. A 4 μL of sample solution was then placed on the grid to adsorb for 1 min. After blotting, 4 μL of 2% uranyl acetate was added to the grid to negatively stain the sample for 1 min. The grid was then blotted and air-dried. Grids were imaged using a T12 Icorr transmission electron microscope operating at 120 kV. Images were captured by an Eagle 4k HS camera.

EV Uptake Assay

To optimize EV concentration for cell uptake assay, UC isolated exosomes (UC Exo) and microvesicles (UC MV) were first stained with 2 μM PKH67 dye in Diluent C (Sigma Aldrich, St. Louis, MO, USA), washed, and incubated separately with HUVEC at varying concentration and duration. The stained EV uptake was examined using fluorescent microscopy and flow cytometry, and optimal uptake condition is determined to be 2.5×10^9 EVs per mL for 24 h. EVs isolated from clinical samples with UC and ExoDFF were then directly incubated with HUVEC as previously described, and the treated HUVEC were trypsinized and examined for inflammatory marker ICAM-1 expression using flow cytometry analysis.

Size Exclusion Chromatography (SEC)

To isolate EVs from plasma by SEC, PURE-EVs columns (HBM-PEV, HansaBioMed Life Sciences, Tallinn, Estonia) were used according to the manufacturer's instructions. The column was first washed three times with 10 mL PBS to eliminate preservative buffer residues and then 500 μL of platelet poor plasma (PPP) was applied onto the column. During the elution,

the column was loaded with PBS (the mobile phase of the SEC column). Fifteen fractions of 500 µL volume each were collected. The first six fractions (3 mL) were discarded as void volume, whereas the rest of the nine fractions were analysed by NTA. EV-rich fractions were pooled together and used to test for EV-induced vascular inflammation.

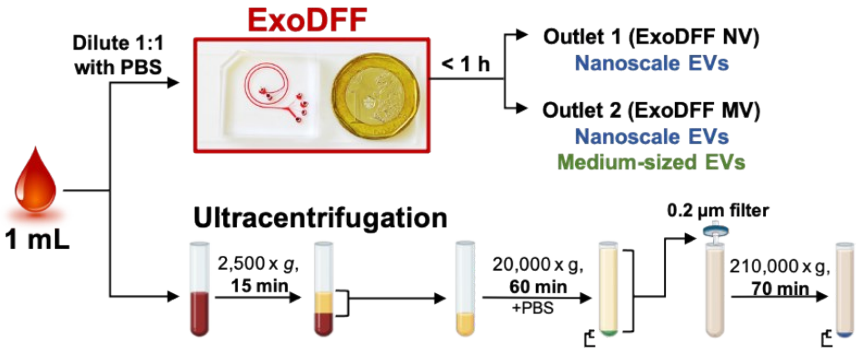


Figure S1. Workflow comparison between the single-step ExoDFF and conventional ultracentrifugation (UC) for isolation of circulating EVs from whole blood. Figure made with BioRender. Photograph shows the spiral device filled with red dye for visualization.

