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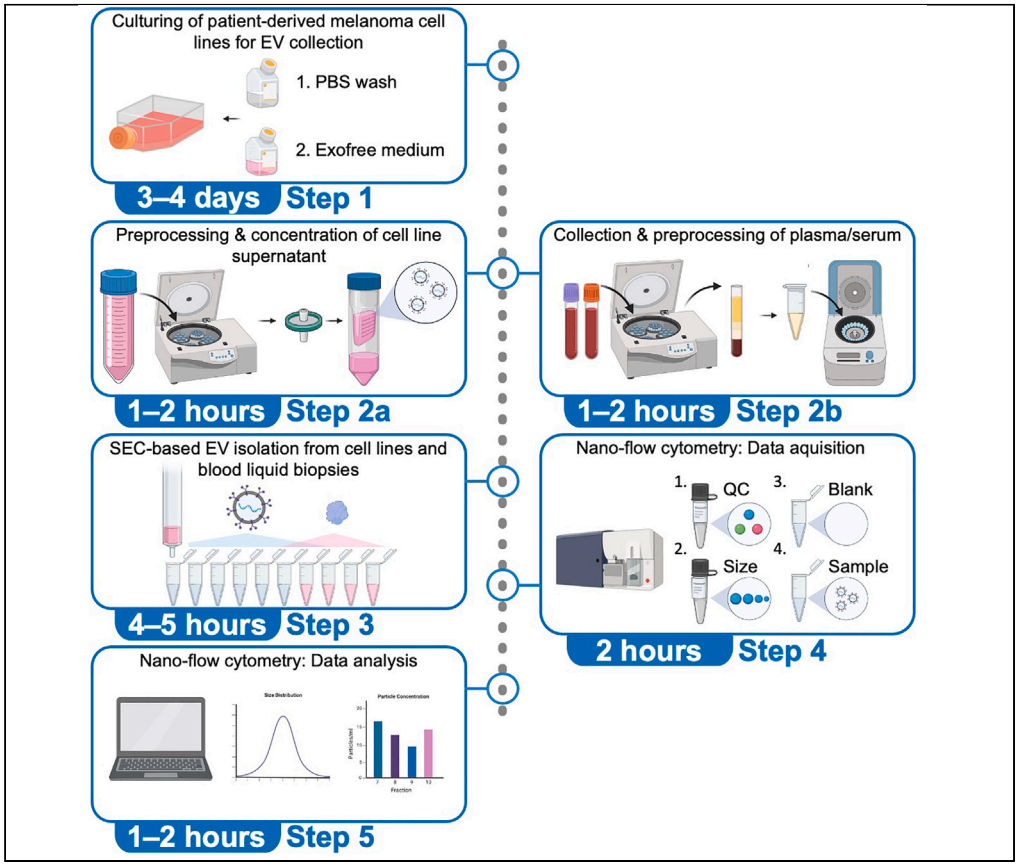
Isolation and detection of extracellular vesicles from melanoma cells and liquid biopsies using size-exclusion chromatography and nano-flow cytometry



Characterization of extracellular vesicles (EVs) holds great promise for biomarker discovery and understanding of diseases, including melanoma, the deadliest skin cancer type. Here, we describe a size-exclusion chromatography method to isolate and concentrate EVs from patient material including (1) patient-derived melanoma cell line supernatants and (2) plasma and serum biopsies. Additionally, we provide a protocol to analyze EVs by nano-flow cytometry. EV suspensions obtained with the presented protocol can be used for several downstream analyses including RNA sequencing and proteomics.

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

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Highlights

Size-based EV isolation by size-exclusion chromatography

EV collection from melanoma-patient-derived cell culture supernatants and blood

Single-particle analysis by nano-flow cytometry

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Isolation and detection of extracellular vesicles from melanoma cells and liquid biopsies using size-exclusion chromatography and nano-flow cytometry

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SUMMARY

Characterization of extracellular vesicles (EVs) holds great promise for biomarker discovery and understanding of diseases, including melanoma, the deadliest skin cancer type. Here, we describe a size-exclusion chromatography method to isolate and concentrate EVs from patient material including (1) patient-derived melanoma cell line supernatants and (2) plasma and serum biopsies. Additionally, we provide a protocol to analyze EVs by nano-flow cytometry. EV suspensions obtained with the presented protocol can be used for several downstream analyses including RNA sequencing and proteomics.

BEFORE YOU BEGIN

EVs are released by almost all cell types and serve as important regulators of cellular homeostasis and intercellular communication.[1] Their function is attributed to the molecular cargo they traffic, such as nucleic acids, proteins, lipids, and metabolites.[2–4] Several studies show involvement of EVs in melanoma biology, including the formation of the metastatic niche or the interaction with the immune system.[5–8] To better understand the function of EVs in oncogenic processes and to explore their potential as (melanoma) biomarkers, the isolation of EVs from (patient-derived) cell lines and blood biopsies is critical. SEC has been proven as a suitable method for EV isolation and has been successfully combined with data-independent acquisition mass spectrometry (DIA-MS) to profile proteins from blood-derived EVs of melanoma patients.

Institutional permissions

The presented study is compliant with all ethical standards involving human participants and is conducted under the ethical approval BASEC Nr. 2017-00494, BASEC Nr. 2014-0425 and BASEC.Nr.2018-00194. Before starting make sure to conform to regulatory standards and acquire the required permissions for working with human cell lines and liquid biopsies from the relevant institutions.

