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A simple, high-throughput method of protein and label removal from extracellular vesicle samples†

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Evidence continues to increase of the clinical utility extracellular vesicles (EVs) as translational biomarkers. While a wide variety of EV isolation and purification methods have been implemented, few techniques are high-throughput and scalable for removing excess fluorescent reagents (*e.g.* dyes, antibodies). EVs are too small to be recovered from routine cell-processing procedures, such as filtration or centrifugation. The lack of suitable methods for removing unbound labels, especially in optical assays, is a major roadblock to accurate EV phenotyping and utilization of EV assays in a translational or clinical setting. Therefore, we developed a method for using a multi-modal resin, referred to as EV-Clean, to remove unbound labels from EV samples, and we demonstrate improvement in flow cytometric EV analysis with the use of this EV-Clean method.

Introduction

Exosomes and ectosomes are small lipid packages released from cells, here referred to under the umbrella term of extracellular vesicles (EVs).[1,2] The majority of EVs have been demonstrated to be ≤100 nm in diameter, with a Power-law distribution ranging from ~25–>1000 nm.[3–5] EVs hold prospect as clinical biomarkers due to their surface and luminal cargo, hypothesized to offer a retrospective snapshot of their parent cell upon their release. Due to their small surface area, the majority of EVs typically express a very low number of copies of any one protein. Current estimates using high-sensitivity, calibrated measurements suggest the majority of EVs express ≤10 protein copies of a protein.[5,6] This small size and limit cargo make isolation, purification, and detection of EVs challenging.

In the most recently reported ISEV survey, which included 196 participants from 30 countries, the most reported EV isolation methods included: ultracentrifugation, density gradient, filtration, size-exclusion chromatography, precipitation, and magnetic bead capture.[7,8] Despite, a wide variety of techniques being utilized to date for EV isolation, a gold-standard, or general consensus, is yet to emerge.[9] One of the main drawbacks of the current methodologies is their lack of high-throughput compatibility, with many techniques being labor intensive and time consuming.[4,10–12] Isolation procedures implemented are also dependent on factors such as, the type of medium *e.g.* plasma, cell culture, the volume of medium *e.g.* μL to L, the downstream analysis technique *e.g.* single-particle methods or bulk methods, and the scale of isolation *e.g.* a couple of samples to hundreds of samples.

A wide variety of detection methods have been utilized for characterizing single EVs.[5,6,13–18] A common analysis technique of interest for translation studies is EV flow cytometry (EV-FC).[6,19] EV-FC has been utilized in a number of forms with some commercially available flow cytometers capable of detecting single-fluorescent molecules.[20] This kind of sensitivity is required to detect limited surface epitope expression due to surface area on the smallest EVs.[21] With instrumentation capable of detecting single-fluorescent molecule, it has become critical that residual or unbound fluorophore is removed from samples prior to analysis. The removal of residual or unbound fluorophore is also a highly rec-

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ommended step for conventional EV-FC with lower sensitivity instrumentation as a means to increase the signal to noise ratio, and remove artefactual populations.[22–24] For this reason, the MIFlowCyt-EV reporting framework; published as a position paper to help standardize reporting of single EV flow cytometry experiments, has specific fields to demonstrate labels are not contributing or being included in EV analysis.[25] Other techniques relying on fluorescence reagents, particularly for membrane labeling, such as microscopy also require wash steps.[5,26]

Currently, there is a gap in EV isolation and purification methods for removal of residual or unbound fluorescent labels that can be applied in a high-throughput format to small volumes, which would retain EV yield without drastically reducing sample concentration, Table 1. While it is possible to titrate antibodies and fluorescent dyes to EVs for high-throughput clinical sample analysis, where samples may be in limited supply, it is neither fast, practical, or cost-effective. Here, we demonstrate an EV-Clean methodology (Fig. 1) as a simple, high-throughput method of EV purification from residual proteins and unbound fluorescent-antibodies, which can be used with μL volumes, with a limited reduction in concentration and does not fractionate EVs into several samples, that is associated with widely used size exclusion methods, and does not require specialized hardware to implement, Table 1. The EV-Clean methodology utilizes a multi-modal resin, Capto Core (GE Biosciences), which has primarily been utilized in a column format and developed for virus purification from biofluids.[27–30]

Table 1 Comparison of previously demonstrated label removed methods for EV-analysis for flow cytometry

	Ultracentrifugation	SEC (qEV columns)	EV-clean
High throughput	–	–	+
Small volume (<50 μL)	+	–	+
Dilution required	–	+	–
Manually intensive	+	+	–
Time intensive	+	+	–
Specialized equipment	+	–	–