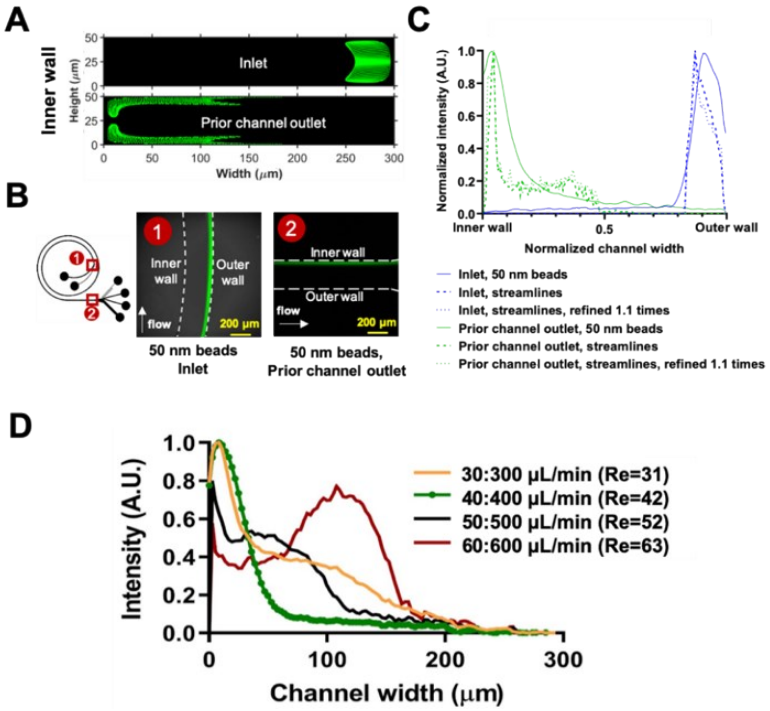


Figure S2. Experimental validation of CFD and flow rate optimization of ExoDFF. (**A**) Cross sectional views of fluid streamlines (mimicking nanoscale EVs) at different channel positions using CFD. **(B)** Top view fluorescent images of 50 nm beads at inlet and prior to channel outlet. **(C)** Comparison between average intensity linescans of 50 nm beads and streamlines at inlet and prior to channel outlet. Streamlines generated from two mesh sizes show similar intensity profile, indicating mesh independence of CFD solution. **(D)** Average intensity linescans of 50 nm beads at different Re indicating optimum flow rate at Re = 42.

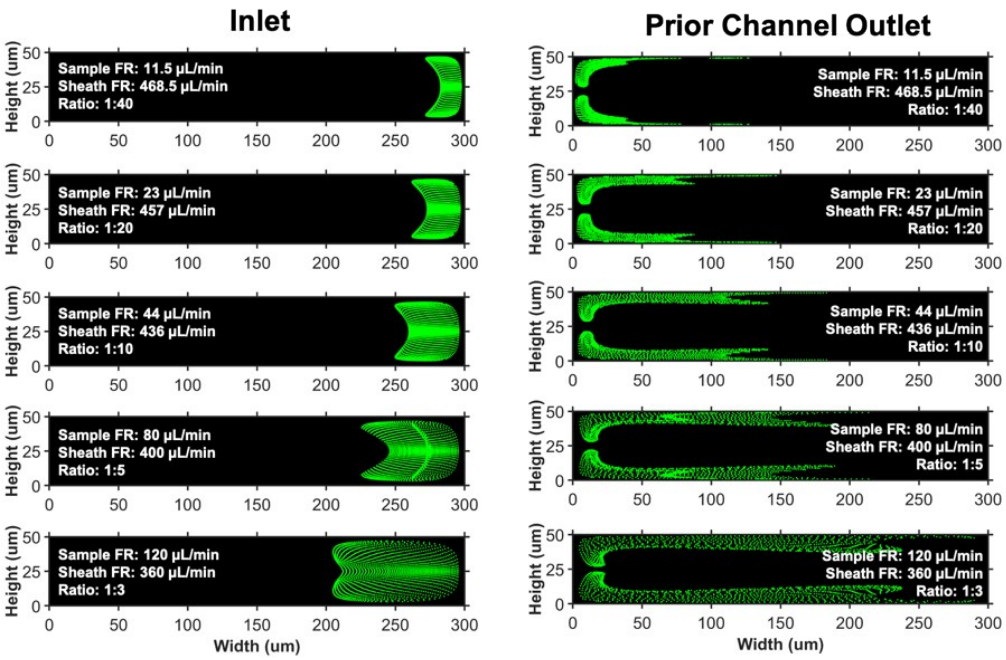


Figure S3. Cross sectional views of fluid streamlines (mimicking EVs) at device inlet and prior channel outlet at different sample to sheath flow rate (FR) ratio using CFD.

200 nm beads	Bead concentration (/mL)	Sample or Eluted volume (µL)	Final EV count	Separation efficiency
Inlet	5.10E+09	1000	5.1E+12	-
O1	4.60E+09	299	1.37554E+12	29%
O2	3.00E+09	757	2.27124E+12	47%
O3	7.37E+08	760	5.59813E+11	12%
O4	3.33E+07	17754	5.91803E+11	12%

Table S1. 200 nm beads concentration, sample elution volume and separation efficiency after ExoDFF isolation.

