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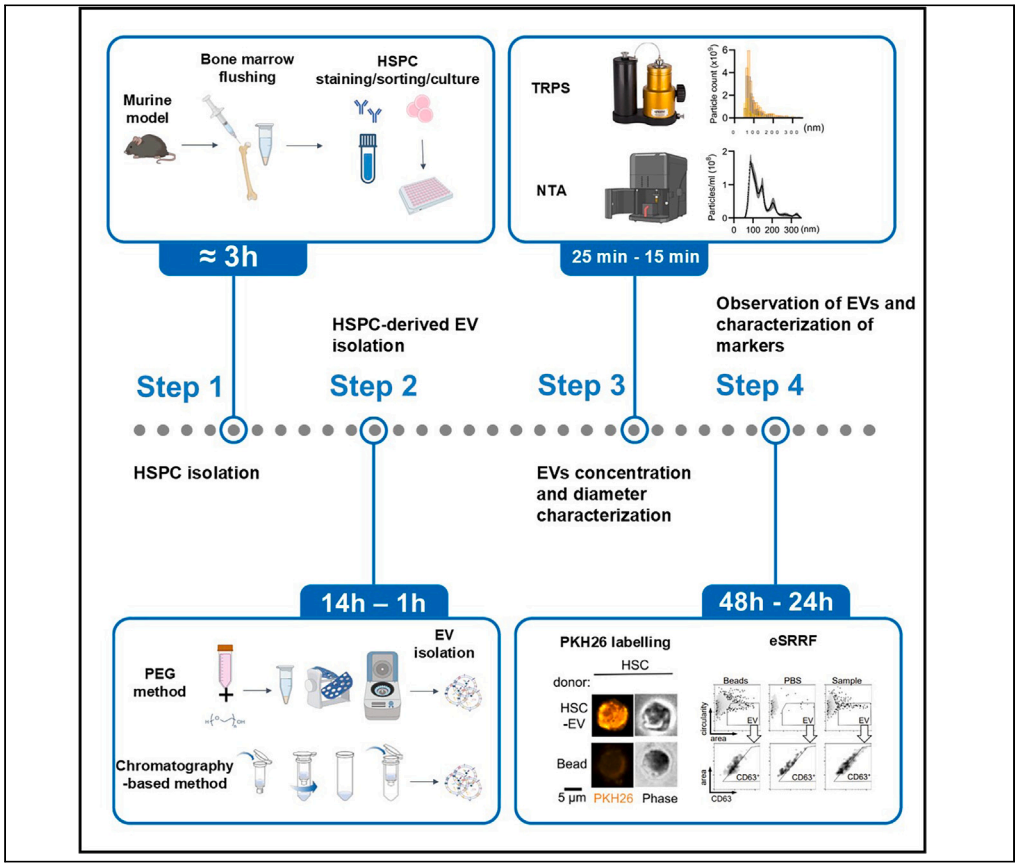
Protocol for the isolation and characterization of murine hematopoietic stem and progenitor cell-derived extracellular vesicles



Hematopoietic stem cells (HSCs) maintain their self-renewal capacity in an autocrine manner through hematopoietic stem and progenitor cell (HSPC)-derived extracellular vesicles (EVs). Here, we present a protocol for the isolation and characterization of EVs from HSPCs starting from an *in vivo* murine model. We describe steps for murine bone marrow isolation, HSPC staining and sorting, HSPC-derived EV isolation, size and concentration characterization, and EV visualization and marker description.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps for investigating HSPC-derived EV surface markers using flow cytometry

Instructions for bone marrow HSPC isolation, staining, and sorting

Guidance on techniques for determining the size and concentration of HSPC-derived EVs

Procedures for characterizing HSPC-derived EVs

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Protocol for the isolation and characterization of murine hematopoietic stem and progenitor cell-derived extracellular vesicles

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SUMMARY

Hematopoietic stem cells (HSCs) maintain their self-renewal capacity in an autocrine manner through hematopoietic stem and progenitor cell (HSPC)-derived extracellular vesicles (EVs). Here, we present a protocol for the isolation and characterization of EVs from HSPCs starting from an *in vivo* murine model. We describe steps for murine bone marrow isolation, HSPC staining and sorting, HSPC-derived EV isolation, size and concentration characterization, and EV visualization and marker description.

For complete details on the use and execution of this protocol, please refer to Bonora et al.[1]

BEFORE YOU BEGIN

Extracellular vesicles (EVs) are membranous vesicles released by all cell types into extracellular space involved in the cell-to-cell communication. EVs play an important role in the communication processes among different cell populations in the bone marrow.[2] The bone marrow is the main site for hematopoiesis, the process responsible for generating all types of blood and immune cells throughout life starting from hematopoietic stem and progenitor cells (HSPCs).[3] Several studies have demonstrated that HSPCs are sensitive to niche derived EV-mediated communication.[4,5]

Our lab recently demonstrated that EVs from hematopoietic stem cells (HSCs) can be utilized in an autocrine manner to enhance their self-renewal capacity.[1] Supplementing HSCs with EVs before bone marrow transplantation (BMT) significantly improves their ability to restore the hematopoietic system. Conversely, disrupting EV production by silencing Rab27a, a key regulator of EV secretion that facilitates multivesicular endosome docking at the plasma membrane, leads to a severe decline in HSC reconstitution potential following BMT.[1]

Moreover, EVs have been studied for their direct involvement and contribution in hematological malignancies development.[6] On these premises, HSPC-derived EVs present an intriguing subject of study to improve our understanding of how EVs influence HSPCs in restoring the bone marrow microenvironment when exposed to malignant conditions, with the goal of developing potential therapies.[7]

The limited research on HSPC-derived EVs may stem from challenges associated with current EV study methods (including ultracentrifugation or ultrafiltration), which typically require a large number of EV-producing cells. However, advancements in technology have enabled the investigation of HSPC-derived EVs by allowing the analysis of EV markers and cargo at the single-vesicle level.

In this protocol we describe techniques such as super-resolution microscopy, and high-resolution single-particle platforms, NanoFCM NanoAnalyzer (nFCM), that have played a crucial role in overcoming these limitations.

Preparation of cell culture medium and reagents

Timing: 15 min; reagents can be prepared in advance and stored at 4°C

1. To prepare 500 mL of FACS buffer (PBS + 2% FBS).
   a. Mix 490 mL PBS without magnesium chloride and calcium chloride with 10 mL FBS.
   b. Store at 4°C in sterile conditions for up to one month.

   Note: Before use, place aliquots on ice.