Materials & methods

Blood collection & ethics

All experiments were performed in accordance with the Guidelines of the National Canacer Institute. Informed consents were obtained from human participants of this study. Experiments were approved by the NIH ethics committee (IRB-approved NIH intramural protocol number 02-C-0064). A blood samples were obtained using EDTA collection tubes. All analyses were performed in a deidentified manner, with IRB-approved NIH intramural protocol number 02-C-0064. Plasma samples were depleted of cells and platelets by two centrifugation steps at 2500*g* at room temperature in a swing-out bucket rotor for 15 minutes with the supernatant isolated. Platelet-poor plasma samples were then stored in low-protein binding tubes (Thermo Fisher Scientific, Waltham, USA) at −80 °C. Samples were thawed at 37 °C for 10 minutes before being used in downstream experiments.

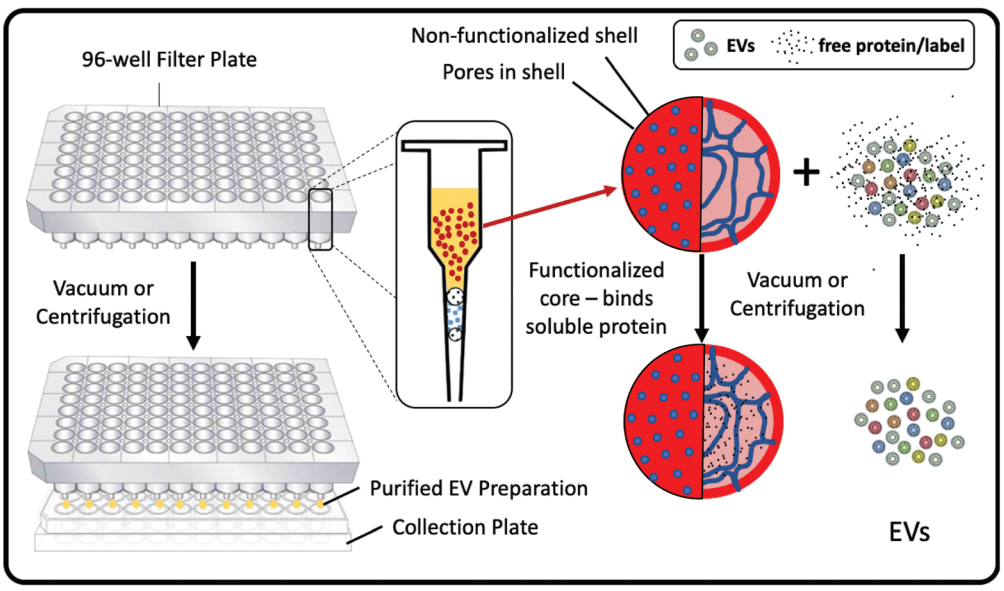

Fig. 1 The proposed utilization of EV-Clean methodology as a high-throughput, scalable, robotic compatible isolation method for EVs from protein and labels. Capto Core 700 packed 96-well filter plates are able to have an EV sample requiring soluble protein or label removal can be added to the top of the well. The resin and sample are mixed and left to incubate. Upon completion of incubation the resin will have intercalating soluble protein/label and the flow through can be collected for downstream application.

Cell culture

The immature dendritic cell line DC2.4 was kindly provided by Kenneth Rock (University of Massachusetts Medical School, Boston, MA) and cultured in phenol red-free RPMI1640 medium supplemented with 10% FBS, 1% L-glutamine, 1% penicillin–streptomycin and 0.1% β-mercaptoethanol (ThermoFisher). For EV-depleted medium preparation, 20% FBS containing RPMI was ultracentrifuged for 18 hours at 100 000*g* at 4 °C in a 45Ti fixed angle rotor using polycarbonate tubes (both from Beckman Coulter). After ultracentrifugation, the top 50 mL of medium suspension were harvested, filtered with 0.2 μm PES filter bottles and stored at 4 °C. Before using for culture, RPMI and L-glutamine, Penicillin–streptomycin and β-mercaptoethanol were added, to achieve the concentrations before mentioned above. To produce DC2.4-derived EVs, cells were cultured for 2–3 days in EV-depleted medium and supernatants harvested before confluence was reached. Supernatants were first depleted of cells, debris and apoptotic bodies by centrifuging at 2500 g for 15 minutes twice. Supernatants were added to 100 kDa Pall Jumbosep concentrators until 5 mL of the harvested supernatant remained. 250 μL of DPBS was added to 250 μL of concentrated EVs. This 500 μL mixture was then loaded onto a qEV Original column with fractions 6–12 collected separately. Each were analyzed using Nanosight and run on an SDS-PAGE gel to confirm prescience of vesicles, before fractions 8 and 9 were combined for downstream experiments.