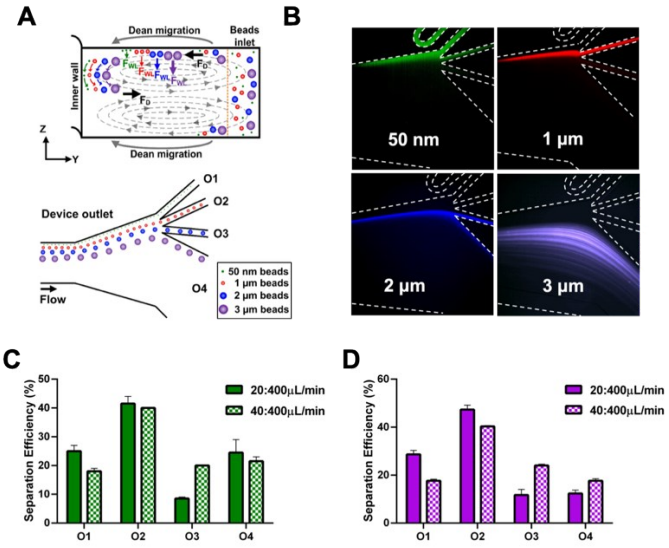


Figure S4. (**A**) Schematic illustration of ExoDFF sorting of different sized beads. (**B**) Corresponding fluorescent images illustrating flow streamlines of the different sized beads (50 nm to 2 µm) at optimized flow rate (Re 42). Beads separation efficiency of (**A**) 50 nm and (**B**) 200 nm beads at different sample:sheath flow rate ratio.

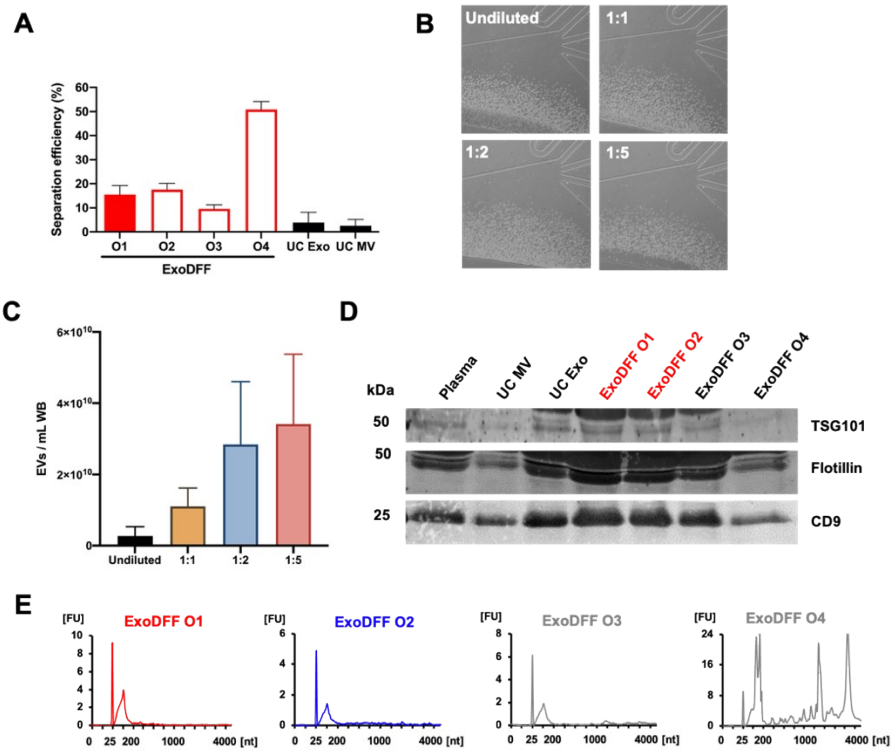


Figure S5. (A) EV separation efficiency from whole blood using ExoDFF (O1 – O4) and UC (n = 3, normalized to sample volume). **B)** High speed images captured at the device outlet illustrating flow of RBCs at different blood dilutions. **(C)** EV yield from ExoDFF NV (O1) from undiluted and diluted (1:1, 1:2 and 1:5) blood samples (n=2). **(D)** Western blot detection of exosomal markers TSG101, flotillin and CD9 from UC and ExoDFF (O1 – O4) isolated EVs. All samples were loaded with equal volume (15 µL) except plasma (0.5 µL). **(E)** Bioanalyzer electropherograms showing the size distribution in nucleotides (nt) and fluorescence intensity (FU) of total RNA from ExoDFF outlets (O1 – O4).