2. To prepare 50 mL of PEG (polyethylene glycol)-based solution.
   a. Mixing all the reagents listed in the table "PEG-based solution" in the "materials and equipment setup" section.
   b. Filter using a 0.2 μm filter unit and store at 4°C.

   Note: Sealed with Parafilm.

   ⚠ CRITICAL: Filter all solutions in contact with EVs with a 0.2 μm filter to prevent contamination.

Institutional permissions

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of the Albert Einstein College of Medicine.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD11b-biotin (dilution 1:100)	BD Biosciences	Cat# 553782; RRID: AB_394773
CD19-biotin (dilution 1:100)	BD Biosciences	Cat# 553784; RRID: AB_395048
CD45R/B220-biotin (dilution 1:100)	BD Biosciences	Cat# 553086; RRID: AB_394615
CD4-biotin (dilution 1:100)	BD Biosciences	Cat# 553045; RRID: AB_394581
NK-1.1-biotin (dilution 1:100)	BD Biosciences	Cat# 553163; RRID: AB_394675
TER-119-biotin (dilution 1:100)	BD Biosciences	Cat# 553672; RRID: AB_394985
CD117 (c-kit)-APC/CY7 (dilution 1:100)	BioLegend	Cat# 105826; RRID: AB_1626278

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD117 (c-kit)-PE (dilution 1:100)	BioLegend	Cat# 105808; RRID: AB_313217
CD150 (SLAM)-PerCp/Cy5.5 (dilution 1:100)	BioLegend	Cat# 115922; RRID: AB_2303663
CD48-Pacific Blue (dilution 1:100)	BioLegend	Cat# 103418; RRID:AB_756140
Ly6G/Ly6C (Gr-1)-biotin (dilution 1:100)	BioLegend	Cat# 108404; RRID: AB_313369
CD127-biotin (dilution 1:100)	eBioscience	Cat# 13-1271-85; RRID: AB_466588
CD135 (Flt3)-biotin (dilution 1:100)	eBioscience	Cat# 13-1351-82; RRID: AB_466599
CD34-APC (dilution 1:100)	eBioscience	Cat# 50-0341-82; RRID: AB_10596826
CD34-FITC (dilution 1:100)	eBioscience	Cat# 11-0341-85; RRID: AB_465022
CD3e-biotin (dilution 1:100)	eBioscience	Cat# 13-0031-85; RRID: AB_466320
CD48-APC (dilution 1:100)	eBioscience	Cat# 17-0481-82; RRID: AB_469408
CD8a-biotin (dilution 1:100)	eBioscience	Cat# 13-0081-85; RRID: AB_466347
CD201 (EPCR)-APC (dilution 1:100)	eBioscience	Cat# 17-2012-82; RRID: AB_10717805
Ly-6A/E (Sca-1)-PE/Cy7 (dilution 1:100)	eBioscience	Cat# 25-5981-81; RRID: AB_469668
Mouse IgM-biotin (dilution 1:100)	eBioscience	Cat# 13-5790-85; RRID: AB_466676
PE mouse anti-human CD63 (dilution 1:100)	eBioscience	Cat# 12-0639-42; RRID: AB_2572565
PerCP/Cyanine5.5 anti-human CD9 (dilution 1:100)	BioLegend	Cat# 312109; RRID: AB_2728251
Streptavidin-APC (dilution 1:100)	eBioscience	Cat# 17-4317-82
Streptavidin-Pacific Blue (dilution 1:100)	eBioscience	Cat# 48-4317-82
CD63 magnetic beads (dilution 1:100)	Thermo Fisher Scientific	Cat# 10606D
CD63-APC (dilution 1:100)	Life Technologies	Cat# A15712; RRID: AB_2534492
Anti-CD63 antibody (dilution 1:100)	Abcam	Cat# ab134045
Goat anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor Plus 488 (dilution 1:100)	Thermo Fisher Scientific	Cat# A32731
Chemicals, peptides, and recombinant proteins		
Animal-free recombinant murine TPO	PeproTech	Cat# AF-315-14-100UG
Recombinant Murine SCF	PeproTech	Cat# 250-03-10UG
StemSpan SFEM	STEMCELL Technologies	Cat# 09600
DAPI	Thermo Fisher Scientific	Cat# 62248
PEG	Merck	Cat# 89510; CAS: 25322-68-3
NaCl 1 M	Sigma-Aldrich	Cat# S7653; CAS: 7647-14-5
Milli-Q water	–	–
PBS pH 7.4 (1×) w/ calcium chloride and magnesium chloride	Gibco	Cat# 10010-023
Heat inactivated FBS	Gibco	Cat# 16140-071
ACK lysing buffer	Gibco	Cat# A10492-01
Nanopore NP150 (70–420 nm)	Izon Science	
Calibration particles CPC100 (100 nm)	Izon Science	–
Calibration particles CPC200 (200 nm)	Izon Science	–
Quality control beads	NanoFCM	–
NanoFCM Silica Nanospheres Cocktail #1	NanoFCM	Cat# S16M-Exo
PKH26	Sigma-Aldrich	Cat# MINI26-1KT
TetraSpeck	Thermo Fisher Scientific	Cat# T7279
ProLong Diamond Antifade Mountant	Thermo Fisher Scientific	Cat#P36965
Experimental models: Organisms/strains		
Mouse C57BL/6, 3–6 months age, male/female	The Jackson Laboratory	JAX: 000664
Other		
5 mL polystyrene round-bottom tube with cell-strainer cap	Falcon	Cat# 352235
MidiMACS Starting Kit (LS)	Miltenyi Biotec	Cat# 130-042-301
SmartSEC Mini EV Isolation System	System Biosciences	Cat# SSEC100A-1
Formvar/carbon-supported copper grid	Sigma-Aldrich	Cat# TEM-FCF200CU50
Software		
GraphPad Prism	GraphPad	https://www.graphpad.com/
FlowJo	BD	https://www.flowjo.com/

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Izon Control Suite Software (v.3.4)	Izon Science	https://support.izon.com/qnano-controlsuite-software
NS XPLORER - v.1.1.0.6	NanoSight NTA software	https://www.malvernpanalytical.com/en/support/product-support/nanosight-range/nanosight-ns300#software
Fiji – ImageJ[8]		Fiji (imagej.net)
NanoJ-eSRRF[9]		https://github.com/HenriquesLab/NanoJ-eSRRF

MATERIALS AND EQUIPMENT

The cell sorting of the HSPCs described in this protocol utilizes the BD FACSAria II Cell Sorter (Becton Dickinson, USA).