BSA removal using Capto Core 700

Samples containing approximately 400 μg of BSA diluted in 75 μL DPBS were aliquoted into PCR tubes containing 100 μL of DPBS-washed CaptoCore 700 (GE Biosciences) Samples were incubated at 4 °C for 30 minutes before the top 75 μL of supernatant was then removed and added to another 100 μL of DPBS-washed EV-Clean, mixed, and incubated for a further 30 minutes at 4 °C.

Plasma protein removal using Capto Core 700

15, 10, and 5 μL of platelet-poor plasma was aliquoted into PCR tubes containing 100 μL of DPBS-washed Capto Core 700. Samples were incubated at 4 °C for 30 minutes before the top 15, 10, or 5 μL of supernatant was then removed and added to another 100 μL of DPBS-washed EV-Clean, mixed, and incubated for a further 30 minutes at 4 °C.

EV CFSE-labeling

CFSE-labelling of DC2.4 EVs was carried out as described previously.[31] Briefly, 15 μL of 1×10^9 DC2.4 EVs, pooled from qEV original columns fraction 8 and 9, were added to 15 μL of 40 μM of CFDA-SE (Thermo Fisher Scientific). This was protected from light and incubated at 37 °C for 2 hours. 70 μL of DPBS was added to the stained sample. This was repeated for each sample. Excess dye removal using size-exclusion chromatography used NAP-5 columns, loading 100 μL of CFSE-stained EVs and collecting fractions 3 and 4 for purified EVs, having a final volume of 500 μL. For dye removal using Capto Core 700, 100 μL of CFSE-stained EVs were added to 100 μL of DPBS-washed EV-Clean in a PCR tube, mixed, and incubated at 4 °C for 30 minutes. The top 100 μL of supernatant was then removed and added to another 100 μL of DPBS-washed Capto Core 700, mixed, and incubated for a further 30 minutes at 4 °C. Post-dye removal all samples were transferred to 1.5 mL low protein binding tubes (Thermo Fisher Scientific) with 1×10^9 200 nm Red FluoSpheres (Thermo Fisher Scientific) added, before being diluted to a final volume of 1 mL for analysis by nanoFACS.

BSA measurements

BSA concentration was measured using a NanoDrop 2000 Spectrometer (Thermo Fisher Scientific, USA). Prior to recording concentration using the NanoDrop, the sensor was rinsed with deionized water and dried with a cotton bud before a baseline reading was taken using DPBS. 2 μL of sample was then placed on the sensor and a concentration reading was recorded three times. Recordings were exported to xml files. Data was plotted using Prism (v8.0.1, GraphPad Software, San Diego, USA). Recordings with negative readings were assumed to be 0 μg mL^{-1}.

SDS page

A 10% tris/glycine/SDS Buffer solution was prepared with 100 mL buffer (Bio-Rad) in 900 mL tissue culture grade water. 10 μL of Bio-Rad Precision Plus ladder were added to Bio-Rad Mini-PROTEAN TGX gels (10- or 15-well). For the neat plasma samples, 15, 10, or 5 μL of plasma was added to 8.25 μL 4× Laemmli Sample Buffer (Bio-Rad, 161-0747) and 0.75 μL 55 mM 2-mercaptoethanol (Gibco, 21985). The entirety (24, 19, or 14 μL) of the sample was added to the wells of the gel. For the samples that had been previously incubated with EV-Clean, 15, 10, or 5 μL of purified sample was added to 8.25 μL 4× Laemmli Sample Buffer and 0.75 μL 55 mM 2-mercaptoethanol. The entirety (24, 19, or 14 μL) of the mixture was added to the wells of the gel. Antibody removal was tested by suspending 0.5 μg of IgG-PE-CD147 (BioLegend, Cat. 306212) and IgG-APC-CD147 (BioLegend, Cat. 306214) in a final volume of 50 μL adding to 100 μL of EV-Clean for 30 minutes. Post-incubation 20 μL of supernatant was added to 8.25 μL 4× Laemmli Sample Buffer (Bio-Rad, 161-0747), 0.75 μL 55 mM 2-mercaptoethanol (Gibco, 21985), and 20 μL of SDS buffer. The final volume of 50 μL was added to each well of a 10 well Bio-Rad Mini-PROTEAN TGX gel.

With a Mini-PROTEAN Tetra System connected to a Bio-Rad Power Pac 1000, SDS-PAGE was run at a constant voltage of 200 V. The stain-free gel was activated and imaged under the Bio-Rad ChemiDoc Touch Imaging System. Gels were analyzed using Image Lab software (v6.0.1, Bio-Rad, Hercules, USA).