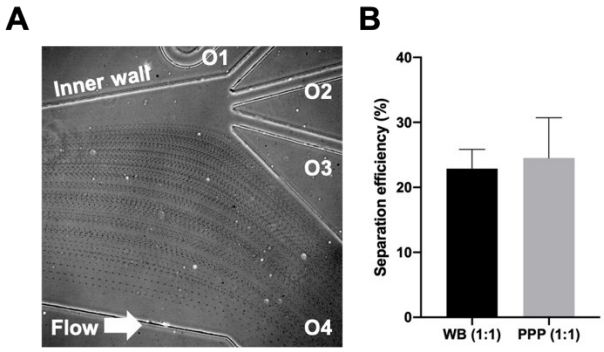


Figure S6: (A) Composite high speed image of platelet-poor plasma (PPP) processing using ExoDFF with platelets (~2-3 um appearing black dots) separating into outlet 3 (O3) and 4 (O4). **(B)** Separation efficiency comparison between diluted whole blood (1:1) and PPP (1:1) using ExoDFF (n=2).

Characteristics	Healthy (n = 5)	T2DM (n = 9)
Age* (Range)	35 (27 - 41)	53 (47 - 65)
HbA1c, %	5.340 (0.107)	7.278 (0.292)
Fasting glucose, mmol/L	5.160 (0.087)	7.667 (0.824)
BP Systolic, mmHg	104.500 (2.118)	125.000 (4.484)
CRP, mg/L	2.260 (0.822)	2.511 (0.686)
Total-C, mmol/L	4.580 (0.331)	3.756 (0.228)
HDL-C, mmol/L	1.240 (0.073)	1.122 (0.047)
LDL-C, mmol/L	3.020 (0.272)	2.1 (0.204)
Triglyceride, mmol/L	0.700 (0.071)	1.167 (0.121)

Average value shown with SEM in parentheses, unless otherwise indicated.

Table S2. Clinical characteristics of healthy (n = 5) and type 2 diabetic mellitus (T2DM) subjects (n = 9).

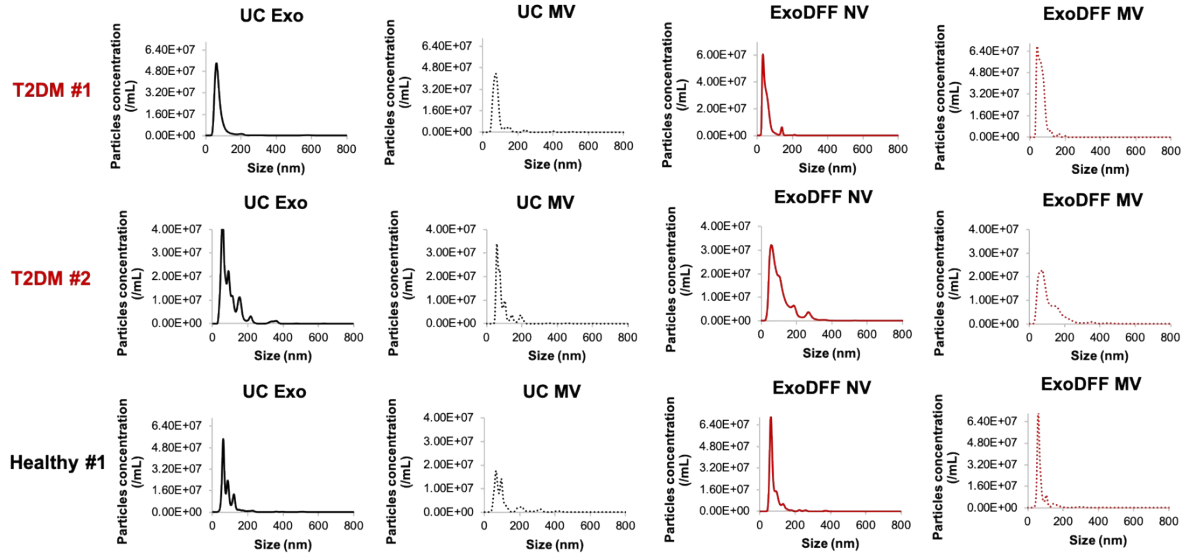


Figure S7. Representative NTA plots of EVs from healthy (n = 1) and T2DM (n = 2) subjects isolated using ultracentrifugation (UC) and ExoDFF.