The EV characterization steps outlined in this protocol are performed using various device/techniques that can work as an alternative for each other's.

- The qNano Gold TRPS Measurement technique (Izon Science, NZ) uses a system in which particles passing through a size-known pore momentarily disrupt the pre-established electric current, creating a blockade. The dimensions and frequency are used to determine particle size and concentration.
- NanoSight NS300 Instrument (Malvern Panalytical, UK) allows a rapid and highly sensitive method for visualizing and characterizing EVs. During NTA measurement, EVs are visualized by the light scattered when they are irradiated with a laser beam. The scattered light is then focused by a microscope onto a camera that records the particles' movement. Ultimately, NTA software tracks the random thermal motion of each particle, known as Brownian motion, to determine the diffusion coefficient, which is used to calculate the size of each particle using the Stokes-Einstein equation.
- Nanoanalyzer (nanoFCM Co., Ltd, China) and the Attune NxT Acoustic Focusing Cytometer (Life Technologies) exploit instead usual cytometer techniques in detecting the fluorescence of the antibodies used to label the EVs under study.
- Flow cytometry techniques to analyze EV membrane markers exploiting antibodies that label well-characterized EV markers (e.g., CD-9 and CD-63).
- Confocal microscopy to evaluate the level of fluorescence of the cells after treatment with PKH26-labeled-EVs.

HSPC-derived EVs can also be observed using transmission electron microscopy (TEM), which enables the identification of specific features of EVs, including a rounded morphology with a central depression and an average diameter ranging from 30 to 200 nm.

Here below the recipes for HSPC cell culture medium and PEG-based preparation for EV isolation.

HSPC cell culture medium

Reagent	Stock concentration	Final concentration	Amount
StemSpan SFEM		–	100 mL
Mouse TPO (Thrombopoietin)	100 μg	50 ng/mL	1 μL
Recombinant Murine SCF	10 μg	50 ng/mL	1 μL

Note: Add the cytokines to the media on the same day as the experiment. Avoid thaw/freeze cycles. The complete medium can be stored at 4°C for a maximum of one week.

PEG-based solution

Reagent	Final concentration	Amount
PEG 8000	8%	8 g
NaCl	1 M	2.92 g
Milli-Q water	–	50 mL

Note: Filter using a 0.2 μm filter unit and sealed with Parafilm. The solution can be stored at 4°C for a maximum of one month.

⚠ CRITICAL: Filter everything that goes in contact with EVs to avoid nanoparticle contamination.

STEP-BY-STEP METHOD DETAILS

Murine bone marrow isolation

Timing: approximately 20 min

This section describes the isolation of murine bone marrow using the flushing technique. Use Morganti et al., 2019 as a reference for visual support with video and pictures of first part of the protocol until "isolation of HSPC-derived EVs."[10]

1. Euthanize the mouse by CO^3 inhalation following institutional guidelines and spray the mouse with 70% ethanol.

Note: This step prevents contamination of the cells without compromising experimental results.

2. Extract the femur and tibia and place them in a 6-well plate filled with 1.5 mL of FACS buffer.
3. Remove the muscles from the bones and cut the ends of the bones allowing the exit of the bone marrow.
4. Harvest the bone marrow from the femur and tibia and flush it using a 3 mL syringe with the FACS buffer.
5. Centrifuge for 5 min at 180 g and discard supernatant.
6. Lyse the red blood cells with 300 μL of ACK Lysing Buffer for 1 min on ice.

⚠ CRITICAL: Avoid keeping cells exposed to ACK for more than 1 min to prevent cell damage.

7. Stop the reaction with 1 mL of FACS buffer and centrifuge for 5 min at 180 g.
8. Resuspend the pellet of bone marrow mononuclear cells with 1 mL of FACS buffer.
9. Filter it using FACS tubes 5 mL Polystyrene round-bottom tube with cell-strainer cap.
10. Centrifuge for 5 min at 180 g and discard supernatant.

HSPC staining

Timing: ≈1 h 20 min

11. Resuspend the pellet with a mix of monoclonal antibodies against lineage markers (Ly6G/Ly6C (Gr-1)-biotin, CD11b-biotin, CD19-biotin, CD45R/B220-biotin, CD4-biotin, NK-1.1-biotin, TER119-biotin, CD135-biotin (also called FLT3-bio), CD127-biotin, CD3e-biotin, CD8a-biotin, Mouse IgM-biotin) resuspended in FACS buffer.

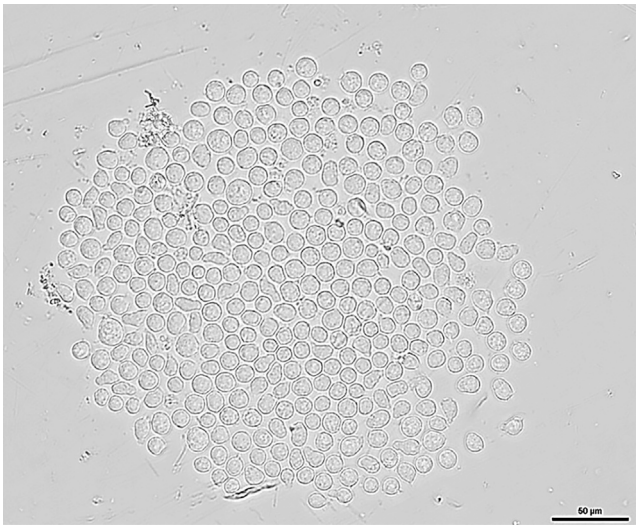

Figure 1. Representative optical microscope image

This figure reports a representative optical microscope (20X) image of sorted HSPCs (scale bar 50 µm), displaying their characteristic rounded morphology.

12. Incubate for 30 min in ice.
13. Wash with 3 mL of FACS buffer.
14. Centrifuge for 5 min at 180 g, discard the supernatant.
15. Enrich stem and progenitor cell fractions using the MidiMACS Separator and Starting Kits provided with LS columns.[11]
16. Resuspend the pellet with a mix of antibodies for HSPC markers (CD117 (c-Kit)-APC/CY7 or CD117-PE, CD150 (SLAM)-PerCP/Cy5.5, CD48-APC or CD48-Pacific blue, CD201 (EPCR)-APC, CD34-APC or CD34-FITC, Ly-6A/E (Sca-1)-PE/Cy7, Streptavidin-APC or Streptavidin-Pacific Blue), all diluted 1:100 FACS buffer.
17. Incubate 30 min on ice.
18. Wash with 3 mL of FACS buffer.
19. Centrifuge for 5 min at 180 g, discard supernatant.
20. Resuspend cells pellet with 300 µL of DAPI solution (1 mg/mL diluted 1:1000 in FACS buffer).