Preparation of cell culture media

1. Prepare cell culture medium for maintenance (a) and EV collection (b). Both media can be prepared ahead of time and kept at 4°C for several weeks.
   a. Cell culture medium for maintenance: Supplement RPMI-1640 medium or any other appropriate cell culture medium with 1% sodium pyruvate (1 mM), 2% L-glutamine (4 mM) and 10% of regular, heat-inactivated fetal bovine serum (FBS).

b. Cell culture medium for EV collection: Supplement RPMI-1640 medium or any other appropriate cell culture medium with 1% sodium pyruvate (1 mM), 2% L-glutamine (4 mM) and 10% of exosome-depleted fetal bovine serum (FBS)

⚠ CRITICAL: Make sure to use exosome-depleted FBS (see key resources table) to avoid contaminations with bovine exosomes in the collected cell supernatant.

2. Sterile filter the supplemented media with a polyethersulfone (PES) membrane filter (0.22 μm) in a clean bench.

⚠ CRITICAL: Since the cell culture media do not contain antibiotics this step is recommended.

General laboratory preparation

3. Prewarm the "cell culture medium for maintenance" and "cell culture medium for EV collection" at 37°C.
4. Cool down the centrifuge (Heraeus Multifuge) and microcentrifuge to 4°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human: melanoma patient-derived cell line M130429	Raaijmakers et al.[9]	N/A
Human: melanoma patient-derived cell line M130219	Raaijmakers et al.[9]	N/A
Human plasma	Blood donation center (CH-8952 Schlieren)	N/A
Human serum	Blood donation center (CH-8952 Schlieren)	N/A
Chemicals, peptides, and recombinant proteins		
RPMI-1640	Sigma-Aldrich, USA	R0883
Heat-inactivated fetal bovine serum (FBS)	Biowest, France	S1810
Exosome-depleted FBS	Gibco, USA	A2720803
L-glutamine (200 mM)	Gibco, USA	25030-24
Sodium pyruvate (100 mM)	Sigma-Aldrich, USA	S8636
Phosphate-buffered saline (PBS)	Gibco, USA	14190144
Dulbecco's phosphate-buffered saline (DPBS)	Gibco, USA	14190144
Sodium hydroxide (NaOH) pellets, 40 g/mol	Merck, Germany	1310-73-2
PlasmoTest™	InvivoGen	rep-pt1
Experimental models: Cell lines		
Human: melanoma cell line M130429	Biobank USZ	N/A
Human: melanoma cell line M130219	Biobank USZ	N/A
Software and algorithms		
NF Profession Version 1.15/1.17	NanoFCM Co. Ltd., China	N/A
Other		
150 cm^2 flask	TPP, Switzerland	90151
15 mL/50 mL conical tubes	TPP, Switzerland	91015/91050
1.5 mL centrifuge tubes	Eppendorf, Switzerland	0030120086
Vacuum Filtration 0.2 μm PES membrane filter	TPP, Switzerland	99500
Millex-HP filter 0.45 μm	Merck Millipore, USA	SLHP033RS
Amicon Ultra-15 filter units	Merck Millipore, USA	SLHP033RS
Plasma collection tubes	BD Biosciences	367525
Serum collection tubes	BD Biosciences	367896
Wide-bore (wide orifice) pipette tips	Starlab, UK	E1011-8618/9618

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
IZON qEV Original / 70 nm Legacy Column	IZON, New Zealand	SP1110150
0.6 mL MaxyClear Snaplock microcentrifuge tube	Corning, USA	MCT-060-C
NanoFCM Quality Control Nanospheres (QC beads), 250 ± 5 nm, 2E+10 particles/mL	NanoFCM Co. Ltd., China	QS2503
Silica Nanospheres Cocktail (size beads), 68–155 nm, 100×	NanoFCM Co. Ltd., China	S16M-Exo
Nano FCM Cleaning Solution, 50×	NanoFCM Co. Ltd., China	17159
NanoFCM Flow NanoAnalyzer	NanoFCM Co. Ltd., China	N/A
Heraeus Multifuge 3S-R refrigerated centrifuge	Thermo Scientific	75004371
Sorvall Heraeus swinging bucket centrifuge rotor	Thermo Scientific	75006445
Microcentrifuge	Eppendorf	5424 R
Microcentrifuge rotor	Eppendorf	5424 R

MATERIALS AND EQUIPMENT

Cell culture medium for maintenance

Reagent	Final concentration	Amount
RPMI-1640 medium	N/A	435 mL[a]
Sodium pyruvate (100 mM)	1 mM (1%)	5 mL
L-glutamine (200 mM)	4 mM (2%)	10 mL
Regular, heat-inactivated FBS	10%	50 mL
Total	**N/A**	**500 mL**

[a]There is no absolute requirement to remove 65 mL from the purchased 500 mL RPMI-1640 medium bottle. However, if the whole bottle is used for medium preparation, this should be done consistently for all cell lines and conditions.

Cell culture medium for EV collection

Reagent	Final concentration	Amount
RPMI-1640 medium	N/A	435 mL[a]
Sodium pyruvate (100 mM)	1 mM (1%)	5 mL
L-glutamine (200 mM)	4 mM (2%)	10 mL
Exosome-depleted FBS	10%	50 mL
Total	**N/A**	**500 mL**

[a]There is no absolute requirement to remove 65 mL from the purchased 500 mL RPMI-1640 medium bottle. However, if the whole bottle is used for medium preparation, this should be done consistently for all cell lines and conditions.

Step 1–2 of the protocol rely on the usage of centrifuges for successful conduction. We used the following models for this purpose (however analogous models work as well).

- A Heraeus Multifuge 3S-R refrigerated centrifuge with a Sorvall Heraeus swinging bucket centrifuge rotor was used for processing of larger volumes in 15 mL–50 mL conical tubes (i.e., for the concentration of cell culture supernatants).
- An Eppendorf microcentrifuge was used for processing smaller volumes (100 µL–1500 µL) in 1.5 mL centrifuge tubes.

Step 3 of the protocol depends on suitable columns for performing SEC. We routinely use the following columns.