Nanoparticle tracking analysis (NTA)

Particle concentration and diameter distribution were characterized by NTA with a NanoSight LM10 instrument (Malvern, UK), equipped with a 405 nm LM12 module and EMCCD

camera (DL-658-OEM-630, Andor). Video acquisition was performed with NTA software v3.2, using a camera level of 14. Three 30 second videos were captured per sample. Post-acquisition video analysis used the following settings: minimum track length = 5, detection threshold = 4, automatic blur size = 2-pass, maximum jump size = 12.0. Exported datasets were compiled and plotted using scripts written in MATLAB v9.3.0 (The MathWorks Inc., USA). Samples were diluted to have a concentration in the region of 1×10^8 to 1×10^9 particles per mL.

Flow cytometry of EVs

Flow cytometric analysis of CFSE EVs was carried out using previously published NanoFACS methodology.[31] Briefly, an Astrios EQ jet-in-air system (Beckman Coulter) was used, configured with 5 lasers (355, 405, 488, 561 and 640 nm wavelength), where SSC can be detected and used as a trigger at laser wavelength except for the 355 nm laser. EV analyses were carried out using a 561-SSC trigger with the 561-SSC voltage and threshold settings adjusted to allow ~10 000 events of background reference noise per second. Samples were loaded and run for 5 minutes until the event rate was stable, and then recorded for 30 seconds. All samples were run at a 0.2 psi differential pressure, monitoring stability closely. Data was acquired using Summit v6 (Beckman Coulter) and analyzed with FlowJo v10.1r5 (TreeStar, USA). CFSE fluorescence data was calibrated using FITC MESF calibration beads using FCM_{PASS} software (v.3.03).[32,33] Full calibration details can be found in the MIFlowCyt-EV report, ESI 1.†[25] Flow cytometric analysis of EV recovery was carried out using a Cytek Aurora (Cytek Biosciences), configured with 4 lasers (405, 488, 561, 640 nm) with a custom modified 405 nm detector. Diameter was calculated for EVs using FCM_{PASS} software (v3.03).[32] Light scatter parameters were calibrated into full calibration details can be found in the MIFlowCyt-EV report, ESI 2.†[25]

Results

Optimal incubation times for protein removal were tested using bovine serum albumin (BSA). These results showed that 30 minutes and 1-hour incubation had minor differences, Fig. 2. After a single incubation with EV-Clean the BSA content dropped from ~4000 μg mL^{-1} to ~700 and ~600 μg mL^{-1}, respectively, Fig. 2. An increase in incubation time to a single 4-hour incubation showed a decrease from ~4000 μg mL^{-1} to no detectable BSA. To determine whether a second, sequential incubation could speed up this process, the use of an additional 30-minute incubation was tested after each of the first incubation times. After the second incubation no detectable BSA was observed after any of the preceding incubation times. We therefore conclude that two 30-minute incubations are sufficient and ergonomic as an incubation period to use for soluble protein removal from EV-containing samples.

The ability of EV-Clean methodology to remove the large amount of soluble protein from a heterogeneous solution was tested using plasma samples with the effects of purification

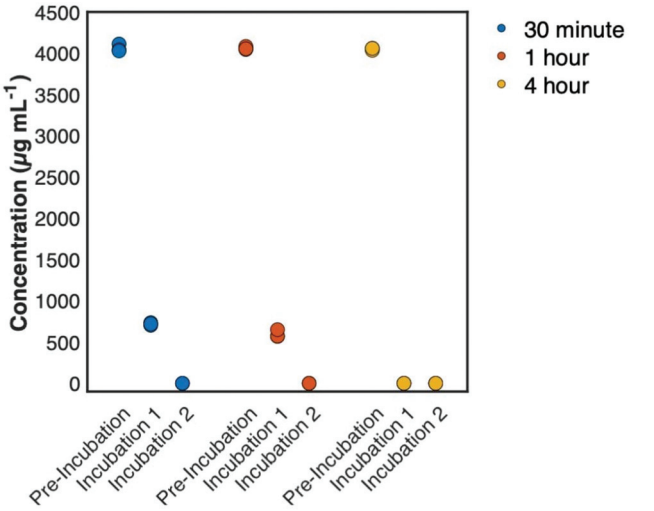

Fig. 2 BSA removal using EV-Clean. The removal of 75 μL purified bovine serum album (BSA) using an initial incubation with 100 μL Capto Core 700 for either 30 minutes, 1 hour, or 4 hours followed by a 30-minute incubation with Capto Core 700.