Sorting of HSPC populations

Timing: ≈40 min

This section describes the workflow to isolate HSPCs (Figure 1 as cells morphology reference) using fluorescent-activated cell sorting (FACS) using BD FACSAria II machine (Becton Dickinson).[10]

21. Load the sample tube onto the flow sorter and set gates as shown in Figure 3 (section "expected outcomes").
22. Use the plate deposition unit of the sorter to place 3,000 HSPCs cells into each of the wells of U-bottom 96-well plates.

Note: Each well having been preloaded with 100 µL of completed StemSpan SFEM media (recipe described above in "materials and equipment" section).

23. Centrifuge for 5 min at 180 g to spin down the sorted cells.

Isolation of HSPC-derived EVs

Timing: ≈14 h (for step 24)

Timing: ≈1 h (for step 25)

This section describes two different techniques to isolate HSPC-derived EVs.

24. PEG-based method.

Note: This section describes a PEG based method to isolate EVs.[12]

a. After 48 h, collect HSPC EV-enriched media and centrifuge at 500 g for 5 min at 22°C.
b. Transfer the supernatant in a new Eppendorf tube.
c. Centrifuge 2000 g for 30 min at 4°C to remove the coarse part in the supernatant.
d. Transfer the supernatant (around 100 μL) in a new 1.5 mL tube.
e. Expose HSPC EV-enriched media to 100 μL 8% PEG 8000 at 4°C for about 12–14 h mixing it on a rotator.
f. Centrifuge the EVs at 14000 g for 1 h at 4°C.
g. Discard the supernatant and resuspend the EV pellet in the desired volume of particle-free PBS (30–40 μL for our experiment).

Note: After isolation, EVs can be stored at −20°C before performing further assays for a maximum of 6 months.

25. SmartSEC Mini EV isolation system.

Note: This section describes how to isolate EVs using a chromatography-based technology that combines all the benefits of size exclusion chromatography (SEC). The SmartSEC Mini EV isolation system is optimized for isolating EVs from 10-100 μL of starting biofluid (Visual and further details support at the link: SmartSEC Mini EV Isolation System | System Biosciences).

a. Collect HSPCs medium after 48 h and centrifuge at 3000 g for 20 min.

Note: An optional second centrifugation at higher speed (10000/20000 g) can be used to remove large vesicles.

b. If necessary, adjust the volume with Isolation Buffer up to 100 μL.
c. Take the SmartSEC mini column, loosen the cap and snap off the button closure.

⚠ CRITICAL: Save the bottom closure for later steps.

d. Place the column into an empty Collection tube and centrifuge at 500 g for 30 s to remove the storage buffer.
e. Remove the cap and add 200 μL of Isolation Buffer to the column.
f. Centrifuge at 500 g for 30 s to wash the beads and discard the collection tube.
g. Place the bottom closure back on the column and apply the EV sample on the top of the column's resin.
h. Put the cap back on and place the column in a new 1.5 mL tube on a rotating platform/mixer.
i. Incubate at 22°C for 30 min with constant mixing.
j. Centrifuge at 500 g for 30 s to collect the first fraction of EVs.

Optional: Add 100 μL of Isolation buffer to the column and repeat steps j to collect a second fraction of EVs.

Note: After isolation, EVs can be stored at −20°C before performing further assays for a maximum of 6 months.

Characterization of EV concentration and size distribution

Timing: ≈2 h (for step 26)

Timing: ≈1 h (for step 27)

This section describes how to measure EVs concentration and size diameter and distributions using Tunable resistive pulse sensing (TRPS) and nanoparticles tracking analysis (NTA) techniques.

26. Tunable Resistive Pulse Sensing (TRPS) technique.

Note: This section describes the particle size distribution analysis of EVs derived from HSPCs, measured by the TRPS system. This technique estimates the size of nanoparticles by their ability to interfere with the electric conductance of a membrane punctuated with nano-sized pores (pore size 100 nm).

a. Assembly the fluid cell and stretch the nanopore to 47 nm. Enter this value in the software and click "Calibrate stretch".
b. Add 75 μL particle-free PBS in the power fluid cell and 35 μL particle-free PBS in the upper fluid cell.
c. Insert the nozzle, close the vent and apply a pressure of 20 mbar and a voltage of 0.1 V.
d. Once a baseline current is established, increase the voltage to reach a current of 100–140 nA in the fluid cell and stretch the nanopore.

⚠ CRITICAL: Every sample must be read with the same voltage, pressure and nanopore stretch.

e. Use an NP150 nanopore (70–420 nm) to measure concentration and size distribution of 35 μL of each EVs sample.
f. Record at least 500 particles with a rate of 100 per sample.

Note: To obtain a significant output, each sample must be run in triplicate.

g. Repeat the previous step with calibration particles carboxylated (CPC) 100 or 200 nm.
h. Analyze the data with Izon Control Suite Software (V3.4).

Note: The readouts of the measurements include particle size (as the diameter of each particle is determined), particle concentration (as the number of particles per volume is measured), and size distribution (as the relative abundance of the particles in the selected range is determined).

Note: Link for q-nano instrument user manual with protocol: qnano-user-manual-QN1-OQ-014.pdf.

27. Nanoparticle Tracking Analysis (NTA) technique.

Note: This section describes how to analyze EVs with NTA, which uses laser light scattering and Brownian motion to determine EVs size and concentration.[1,13]

a. Turn on the Nanosight NS300 equipped with an automated syringe pump and a 488 nm laser.
b. Set the instrument temperature to 25°C and the pump speed to 30 (equivalent to 1 mL in 30 s).
c. Before reading the sample, clean the flow cell of the machine with filtered water, injecting some drops slowly to check the flow and remove any air bubbles.
d. Load 1 mL of EV-free PBS into the instrument and wash the pump and instrument by flowing 1 mL of EV-free PBS through slowly.
e. Repeat step d twice.
f. Dilute each HSPC-derived EVs sample in EV-free PBS to a final volume of 1 mL for insertion into the machine.
g. Record five independent measurements using the NS XPLORER software.

Note: Setting the "flow rate" to 5, to obtain a reliable reading of the samples confirming the absence of contaminants.

h. After the readings, check the "quality" window in the software to ensure the green color appears, confirming that everything respected the set ranges.
i. Observe the final output in NS XPLORER.

Note: The output is represented as averaged finite track length adjustment (FTLA) concentration/size.