- IZON qEV Original / 70 nm Legacy Column

Alternatives: SEC columns can also be prepared manually for example using Sepharose resins.[10]

Step 4–5 of this protocol require the usage of the following specialized equipment.

- Following SEC a NanoFCM Flow NanoAnalyzer device was used for EV characterization such as particle number and size measurements. Obtained data was analyzed with the NanoFCM software NF Profession Version 1.15.

Alternatives: There are other methods available for basic EV characterization similar to nano-flow cytometry. For example, particle number and size can also be determined by nano-particle tracking analysis (NTA)[11] or tunable resistive pulse sensing (TRPS) measurements.[12]

STEP-BY-STEP METHOD DETAILS

This part of the protocol describes the collection and processing of patient-derived melanoma cell line supernatants (conditioned media) as well as blood liquid biopsies (plasma or serum). It is followed by a detailed procedure for EV isolation from the above-mentioned samples. The last part of the protocol outlines EV characterization by nano-flow cytometry, which allows us to determine particle number and size. The resulting isolated EVs can be used for further downstream processing such as immunostainings, TEM-based visualization, RNA sequencing or proteomic profiling.

Culturing of patient-derived melanoma cell lines for EV collection (step 1)

Timing: 3–4 days

1. Prior to EV collection from patient-derived melanoma cell lines, test the cells for mycoplasma contamination. For this protocol cells were tested with PlasmoTest™ (see key resources table), however there are several other methods available to test for mycoplasma.[13]
2. Culture the melanoma cells in a 150 cm^2 flask (see key resources table) in 25–30 mL of pre-warmed "cell culture medium for maintenance" in a cell incubator (37°C, 5% CO_2) (One 150 cm^2 cell culture flask typically yields particles in the range of 10^9 particles/mL).
3. Once cells have reached 50%–70% confluence, wash the cells with standard phosphate-buffered saline (PBS) to remove traces of FBS originating from the cell culture media:
   a. Aspirate cell culture medium.
   b. Add 10 mL PBS and slightly sway the flask.
   c. Immediately remove PBS.
   d. Repeat steps **a.–c.** two more times.

Note: The confluency depends on the proliferation rate of cell lines. For slow growing cells aim for confluency of 70%.

⚠ CRITICAL: It is important to wash off the "cell culture medium for maintenance" to avoid carry-over of exosomes contained in the FBS

4. Add 25–30 mL of "cell culture medium for EV collection" and grow the cell in a cell incubator for 48–72 h.

⚠ CRITICAL: Avoid cell confluency above 80% to avoid contamination with EVs from apoptotic cells. Furthermore, maintain the same incubation time for EV collection across experiments to ensure reproducibility.

5. Collect the cell supernatant (containing the secreted EVs) by pipetting it into a 50 mL conical tube. At this point, the cells can be discarded or further cultured for other experiments.

Note: The collected supernatant contains the EVs that were secreted by the melanoma cells into the "cell culture medium for EV collection". Further processing steps will be required for cleanup and concentration before isolation of melanoma-derived EVs by SEC.

Preprocessing and concentration of cell line supernatant (step 2a)

Timing: 1–2 h

6. Cool down centrifuge (Heraeus Multifuge) to 4°C prior to processing and concentration of cell culture supernatants.
7. Centrifuge the 50 mL conical tubes containing c.a. 25–30 mL of cell culture supernatant (500 *g*, 5 min, 4°C) to remove cellular debris.
8. Carefully pipet the cell culture supernatant (containing the secreted EVs) into a new 50 mL conical tube without touching the pellet.

⚠ CRITICAL: Work as fast as possible to avoid the cell pellet to dissociate. You may leave a minimal amount of supernatant above the pellet to avoid disturbing the pellet.

9. Centrifuge the collected cell culture supernatant (3000 *g*, 20 min, 4°C) to remove larger microvesicles (MVs) and enrich for smaller MVs and exosomes.[14]
10. Carefully pipet the cell culture supernatant into a new 50 mL conical tube without touching the pellet.
11. Filter the collected melanoma cell culture supernatant through a 0.45 μm PES filter using a syringe.

Note: The cell culture supernatant is now ready for concentration.

12. For concentration of cell culture supernatants use Amicon Ultra-15 filter units with a cut-off of 100 kDa. Prior to concentration, wash the Amicon Ultra-15 filter units with PBS:
    a. Add 15 mL of PBS in the Amicon Ultra-15 filter unit and centrifuge until the PBS has passed through the filter into the collection tube (4000 *g*, 3 min, 4°C).

⚠ CRITICAL: It is essential to accurately balance Amicon Ultra-15 filter units in the centrifuge. An unbalanced rotor may cause damage to the surroundings and/or the rotor of the centrifuge.

    b. After centrifugation remove the Amicon Ultra-filter unit from the collection tube, decant the PBS, and immediately proceed to the concentration of the cell supernatant.
13. Put the Amicon Ultra filter unit back onto the same collection tube and pipet 15 mL of processed cell culture supernatant onto the filter.
14. Centrifuge (4000 *g*, 6 min, 4°C) until the cell culture supernatant has flown through and 500 μL of concentrated melanoma cell line supernatant remain in the filter unit.

Note: The centrifugation time depends on the viscosity of the cell culture supernatant and the position of the tube in the centrifuge, but typically ranges between 6–10 min (see troubleshooting problem 1).

15. Discard the flow-through by decanting.
16. Repeat step 13–16 with the rest of the cell culture supernatant to obtain a final volume of 500 μL of concentrated cell line supernatant.
17. Transfer 500 μL of the concentrated melanoma cell line supernatant into a 1.5 mL centrifuge tube.

Note: The concentrated melanoma cell line supernatant is now ready for EV isolation by SEC.

Pause point: Concentrated melanoma supernatants can be stored at −80°C until EV isolation by SEC.