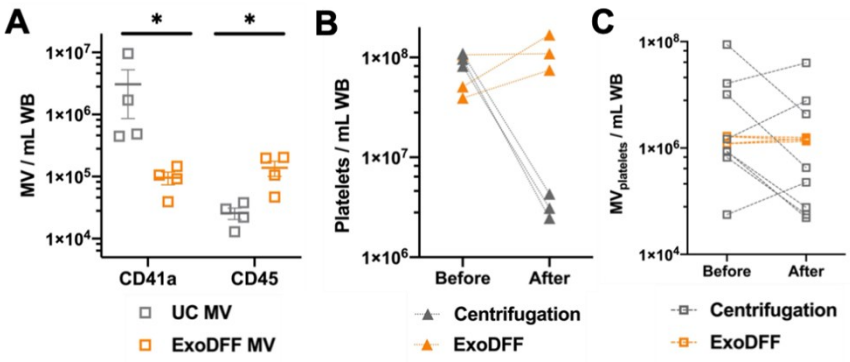


Figure S8. (A) Quantification of platelet-derived (CD41a+) and leukocyte-derived (CD45+) microvesicles isolated from UC MV and ExoDFF MV. (**B**) Significant platelet loss after high speed centrifugation (20,000 g, 45 min) in PRP samples as compared to ExoDFF (O3 – O4). (**C**) Significant variations in platelet-derived microvesicles in PPP samples after high speed centrifugation (20,000 g, 45 min) as compared to ExoDFF MV.

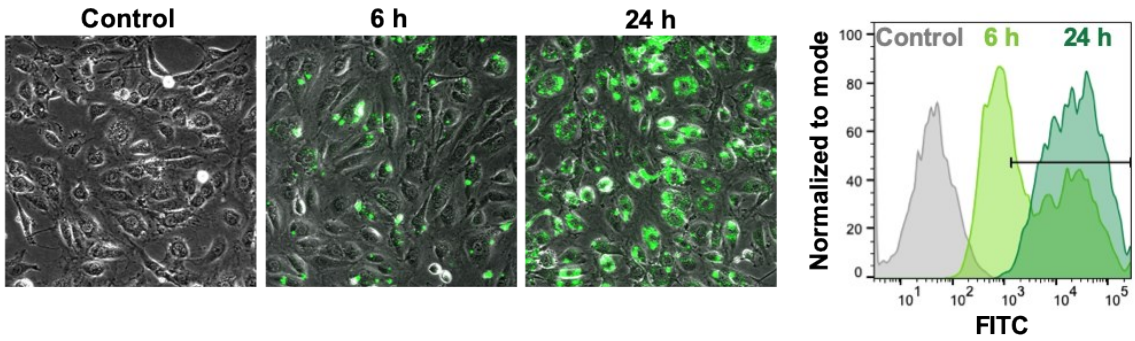


Figure S9. Representative bright-field and fluorescence overlaid images of HUVEC monolayer incubated without (control) and with fluorescence-labelled EVs (PKH67) after 6 and 24 h**.** Flow cytometry analysis of EVs uptake by HUVEC after 6 and 24 h.