Visualization and marker characterization of HSPC-derived EVs

Timing: ≈24 h (for step 28)

Timing: ≈20 min for PKH26 staining (for step 29)

Timing: ≈48 h for HSPC-derived EV treatment (for step 29)

Timing: ≈24 h; image processing may require from several minutes to several hours per image, depending on the timelapse size and the computer performance capabilities (for step 30)

Timing: ≈30 min, including alignment and calibration of the instrument (for step 31)

This section describes different techniques to visualize EV and also label and characterize specific EV markers.

28. Flow cytometry technique to investigate HSPC-derived EVs surface markers.

Note: This procedure describes how to enrich the bulk EV populations on magnetic particles, in order to make them detectable by flow cytometry.

a. Resuspend the magnetic CD-63 beads by mixing for 10 min or vortexing for 30 s.
b. Transfer 20 μL magnetic beads into a 0.5 mL tube and wash them with 200 μL of Isolation Buffer, mix well.

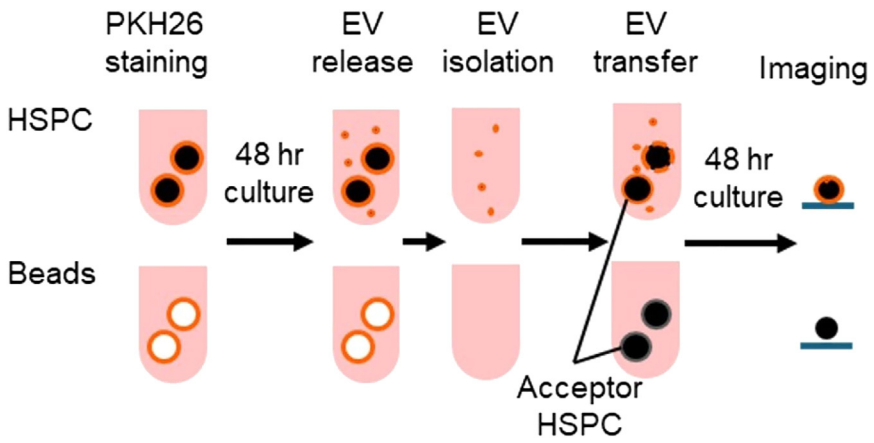

Figure 2. Experimental strategy of EV transfer between donor- and acceptor-HSPCs

Donor HSPCs (black) or compensation beads (white) were sorted into FBS-free media, and stained with the lipid marker, PKH26 (orange). Isolated EVs were then supplemented to freshly isolated acceptor HSPCs, and EV-related PKH26 signal (orange) was determined by fluorescence microscopy. Images adapted from Bonora et al., 2024.[1]

c. Place the tube on the magnet for 1 min to allow the beads to translocate to the tube wall, then discard the supernatant.
d. Remove the tube from the magnet.
e. Add a pre-enriched EVs solution titrated with Isolation Buffer (100 μL final volume) to the magnetic beads.
f. Mix well.
g. Incubate and mix the tube for 12–14 h at 4°C on a rotator.
h. Spin the tube to collect the sample at the bottom of the tube.
i. Wash the bead-bound EVs by adding 300 μL of Isolation Buffer.
j. Mix gently by pipetting (do not vortex).
k. Place the tube on the magnet for 1 min and discard the supernatant.
l. Remove the tube from the magnet, and add 400 μL of Isolation Buffer.
m. Mix gently by pipetting (do not vortex).
n. Place the tube on the magnet for 1 min and discard the supernatant.
o. Resuspend all the EVs in 35 μL of isolation buffer and incubate with CD63 magnetic beads.
p. Isolate CD63-positive EVs and label them with anti-CD63 or anti-CD9 monoclonal antibodies for 30–45 min at 22°C shaking them.
q. Create a negative control by staining PBS (vehicle) instead of EVs.
r. Wash the bead-bound EVs by adding 300 μL of Isolation Buffer.
s. Mix gently by pipetting (do not vortex).
t. Place the tube on the magnet for 1 min and discard the supernatant.
u. Resuspend in a desired volume of isolation buffer.
v. Run the samples in a flow cytometer (e.g., Attune NxT Acoustic Focusing Cytometer) and analyze the data using FlowJo 10 software.[10]

29. PKH26 staining to visualize HSPC-derived EVs internalized by freshly-isolated HSPCs.

Note: This section describes how to stain HSPCs with the lipid marker PKH26 to be able to observe the transfer of HSPC-derived EVs (experimental strategy reported in Figure 2).

a. Place a suspension of single HSPCs in a conical bottom polypropylene tube.
b. Wash once using medium without StemSPAN SFEM medium.
c. In a second tube, plate compensation beads in a number equivalent to the HSPCs.

Note: This will serve as a negative control.

⚠ CRITICAL: Use beads as a negative control since PKH26 can spontaneously generate fluorescent particles of a size comparable to EVs.

d. Centrifuge both tubes 400 g for 5 min and carefully aspirate the supernatant.

Note: Avoid removing cells or beads, but do not leave more than 25 μL of supernatant in the tube.

e. Prior to staining, prepare a 2× Dye Solution (4 μM) in Diluent C by adding 4 μL of the PKH26 ethanolic dye solution to 1 mL of Diluent C in a polypropylene centrifuge tube and mix well.
f. Rapidly add the 50 μL of cell or beads suspension to 50 μL of 2 Dye Solution and immediately mix the sample by gentle pipetting.
g. Incubate the suspension for 5 min with periodic mixing.
h. Stop the staining by adding an equal volume (100 μL) of StemSpan SFEM medium and incubate for 1 min.
i. Centrifuge at 400 g for 10 min at 20°C–25°C.
j. Carefully remove the supernatant.
k. Resuspend the pellet in 200 μL of StemSpan SFEM medium.
l. Transfer to a fresh sterile conical polypropylene tube.
m. Centrifuge at 400 g for 5 min at 22°C.
n. Wash the cell pellet 2 more times with 200 μL of SpemSpan SFEM medium.
o. Plate the stained cells or beads in 96-well plate and culture them with SpemSpan SFEM medium.
p. After 48 h of culture, collect the medium and isolate EVs following the protocol described in steps 17-20.
q. Determine the concentration of particles in EV preparation by NTA or TRPS.
r. Add stained EVs to freshly sorted HSPCs.