Collection and preprocessing of plasma or serum (step 2b)

Timing: 1–2 h

Note: To reduce shear forces on the sample use wide-bore (wide orifice) pipette tips. This is not required, but recommended, when gentle handling of the sample is desired.

18. Centrifuge blood collected in plasma and serum collection tubes immediately upon blood draw (1600 *g*, 10 min, 22°C). In our biobank blood is routinely collected in 10 mL plasma or serum collection tubes (see key resources table). Since one EV isolation only requires 250 μL of blood, excess plasma or serum can be stored at −80°C after preprocessing.

⚠ CRITICAL: Temperature and time till processing can have a profound impact on EV biology. To ensure reproducibility, process blood samples immediately after blood draw. Furthermore, the different coagulation agents inside collection tubes influence EV production.[15] When planning your study, inform yourself on the best option for your experiment.

19. Transfer the plasma or serum supernatants into a fresh 1.5 mL centrifuge tube.

⚠ CRITICAL: Avoid touching the buffy coat layer

20. Perform a second centrifugation step (3000 *g*, 10 min, 22°C) and transfer the plasma or serum into a fresh 1.5 mL centrifuge tube. A second centrifugation step is recommended by the International Society on Thrombosis and Haemostasis (ISTH) to remove platelets.[16]

Pause point: At this step plasma or serum samples can be frozen at −80°C. However, it is recommended to reduce freeze-thaw cycles to a minimum, as they affect EV structure and stability.

21. To prepare for the processing of the blood liquid biopsies cool a microcentrifuge to 4°C.
22. If plasma and serum were frozen, thaw the samples on ice for 30 min.
23. Pipet 250 μL of the plasma or the serum into a 1.5 mL centrifuge tube. It is possible that the plasma or serum is still cloudy after preprocessing, in that case see troubleshooting problem 2.
24. Centrifuge plasma or serum supernatants in a microcentrifuge (1500 *g*, 10 min, 4°C).
25. Transfer the supernatant to a fresh 1.5 mL centrifuge tube without touching the pellet.
26. Centrifuge plasma or serum into a microcentrifuge (10,000 *g*, 30 min, 4°C).

Note: Centrifugation at 10,000 *g* will remove larger MVs. Omit steps 26–27 if heterogenous EV populations (e.g., containing larger MVs) are desired.[17]

27. Transfer the supernatant into a fresh 1.5 mL centrifuge tube without touching the pellet.

Note: The plasma and serum samples are now ready for EV isolation.

SEC-based EV isolation from cell lines and blood liquid biopsies (step 3)

Timing: 4–5 h

SEC isolates EVs based on their size by filtering them through a porous resin. Here, we use a resin with a pore size of 70 nm that allows proteins and particle below that cut-off to enter the pores, which reduces their flow rates. In contrast, EVs are excluded from the pores and elute in earlier fractions. Typically, EVs are enriched in fraction 7–10 (see Expected Outcome).

Note: When working with a new (melanoma) cell line, determine which fractions are enriched in EVs. For a given cell line and condition, SEC is highly reproducible, thus fractions can be collected in pools, once the elution range of EVs has been determined.

28. If IZON columns are stored at 4°C, take out a column the night before EV isolation and put it in a Styrofoam box to allow for slow equilibration to the operational temperature range of 18°C–24°C.
29. Before you begin, sterile filter DPBS through a 0.2 μm membrane filter and degas the buffer with a vacuum pump (or alternatively a vacuum chamber) for at least 1 h at room temperature.

Note: Gases that are dissolve in the buffer can cause bubble formation at nucleation sites in the porous matrix, which can negatively impact separation by SEC.

30. Prepare an appropriate column holder/rack and align pre-labelled 1 mL/5.0 mL collection tubes in the order of elution.
31. Remove the top column cap.

⚠ CRITICAL: To ensure consistent elution profiles, do not remove the caps before the column has reached operational temperature.

32. Attach the IZON qEV original (70 nm) column to column holder, insert the buffer reservoir on top of the column and prepare a container for the collection of DPBS washes.
33. Remove the bottom column cap and allow the buffer to run through the column matrix. Let it flow into the collection container.
34. As soon as the last drop has run into the filter, add 20 mL (i.e., fill the entire buffer reservoir) of degassed DPBS on top of the column and let the buffer flow into the collection container.

⚠ CRITICAL: It is important to properly wash columns with 20 mL of degassed DPBS as they contain sodium azide as a bacteriostatic agent. Any remaining sodium azide may destroy eluted EVs (see troubleshooting problem 3).

35. Add the sample:
    a. Cell culture supernatants: Carefully pipet 500 μL processed and concentrated cell culture supernatant onto the column and let the void volume flow into a 1.5 mL centrifuge tube below the column.
    or
    b. Plasma or serum samples: Carefully pipet 200 μL of processed plasma or serum onto the column. As soon as the sample has run into the frit, add 300 μL of degassed DPBS and let the void volume flow into a 1.5 mL centrifuge tube below the column.

Note: Make sure the sample is at room temperature before loading it onto the column.

36. Wait until the entire sample volume has been absorbed by the column, then add another 500 μl of degassed buffer onto the column and continue to collect the void volume into a fresh 1.5 mL centrifuge tube (Figure 1A).
37. Repeat step 36 collecting the eluting 500 μL fractions in separate 1.5 mL centrifuge tubes (Figure 1B).