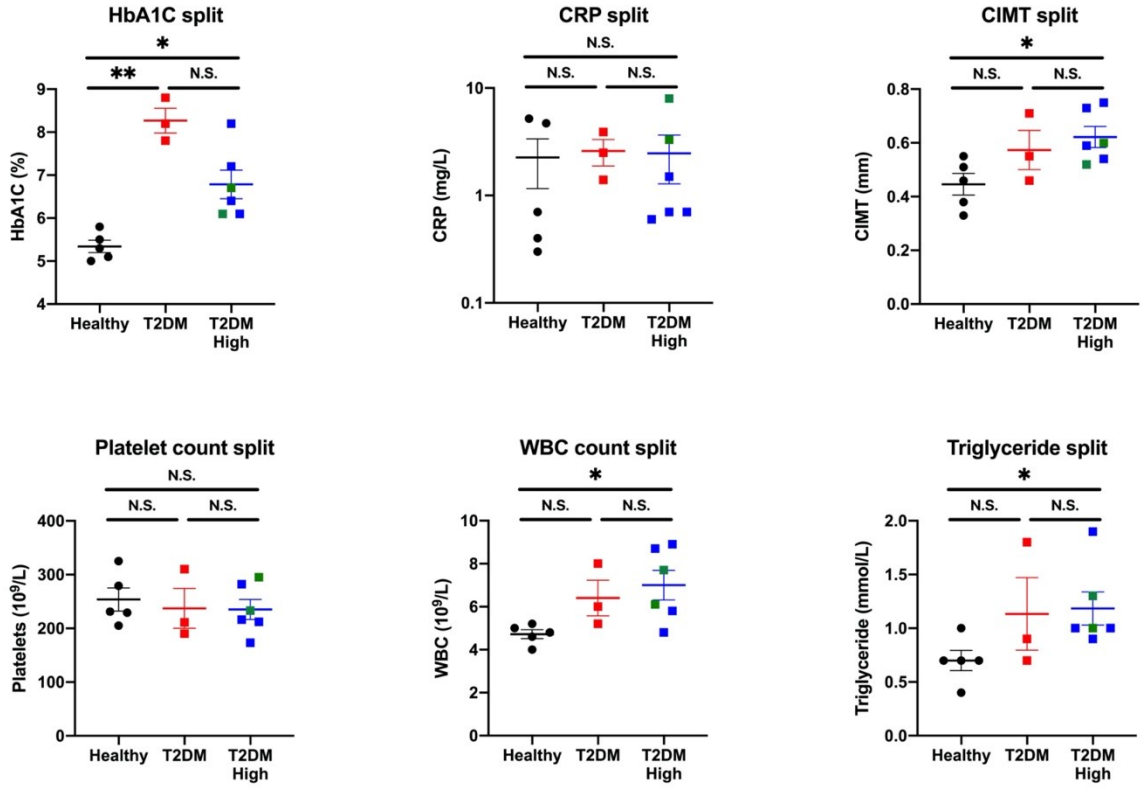


Figure S10. Clinical characteristics comparison of healthy (n = 5), T2DM (n = 3) and high risk T2DM (n = 6) subjects in terms of (**A**) HbA1C, (**B**) C-reactive proteins (CRP), (**C**) carotid intima-media thickness (CIMT), (**D**) platelet count, (**E**) white blood cell (WBC) count and (**F**) triglyceride levels.

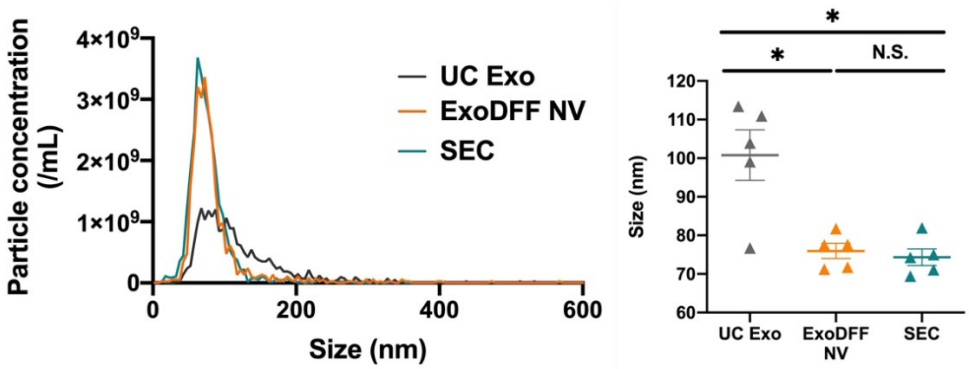


Figure S11. NTA size distribution profile (*left)* and EV size comparison (*right,* n = 5) of EVs isolated using ultracentrifugation (UC), ExoDFF and size exclusion chromatography (SEC).