Note: The optimal amount of EVs per recipient HSPCs should be determined experimentally. A minimum of 1.5 × 10^6 EVs per recipient HSPC is recommended.

s. Administer an equivalent volume of bead-derived preparation to a separate batch of HSPCs as a control.
t. After 24 h, refresh the medium and repeat EV administration following the same protocol.
u. At 24 h after the second administration, detect PKH26 signal in HSPCs by fluorescence microscopy.

30. Super-resolution radial fluctuation imaging for analysis of EV markers.

Note: This section describes how to test the content of EV markers at single EV levels, using enhanced super-resolution radial fluctuations (eSRRF) microscopy that has been demonstrated to resolve structures close to 100 nm.[14]

a. Prepare the microscope slide be performic acid wash.
b. Submerge microscope slide in 1 N HCL at 50°C for 12–14 h.
c. Wash the microscope slide twice with EV free-PBS.
d. Dry the slide under a tissue culture hood.
e. Add one drop (approximately 100 μL) poly-lysine and rinse in the center of the microscope slide for 60 min a 37°C.
f. Remove the poly-lysine and rinse off excess with EV-free PBS.
g. Add 100 μL of EV preparation to the poly-lysine-coated slide.
h. Incubate for 60 min at 37°C to allow adhesion.
i. Repeat the procedure with 100 μM fluorescent beads and EV-free PBS as positive and negative controls for EV detection.

j. Fix EVs with 2% PFA for 10 min.
k. Block the slide with filtered 5% BSA for 60 min at 22°C.
l. Incubate with the primary antibody anti-CD63 diluted 1:100 in filtered 5% BSA and incubate for 12–14 h at 4°C.
m. Wash three times with filtered PBS, then incubate with the second antibody goat anti-rabbit conjugated with Alexa 488 fluor.
n. Add 50 μL of Prolong Diamond Antifade Mountant, cover with a coverslip, and let the mounting medium dry for 60 min at 22°C.
o. Install the sample on a wide-field microscope equipped with an oil immersion objective (numerical aperture ≥ 1.4).
p. Adjust settings to achieve a pixel size below 100 nm.

Note: Ideally this should be achieved using a 100× oil immersion objective. Alternately, images could be acquired using 60× oil objective and adding an additional 1.5× magnification.

q. Focus on the EV plane and acquire timelapses with varying frame numbers (e.g., 100, 200, 500 and 1000 frames) to determine the optimal number.
r. Export timelapses at TIFF stacks and import them into ImageJ.
s. Process the pictures using the NanoJ set of plug-ins available at: https://github.com/HenriquesLab/NanoJ-eSRRF9.
t. On the first access to the dataset, start by loading the smallest timelapse available.
u. Use the "eSRRF - Estimate Number of Frames" plugin to calculate the optimal number of frames required for reconstruction.
v. Compare the calculated optimal number of frames with the total number of frames in timelapse:
    i. If the calculated number matches the actual number of frames in the timelapse, repeat the analysis using the next larger timelapse.
    ii. If the calculated optimal number is smaller than the actual size of the timelapse, save this value for all the subsequent steps.
w. Estimate the optimal reconstruction parameters. Load a sample timelapse into ImageJ and run the "eSRRF - Parameters Sweep" plugin to determine the ideal values for radius and sensitivity.
x. Perform eSRRF analysis on all desired timelapses using the frame number and parameters calculated in the previous steps.

31. NanoFCM analysis.

Note: This section describes how to use a recently developed high-resolution single-particle platform, NanoFCM NanoAnalyzer (nFCM), to investigate the expression of CD63 on HSPC-derived EVs.[1,15]

a. Incubate 2×10^8–2×10^9 of purified EVs with CD63-APC primary antibody for 1 h at 37°C on a rotator, protected from light.
b. Remove unbound antibodies by washing 6–8 times with 500 mL of EV-free PBS.
c. Align the NanoAnalyzer using polystyrene QC beads (nanoFCM Inc.) according to the manufacturer's instructions.
d. Calibrate the NanoAnalyzer using size and concentration standard nanospheres (nanoFCM Inc.) as per the manufacturer's instructions.
e. Dilute each sample in filtered PBS to the optimal range for measurement (1 × 108 particles/mL).
f. Measure EV samples for 1 min (to collect 200–800 events) with the following parameters: laser power of 15 mW as excitation source, constant pressure of 1 kPa, and at an event rate between 2,500 and 12,000 events/min (as recommended by the manufacturer).

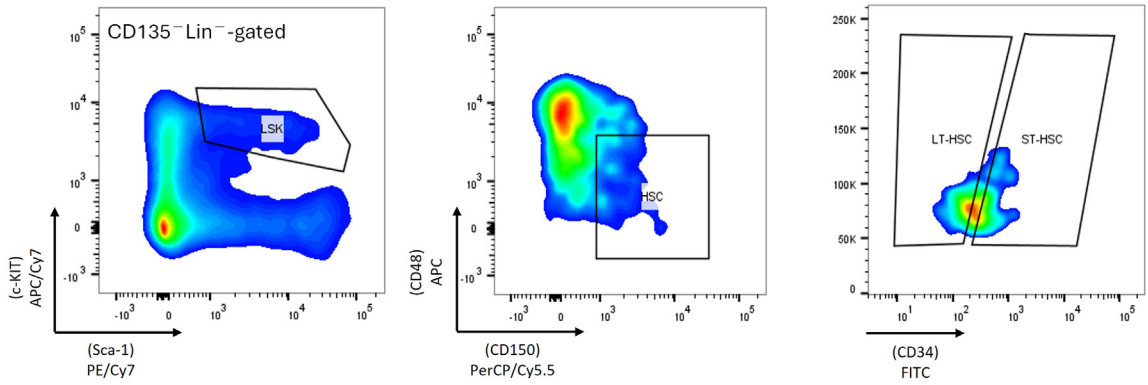

Figure 3. Representative gating strategy to purify HSPCs

From left to right the graphs represent how to select and purify: Lineage, Lin; LSK, $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$; LT, long term; ST, short term.

g. Measure empty staining reactions (filtered PBS) as described in step 78.
h. Analyze data with FlowJo 10 (Becton Dickinson).

EXPECTED OUTCOMES

Mouse bone marrow cell preparation

The cell suspension obtained from a healthy mouse should appear pinkish and slightly cloudy. After the first centrifugation step, the cell pellet will look reddish. However, following the red blood cells lysis step and centrifugation, the supernatant should be clear, and the pellet will be whitish with red edges, ready for filtration.

Sorting of HSPCs

The representative flow cytometric plots for a typical HSPCs cell sorting from a C57BL/6 mouse model resemble the gating shown in Figure 3.