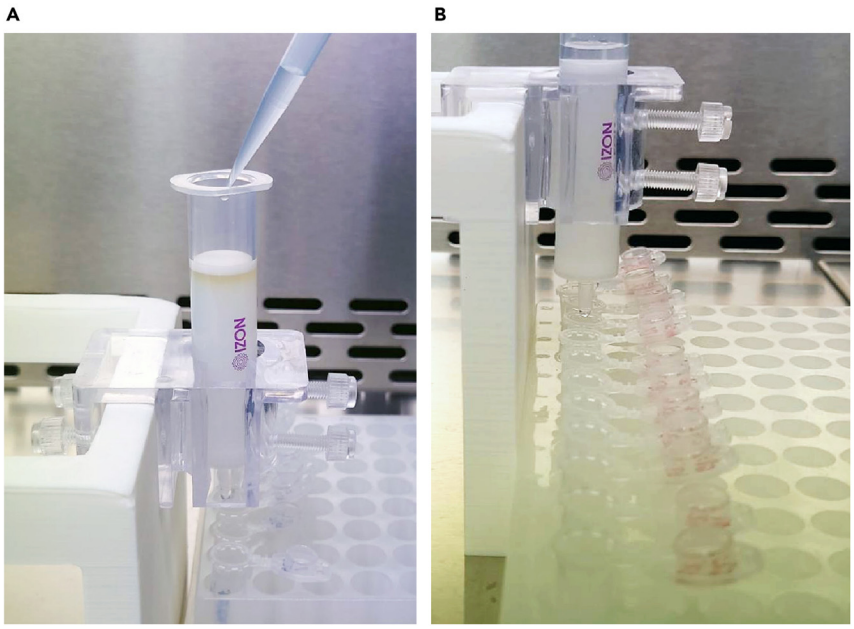

Figure 1. SEC-based isolation of EVs from patient-derived melanoma cell line supernatants

Note: See left and right columns of Table 1 for guidance on the expected fraction content.

However, it is recommended to determine the EV-containing fractions (by nano-flow cytometry, nanoparticle tracking analysis (NTA) or similar methods) for your specific experimental condition, as elution profiles may vary depending on the sample type and experimental setup.

38. When running more than one sample on a column wash with at least 20 mL of DPBS between samples.
39. Cleaning step: When required rinse the column with 5 mL of 0.5 M NaOH and wash with 30–40 mL of DPBS (measure PH of elution to check whether the wash was sufficient). IZON qEV original Columns can be used up to five times.
40. Pool the EV-containing (e.g., fractions 7–10) for further downstream processing.

Note: If EV-containing fractions are known, they can already be pooled during acquisition (Table 1). When working with a new (melanoma) cell line determine particle concentration and size of the different fractions (see "Nano-flow cytometry") to identify fractions enriched in EVs.

41. Store obtained EV-containing fractions at 4°C, when used for downstream analysis (including nanoflow cytometry) within the next 24 h or freeze fractions at −80°C for longer-term storage.

⚠ CRITICAL: During the entire EV isolation procedure it is important to never let the column run dry as this will negatively impact the SEC performance.

Nano-flow cytometry: Data acquisition (step 4)

Timing: 2 h

This part of the protocol describes the quantification of single-particle concentration and size. The NanoFCM Flow NanoAnalyzer (NanoFCM Co. Ltd., China) allows quantification of particles at the

Table 1. Overview of SEC fraction volumes and content

Fractions	Volume	M130429/M130219 cells
Fraction 1–6	3 mL	Void volume
Fraction 7–10	2–3 mL	EV containing fractions
Fraction 11–32	11 mL	Free protein containing fractions

nanoscale between 40–1000 nm. EVs are quantified by means of a highly sensitive side-scatter channel.

Note: In addition, the NanoFCM has two in-built lasers that allow fluorescent-based detection of particles. Thus, EVs may be stained with antibodies of interest prior to data acquisition on the NanoFCM.

42. Prepare control samples, standards and cleaning samples:
    a. Prepare NanoFCM Quality Control Nanospheres (QC bead) solution as a concentration standard and for initial system alignment: Add 1 μL QC beads in 99 μL deionized water (1:100 dilution) in a 0.6 mL MaxyClear Snaplock microcentrifuge tube.
    b. Prepare Silica Nanospheres Cocktail (size bead) solution for size calibration (68 nm, 91 nm, 113 nm, 155 nm). Add 1 μL in 99 μL deionized water (1:100 dilution) in a 0.6 mL MaxyClear Snaplock microcentrifuge tube.
    c. Prepare the cleaning solution for washing of the sample line: Add 150 μL Nano FCM cleaning solution in a 0.6 mL MaxyClear Snaplock microcentrifuge tube.
    d. Prepare a negative control a control for background correction: Add 100 μL of DPBS in a 0.6 mL MaxyClear Snaplock microcentrifuge tube.

Note: Keep the samples on ice when not acquiring

43. Thaw the experiment samples (single fractions or pooled EV fractions (e.g., fractions 7–10)) on ice while performing the setup of the nanoFCM.
44. Start-up and initialize the fluidics of the NanoFCM machinery.

⚠ CRITICAL: Make sure to use 0.6 mL MaxyClear Snaplock microcentrifuge tubes that are compatible with nanoFCM acquisition.

45. Once the fluidics are initialized, switch on the single-photon counting modules (SPCM) detector.
46. Load the MaxyClear Snaplock microcentrifuge tube with the QC bead solution into the machine and boost the QC bead solution for 45 s (see Methods video S1).

⚠ CRITICAL: To ensure proper pressurization each sample needs to be boosted for 45 s prior to data acquisition for 1 min.

47. Align the lasers until optimal signal intensities/events for the side scatter are achieved:
    a. Optimal value for side scatter intensity: 1000–1500.
    b. Optimal number of events: ≥ 30.

⚠ CRITICAL: During optimal alignment ensure that intensity histograms represent a uniform and narrow peak. Otherwise, manually adjust horizontal and vertical position of the optical lenses or optimize laser focusing lens alignment turning the knobs of the lasers.

48. Once aligned, switch to "auto-sampling" to keep pressure stable throughout use. The auto-sampling pressure range should be between 0.4–1.5 kPa.
49. Acquire QC beads for 1 min and save the data as NFA file.