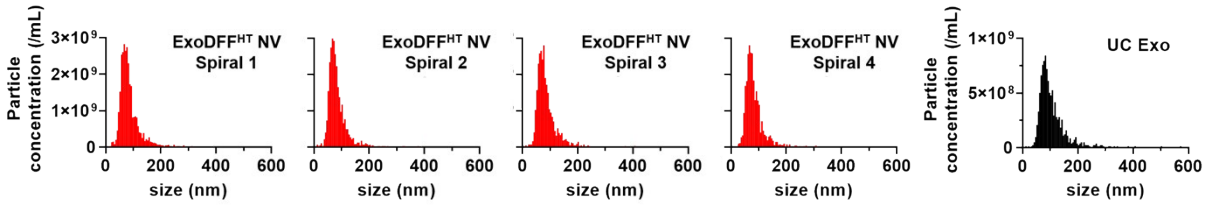
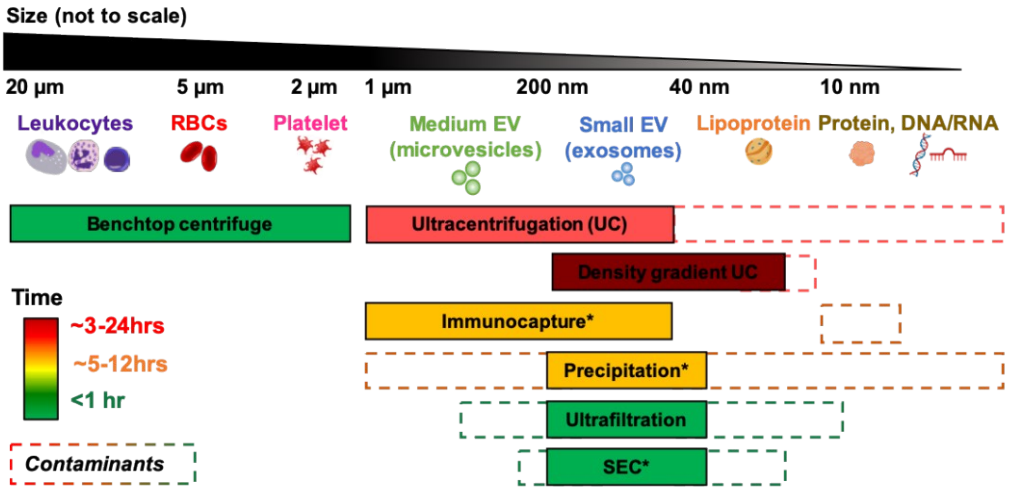


Figure S12. Representative NTA plots of EVs from each spiral of high throughput ExoDFF (ExoDFF^{HT} NV) and UC as control.

A



B

Methods	Differential Ultracentrifugation	Density Gradient Ultracentrifugation	Immunocapture	Precipitation	Ultrafiltration	Size Exclusion Chromatography
Principles	Sequential centrifugation to sediment cells, platelets, and EV at different rpm	Density-based EV fractionation in discontinuous density cushion or continuous density gradient	EV capture through binding with immobilized recognition elements including antibodies, aptamers	EV precipitation using water-excluding polymer which decreases EV solubility in medium	Size based separation through a porous membrane	Size dependent elution profile through a column packed with porous polymeric beads
Advantages	-Gold standard -Large capacity -Large dynamic range	-Low protein contamination -High purity -Large capacity	-High specificity -High purity	-Easy to use -High scalability	-Fast method -High scalability	-Low protein contamination -High reproducibility
Disadvantages	-Poor repeatability -High equipment cost -Low scalability	-Poor repeatability -High equipment cost -Low scalability	-High reagent cost -Low scalability -Low capacity	-Coprecipitation of protein and polymer precipitant	-Membrane clogging -Single size cutoff -Shearing of EV	-High dilution -Medium scalability
Contaminants	Lipoproteins, protein aggregates	Negligible	-Soluble proteins	MV, lipoproteins, protein aggregates	Lipoproteins	Lipoproteins
EV recovery (%)	~ 2 - 80	~10	~ 60 - 90	~ 90	~ 10 - 80	~ 40 - 90
Assay time (h)	~ 3 - 9	~ 16 - 90	~ 4 - 20	~ 0.3 - 12	~ 0.5	~ 0.3
Sample volume	mL - L	µL - mL	µL	µL - mL	µL - mL	µL - mL

Figure S13. Conventional methods for isolation of EVs. (A) Schematic overview and (**B)** table comparison on separation principles and various performance metrices for different methods.