Characterization of EV size and concentration after PEG-based isolation using TRPS and NTA techniques

Using TRPS and NTA techniques, EVs are found in the range of 30 nm–200 nm, with variable concentration depending on the volume of collected medium, as shown in Figures 4A and 4B. Each histogram of the replicates for each sample analyzed should show consistency across the different machine readings.

Observation of HSPC-derived EV internalization through PKH26 labeling

Using fluorescence microscopy, freshly sorted HSPCs treated with EVs isolated from previously stained HSPCs with PKH26 dye should exhibit higher fluorescence compared to the control cells stained with beads only, as shown in Figure 5.

Super-resolution radial fluctuation imaging analysis of HSPC-derived EVs

The enhanced super-resolution radial fluctuation imaging (eSRRF) technique, used on HSPC-derived EV samples (right), along with beads alone (left) and empty PBS (middle) as controls, should provide an output showing the expected area and circularity of the EVs under analysis, together with the positivity for CD63 (or CD9) EV membrane markers (Figure 6).

LIMITATIONS

HSPC differentiation

EV isolation is typically performed 72 h after cell seeding in a medium supplemented with exosome-depleted FBS. However, HSPCs begin differentiating into various blood lineages within 48 h upon

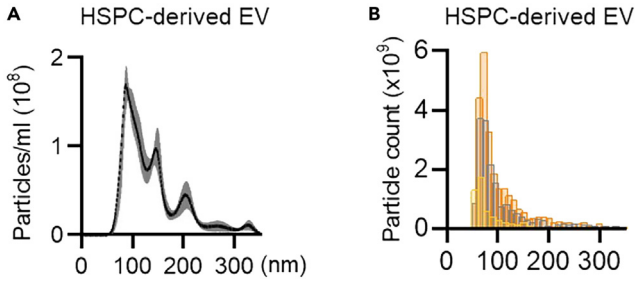

Figure 4. Representative charts of EVs quantification and size measurement with NTA and TRPS techniques (range 30-150 nm)

(A) Representative NTA result for HSPC-derived EVs concentration and size distribution.

(B) Representative histogram of HSPC-derived EVs concentration and size distribution with TRPS: the three different colors represent a sample reading in triplicate. Images adapted from Bonora et al., 2024.[1]

exposure to microenvironmental cues and growth factors. Consequently, in this study, HSPC-derived EV isolation was carried out 48 h post-cell sorting to minimize contamination from EVs released by differentiated progeny. Despite maintaining strict timing, early differentiation of HSPCs before the 48-h time point remains possible, and therefore, the collected EVs may not be exclusively derived from undifferentiated HSPCs.

Potential inaccuracies in EV size measurements

The qNano Gold TRPS Measurement System (Izon Science, NZ) uses the Tunable Resistive Pulse Sensing (TRPS) technology for nanoparticle analysis, detecting and characterizing single particles

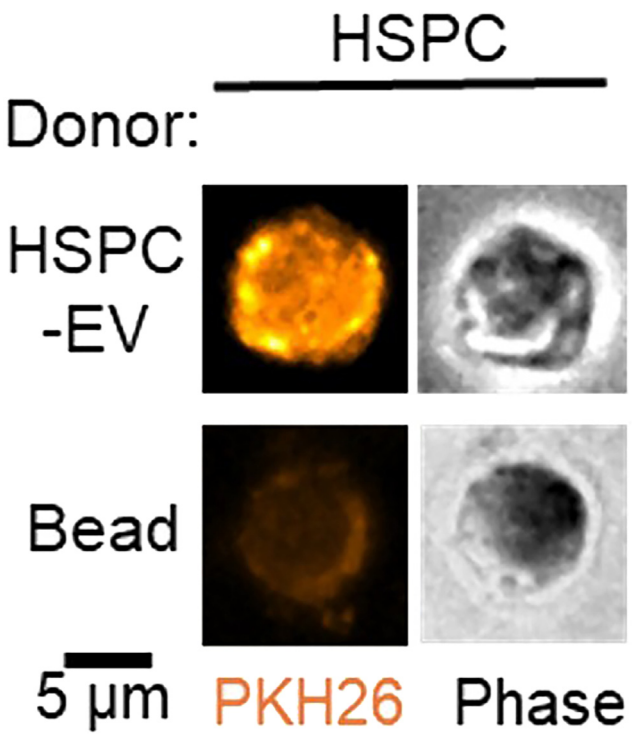

Figure 5. EVs marked with PKH26 label

Treated HPSCs with HPSC-derived EVs previously marked with PKH26 dye (upper part) and control with beads (lower part). Images adapted from Bonora et al., 2024.[1]

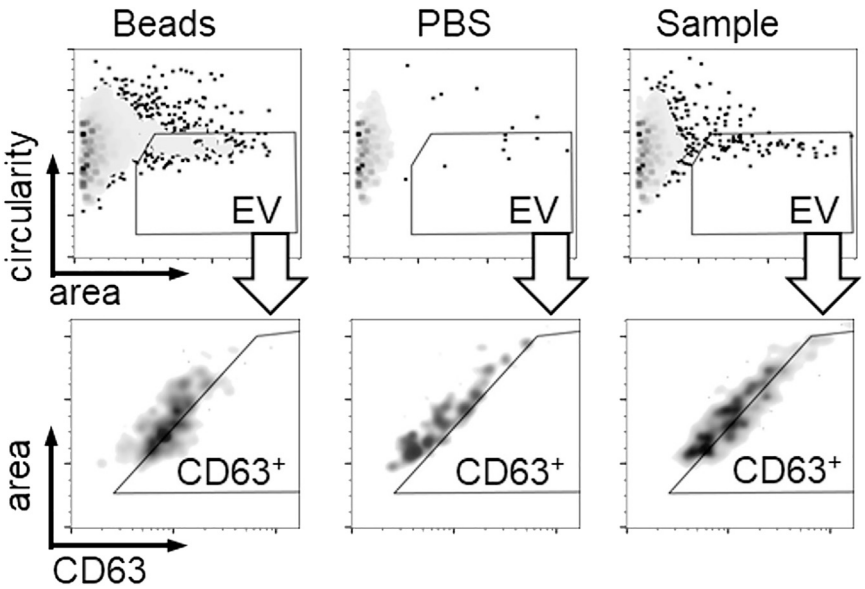

Figure 6. Representative eSRRF graph to analysis EVs markers

Area, circularity and positivity for the CD63 marker in HPSC-derived EVs using eSRRF technique compared to beads and PBS as positive and negative controls, respectively; Images adapted from Bonora et al., 2024.[1]

passing through a nanopore. However, potential aggregation of particles may result in inaccurate size measurements of EVs, as the system may read two aggregated EVs as a single larger one.