⚠ CRITICAL: Once a sample is acquired on the NanoFCM must be saved manually as an NFA file prior to boosting the next sample, as otherwise the data is lost.

50. Unload the MaxyClear Snaplock microcentrifuge tube with the QC bead solution.
51. Load the MaxyClear Snaplock microcentrifuge tube with the cleaning solution and boost the cleaning solution for 45 s.
52. Remove the MaxyClear Snaplock microcentrifuge tube with the cleaning solution from the inlet.
53. Briefly dip the inlet in a MaxyClear Snaplock microcentrifuge tube with deionized water to clean the sample line.

⚠ CRITICAL: Removal of remaining cleaning solution from the inlet is important to avoid EV lysis in the following samples.

54. Load the MaxyClear Snaplock microcentrifuge tube with the size bead solution and boost for 45 s.
55. Acquire the size bead solution for 1 min and save the data as NFA file.
56. Unload the MaxyClear Snaplock microcentrifuge tube with the size bead solution.
57. Repeat the washing steps (51–53).
58. Load the MaxyClear Snaplock microcentrifuge tube with filtered DPBS and boost for 45 s.
59. Acquire PBS for 1 min and save the data as NFA file.
60. Unload the MaxyClear Snaplock microcentrifuge tube with PBS.
61. Repeat the washing steps (51–53).

Note: The NanoFCM is now ready for data acquisition of your samples.

62. Load the MaxyClear Snaplock microcentrifuge tube with the sample and boost for 45 s.
63. Acquire the sample for 1 min and save the data as NFA file.

⚠ CRITICAL: event should not exceed 12000 events/min otherwise swarming effects occur. You may need to dilute your sample with DPBS to not exceed this limit (see troubleshooting problem 4).

64. Unload the MaxyClear Snaplock microcentrifuge tube with the sample.
65. Repeat the washing steps (51–53) before measuring the next sample.

Note: if particle concentration is too low this could mean that your sample is too diluted or that the sample line has been clogged (for advice on the latter see troubleshooting problem 5).

66. After the last sample: Load again the MaxyClear Snaplock microcentrifuge tube with the QC bead solution and boost for 45 s.
67. Acquire the QC bead solution for 1 min and save the data as NFA file.

Note: Check that the recording of the QC bead solution is comparable to the initial acquisition.

68. When done, shut down NanoFCM.

Nano-flow cytometry: Data analysis (step 5)

Timing: 1–2 h

This part of the protocol describes the analysis of particle and size measurements by nano-flow cytometry. Data analysis is performed with the *NanoFCM software NF Profession version 1.15/1.17* (see Methods video S2).

69. Load the data to be analyzed into the NanoFCM NF Profession software by opening the entire folder.

Note: All files to be analyzed should be located in the same folder.

⚠ CRITICAL: The name of the folder with data files should not include any dots, as otherwise the file will not be opened.

70. Open the file with the acquired QC beads.
71. Automatically set the side scatter threshold for the QC beads by clicking on "**AUTO**" (Auto-threshold).
72. Click on "**Count STD**" to set the standard.

Note: the QC beads serve as the concentration standard for acquired particles.

73. Open the file with the acquired size beads.
74. Automatically set the threshold by clicking on "**AUTO**" (Auto-threshold).

Note: If required, manually alter the baseline to reduce for background signals for each channel using the "Signal Processing Control" tab. Usually the side scatter channel intensity is set to 35 when blank level of the side scatter channel intensity is ~70.

75. To generate a size calibration curve, open the calibration panel "**Size MESF**" and choose the S16M-exo standard to fit the curve. Click on "find peak".
76. Open the file with the acquired DPBS sample.
77. Automatically set the threshold for DPBS by clicking on "**AUTO**" (Auto-threshold).

Note: The DPBS sample serves as the blank control and is used to subtract unspecific background.

78. Click on "**Count Blank**" to set the background and save the DPBS background (size distribution window "**B**") to the settings.
79. Open the file with sample data and subtract the saved DPBS background from the sample with blank correction (size distribution window "**BC**").
80. The quantified data is now present as a size and concentration report.
81. There are different options for file export:
    a. Export the file as PDF report, which includes information about sample size distribution (nm) and the particle concentration (particles/mL).
        i. Make sure to adjust the concentration of the beads by the concentration of the QC beads you used by clicking into the window. Enter your sample dilution to get the final particle concentration displayed.
    b. Save the files as FCS files, which allows you to import the files to alternative programs such as FlowJo for subsequent analysis.

⚠ CRITICAL: The FCS files are exported with the threshold that has been applied to the data. Data points below the threshold will be lost.

⚠ CRITICAL: Samples including blank control must be acquired with the same instrument settings and thresholds set during analysis.

EXPECTED OUTCOMES

Here, we offer a protocol that describes the isolation and single-particle characterization of EVs from patient-derived melanoma cells and blood liquid biopsies. Applying the described SEC

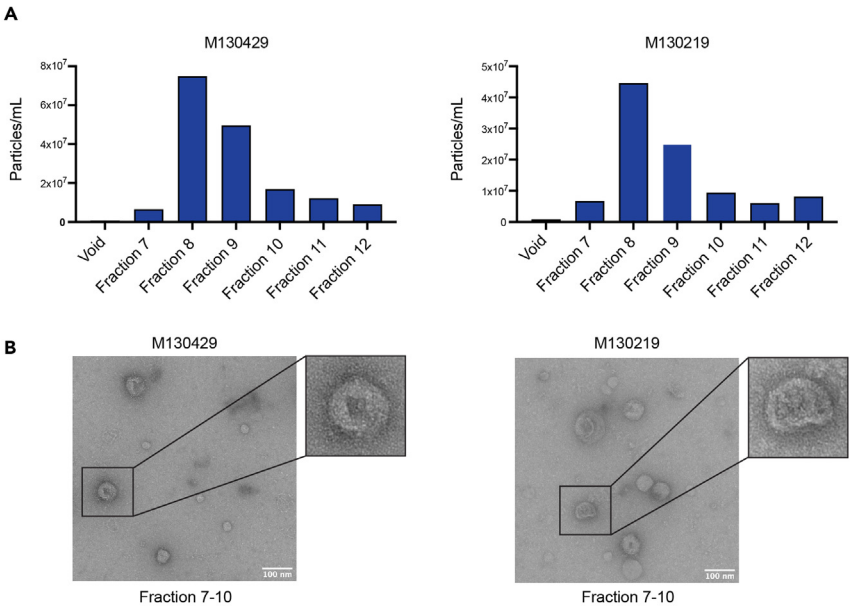

Figure 2. Single fraction particle analysis and TEM-based characterization of EVs isolated from 500 μL of cell line supernatant