TROUBLESHOOTING

Problem 1

Blockages during the measurement of HSPC-derived EVs.

The qNano Gold TRPS Measurement System (Izon Science, NZ), the NanoSight NS300 Instrument (Malvern Panalytical, UK) and the Nanoanalyzer (nanoFCM Co., Ltd, China) are used for HSPC-derived EVs characterization utilize distinct technologies - a nanopore system, a flow chamber system and a proper microfluidic system, respectively. A problem that can be caused by the structure of these systems in the formation of clogs made by sample aggregation.

Potential solution

To ensure optimal performance and prevent blockages in their components, sterile filtered PBS must be used. Additionally, these instruments have specific operating parameters recommended by their manufacturers, and serial dilution of the HSPC-derived EVs samples may be necessary to maintain accurate measurements.

Problem 2

PEG could create aggregates since its polymeric nature, this intrinsic feature can provide biased characterization of EVs after PEG isolation method.

Potential solution

Due to the low amount of EVs isolated from HSPC cells, using PEG at 8% might be excessive. To optimize PEG concentration, a titration of PEG based on the initial amount of cells could be performed to better adjust the polymer quantity needed for EV collection without disturbing the subsequent characterization.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Keisuke Ito (keisuke.ito@einsteinmed.edu).

Technical contact

Further information and requests for technical information should be directed to and will be fulfilled by the technical contact, Federica Zanotti (federica.zanotti@einsteinmed.edu).

Materials availability

This study did not generate new unique materials or reagents.

Data and code availability

This study did not generate new databases or code.

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AUTHOR CONTRIBUTIONS

F.Z., I.Z., M.B., and C.M. conceived and optimized the protocol. F.Z. and I.Z. wrote the original draft. M.B., C.M., B.Z., L.F., P.P., and K.I. contributed by reviewing, editing, and supervising the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Bonora, M., Morganti, C., van Gastel, N., Ito, K., Calura, E., Zanolla, I., Ferroni, L., Zhang, Y., Jung, Y., Sales, G., et al. (2024). A mitochondrial NADPH-cholesterol axis regulates extracellular vesicle biogenesis to support hematopoietic stem cell fate. Cell Stem Cell *31*, 359–377.e10. https://doi.org/10.1016/j.stem.2024.02.004.

2. Laurenzana, I., Trino, S., Lamorte, D., Girasole, M., Dinarelli, S., De Stradis, A., Grieco, V., Maietti, M., Traficante, A., Statuto, T., et al. (2021). Analysis of Amount, Size, Protein Phenotype and Molecular Content of Circulating Extracellular Vesicles Identifies New Biomarkers in Multiple Myeloma. Int. J. Nanomedicine *16*, 3141–3160. https://doi.org/10.2147/IJN.S303391.

3. Morrison, S.J., and Scadden, D.T. (2014). The bone marrow niche for haematopoietic stem cells. Nature *505*, 327–334. https://doi.org/10.1038/nature12984.

4. Goldberg, L.R. (2021). Extracellular Vesicles and Hematopoietic Stem Cell Aging. Arterioscler. Thromb. Vasc. Biol. *41*, e399–e416. https://doi.org/10.1161/ATVBAHA.120.314643.

5. Morganti, C., Bonora, M., and Ito, K. (2024). Metabolism and HSC fate: what NADPH is made for. Trends Cell Biol. https://doi.org/10.1016/j.tcb.2024.07.003.

6. Caivano, A., Laurenzana, I., De Luca, L., La Rocca, F., Simeon, V., Trino, S., D'Auria, F., Traficante, A., Maietti, M., Izzo, T., et al. (2015). High serum levels of extracellular vesicles expressing malignancy-related markers are released in patients with various types of hematological neoplastic disorders. Tumour Biol. *36*, 9739–9752. https://doi.org/10.1007/s13277-015-3741-3.

7. Kalluri, R., and LeBleu, V.S. (2020). The biology, function, and biomedical applications of exosomes. Science (New York, N.Y.) *367*, eaau6977. https://doi.org/10.1126/science.aau6977.

8. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682. https://doi.org/10.1038/nmeth.2019.

9. Gustafsson, N., Culley, S., Ashdown, G., Owen, D.M., Pereira, P.M., and Henriques, R. (2016). Fast live-cell conventional fluorophore nanoscopy with ImageJ through super-resolution radial fluctuations. Nat. Commun. *7*, 12471. https://doi.org/10.1038/ncomms12471.

10. Morganti, C., Bonora, M., and Ito, K. (2019). Improving the Accuracy of Flow Cytometric Assessment of Mitochondrial Membrane Potential in Hematopoietic Stem and Progenitor Cells Through the Inhibition of Efflux Pumps. J. Vis. Exp. e60057. https://doi.org/10.3791/60057.

11. Liu, R.F., Li, J.Q., Hou, R.X., Wang, R., and Zhang, K.M. (2015). Impact of BMMSCs from different sources on proliferation of $CD34^+$ cells. Genet. Mol. Res. *14*, 474–482. https://doi.org/10.4238/2015.January.23.22.

12. Rider, M.A., Hurwitz, S.N., and Meckes, D.G., Jr. (2016). ExtraPEG: A Polyethylene Glycol-Based Method for Enrichment of Extracellular Vesicles. Sci. Rep. *6*, 23978. https://doi.org/10.1038/srep23978.

13. Kowkabany, G., and Bao, Y. (2024). Nanoparticle Tracking Analysis: An Effective Tool to Characterize Extracellular Vesicles. Molecules *29*, 4672. https://doi.org/10.3390/molecules29194672.

14. Laine, R.F., Heil, H.S., Coelho, S., Nixon-Abell, J., Jimenez, A., Wiesner, T., Martínez, D., Galgani, T., Régnier, L., Stubb, A., et al. (2023). High-fidelity 3D live-cell nanoscopy through data-driven enhanced super-resolution radial fluctuation. Nat. Methods *20*, 1949–1956. https://doi.org/10.1038/s41592-023-02057-w.

15. Tian, Y., Gong, M., Hu, Y., Liu, H., Zhang, W., Zhang, M., Hu, X., Aubert, D., Zhu, S., Wu, L., and Yan, X. (2020). Quality and efficiency assessment of six extracellular vesicle isolation methods by nano-flow cytometry. J. Extracell. Vesicles *9*, 1697028. https://doi.org/10.1080/20013078.2019.1697028.