(A) Bar plots representing the particle concentration (particles/mL) quantified by nano-flow cytometry of single fractions isolated by SEC from 500 μL of cell line supernatant of M130429 and M130219 melanoma cells. Fraction representing the void volume and fractions 7, 8, 9, 10, 11, 12 are shown. EV-containing fraction 7–10 are used for further downstream analysis.

(B) Transmission electron microscopy (TEM) images of EVs of pooled fractions 7–10 of M130429 and M130219 melanoma cells. Black boxes represent a close-up of a single exosome. Scale bars = 100 nm.

isolation and nano-flow cytometry analysis yields SEC fractions that are enriched in EVs. For melanoma cells these are typically fractions 7–10 (Figure 2A). Transmission electron microscopy (TEM) analysis of the pooled EV fractions reveals the characteristic cup-shaped form of exosomes in melanocytic (M130429) and mesenchymal (M130219) melanoma cells derived from the same patient (Figure 2B). Typically, the particle size of pooled EV-containing fractions should range from 50 – 200 nm, which is the usual distribution of exosomes. The measured size distribution of the pooled-EV containing fractions (fractions 7–10) of M130429 and a M130219 melanoma cells are shown (Figures 3A and 3B). Additionally, the expected particle concentration of the pooled EV fractions accounts to approximately 1×10^9 particles/mL from an original input of 30 mL of medium (Figure 3B) and 4×10^8–6×10^9 particles/mL from an input of 200 μL blood. Additionally, exosome markers such as CD9, CD63 and CD81 are expected to be detectable on the surface of the EVs[1] (Figure 3C).

LIMITATIONS

Isolation of EVs by SEC is very gentle on the sample and yields preparations of highly pure EVs that maintain their native structure. However, there are some limitations compared to other EV isolation techniques such as differential ultracentrifugation (UC) and asymmetrical flow field-flow fractionation (A4F) in terms of sample heterogeneity, dilution and time requirements, which are further detailed in the following paragraphs.

Sample heterogeneity

Since SEC separates EVs based on size, EV-containing fractions may contain different EV subpopulations. Although exosome markers such as the tetraspanins CD63, CD81 and CD9 indicate the presence of exosomes these markers are heterogeneously present on single vesicles.[18–20] In addition, SEC may co-purify smaller microvesicles, which have an overlapping size range with exosomes.

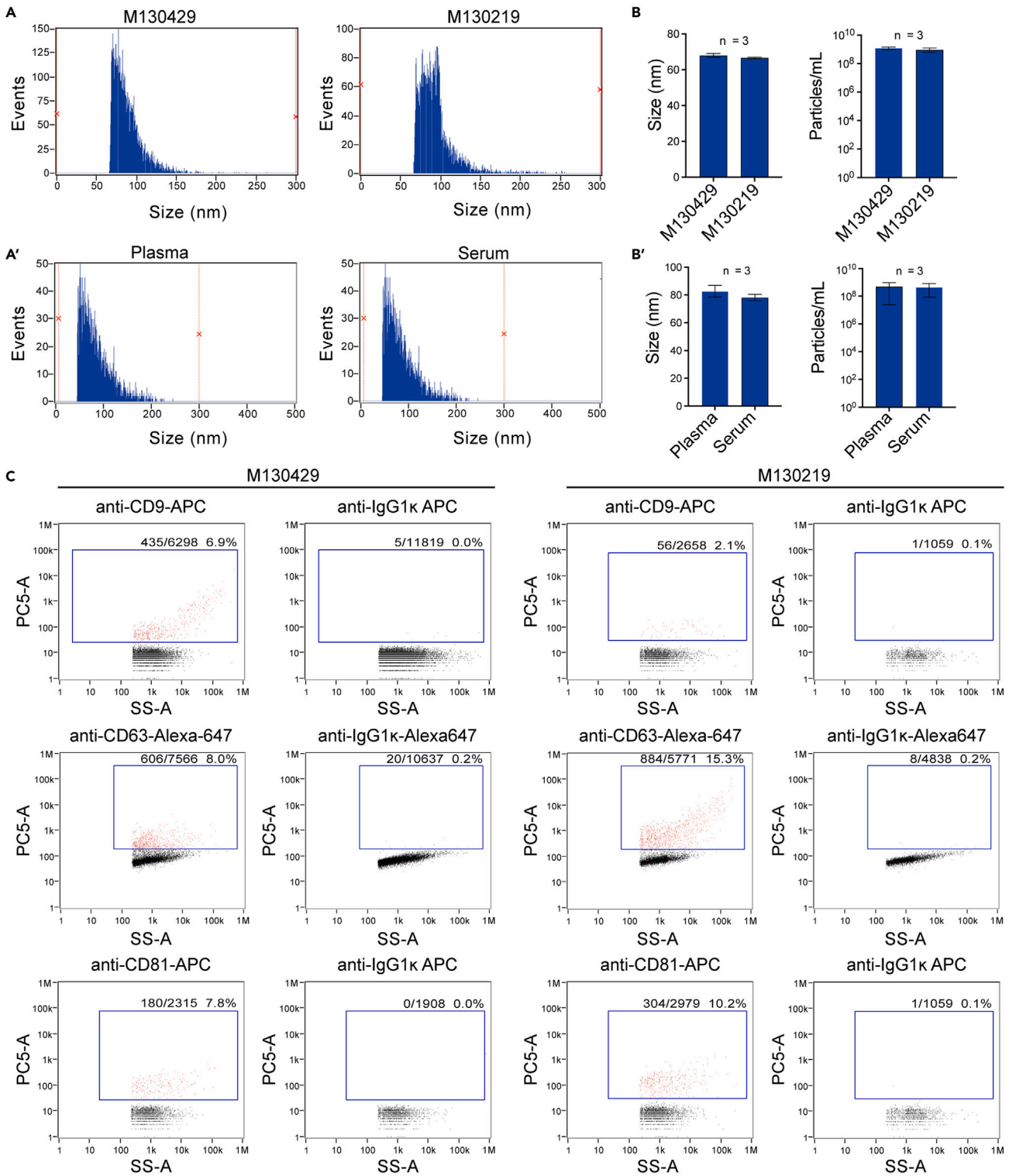

Figure 3. Nano-flow cytometry-based characterization of EVs isolated from 500 μL of patient-derived melanoma cell line supernatant and 200 μL melanoma blood using SEC

(A and A′) Size profiles and mean particle size (nm) and particle concentration (particles/mL) of isolated EVs acquired by nano-flow cytometry. Size profiles are derived from one representative EV sample of M130429 and M130219 melanoma cells (A) and of plasma or serum biopsies (A′), respectively.

Figure 3. *Continued*
(B and B′) Mean particle size and particle concentration of M130429 and M130219 samples (B) and plasma or serum samples (B′) are represented as mean ± SD of three independent biological replicates (n = 3).
(C) Representative dot plots of antibody labeling against exosome-specific tetraspanins CD63, CD9 and CD81 on EVs derived from M130429 and M130219 melanoma cells. EVs were stained for 30 min at 4°C in the dark and purified by SEC to remove unbound antibody prior to data acquisition. Antibodies anti-CD63-Alexa-647, anti-CD9-APC and anti-CD81-APC were acquired in the PC5 channel. For each sample the appropriate isotype control (IgG1κ-Alexa-467 and IgG1κ-APC) was acquired.

Therefore, combining SEC with orthogonal methods such as affinity purification is needed for in-depth study of EV subpopulations. Alternatively, the A4F technology allows for more granular separation of EVs.

Sample dilution

Using SEC, EVs are eluted using DPBS, which results in dilution of the initial sample input, which is a clear disadvantage compared to UC. To overcome this limitation eluted Fraction 7–10, known to contain EVs of interest, may be further concentrated to enrich for intact EVs and reduce sample volume to an appropriate volume for downstream processing, such as immunoblotting, RNA-sequencing or proteomic profiling.

Time requirement for SEC

If several samples have to be processed, SEC is time-consuming. In comparison to UC, where multiple samples may be processed at the same time, SEC samples must be processed sequentially.

TROUBLESHOOTING

Problem 1

When concentrating cell culture supernatants in Amicon Ultra-15 filter units, the centrifugation time depends on the viscosity of the cell culture supernatant and it is thus possible that the concentration accidentally overshoots the desired 500 µL volume required as SEC input.

Potential solution

If that occurs, simply add the missing volume (difference to a total of 500 µL) by pipetting fresh "Cell culture medium for EV collection" onto the Amicon Ultra-15 filter unit, because it is recommended to use the same input volume (i.e., 500 µL) for SEC across all samples to avoid differences in the elution profiles.

Problem 2

Despite the double centrifugation the plasma or serum are still cloudy, or such plasma or serum has been obtained by the biobank.

Potential solution

In such cases it is recommended to add an additional centrifugation step to remove some of these impurities. Centrifuge the sample in a microcentrifuge (500 *g*, 5 min, 4°C) and transfer the supernatant to a fresh 1.5 mL centrifuge tube without touching the pellet.

Problem 3

A low yield of EVs following SEC may arise from insufficient washing before sample loading.

Potential solution

To prevent low yield of EVs following SEC, the column should be thoroughly washed with at least 20 mL of DPBS prior to loading the first sample.

Problem 4

Swarming effects of particles acquired by the nano-flow cytometer occurs when particle concentration is too high. Consequently, individual events can no longer be captured as single particles resulting in erroneous data interpretation.

Potential solution

Acquired events/minute by the nano-flow cytometer should not exceed 12000 events. If the captured event number exceeds 2000 events in the first 10 s of data acquisition, we recommend diluting the sample in DPBS starting with a small dilution such as 1:5 or 1:10.

Problem 5

Cleaning of the sample line by boosting the cleaning solution for 45 s following each sample acquisition is essential. Despite this washing step, the sheath flow can get disturbed due to overloading of the system with too many particles or contaminants. If the sheath flow is disturbed due to a clogged sample line, particles may no longer be detected.

Potential solution

Once the sample line is clogged, the cleaning solution is not sufficient to clear the sheath flow. In such situations, we suggest to load a MaxyClear Snaplock microcentrifuge tube with 1 M NaOH and boost the sample for 10 min with the sheath flow closed. Once you have verified that particles are detected again normally, make sure to re-align the lasers anew with QC beads, before continuing sample acquisition.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mitchell P Levesque (Mitchell.Levesque@usz.ch).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze any dataset or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102365.

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AUTHOR CONTRIBUTIONS

E.L. and V.L. conducted the experiments, analyzed the data, and wrote the manuscript. E.L. and M.P.L. designed and supervised the study. All authors revised the manuscript.

DECLARATION OF INTERESTS

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