observed by SDS-PAGE, Fig. 3. 15 μL of plasma after a single 30-minute incubation with Capto Core 700 shows a significantly reduced signal when compared to neat plasma. This reduction in protein content shows no observable bias in protein size with all observable protein ≲300 kDa showing depletion. A second incubation again further depletes all observable protein with only faint bands visible at ~13, 50, 65, 185, 311 kDa. This was repeated for 10 μL and 5 μL of plasma. With 5 μL of plasma bands are only faint bands were only

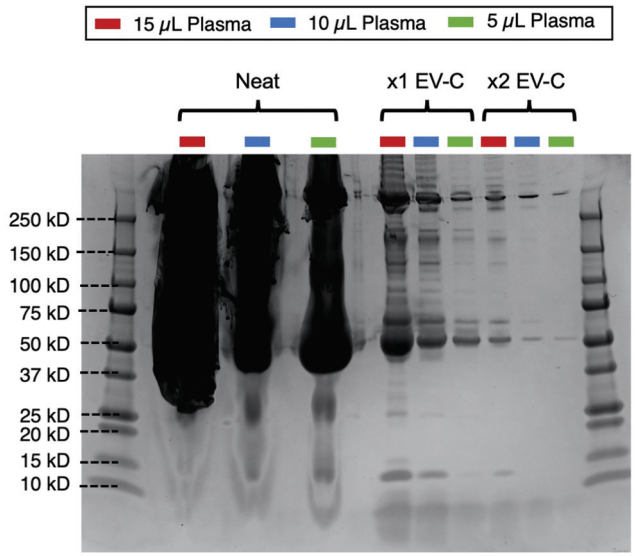

Fig. 3 Protein removal from plasma. The ability of the EV Clean (EV-C) methodology using Capto Core 700 to remove protein from plasma was tested using 15 μL (red), 10 μL (blue), and 5 μL (green) of neat platelet-depleted plasma with Capto Core 700 for one incubation and two incubations for 30 minutes.

visible at ~50 and 311 kDa indicating the majority of small proteins can be depleted from plasma with 100 μL of EV-Clean and two 30-minute incubations.

The EV-Clean methodology was next tested for its ability to remove fluorescently-conjugated IgG antibodies. R-Phycoerythrin (R-PE) and allophycocyanin (APC) were chosen due to their large size, 250 and 105 kDa, respectively. Both R-PE-IgG1 and APC-IgG1 were significantly depleted when incubated with Capto Core 700, Fig. 4A. Along with antibodies, molecular labels are used for staining EVs. The ability of Capto Core 700 to remove excess CFSE from stained EV samples compared to a previously published method using size exclusion chromatography (SEC) using nanoFACS. The instrument reference noise, measured with PBS, had a median brightness of 48–49 fluorescein (FITC) molecules of equivalent soluble fluorophore (MESF) units. EVs without the removal of excess CFSE label resulted in the cytometer noise being raised to a median brightness of 437 FITC MESF units, Fig. 4B. By removing the excess CFSE, the noise level remained low with the SEC and EV-Clean purification methods having a median brightness of 58 and 54 FITC MESF units, respectively, Fig. 4C and D.

Finally, the effect of EV-Clean metholodology on the detectable EV concentration after removing unbound label was evaluated, Fig. 5. Recovery was assessed by gating single EVs between 115 to 200 nm, Fig. 5A. After one incubation the detectable concentration was 75%, with a subsequent reduction after a second incubation to 51%. In summary, the use of EV-Clean demonstrates a 75% EV concentration after each incubation and >95% label removal, without dilution, for each incubation.

Discussion

We have demonstrated that EV-Clean methodology is able to significantly deplete soluble proteins from heterogeneous samples such as plasma in a form-factor that is compatible with 96-well plates and robotics. This methodology requires up to just two 30-minute incubations to achieve substantial protein-depletion. Furthermore, we have demonstrated that the detectable EV concentration using flow cytometry is ~75% using EV-clean, making it a useful tool for removing soluble labels and other non-EV molecules from heterogenous samples and biofluids. Due EV-clean protein removal being a multi-mode process of intercalation and affinity capture, it is possible that EV recovery is higher in samples containing more proteins than the size-exclusion purified samples that were tested. We have also demonstrated the use of the EV-Clean methodology for EV-labelling is beneficial for high-sensitivity analysis techniques, such as flow cytometry, where the removal of excess label can reduce sensitivity and several samples need to be prepared simultaneously. While this proposed method offers potential for high-throughput purification applications with small samples, it may be less suitable for large volume applications such as tissue culture supernatants.

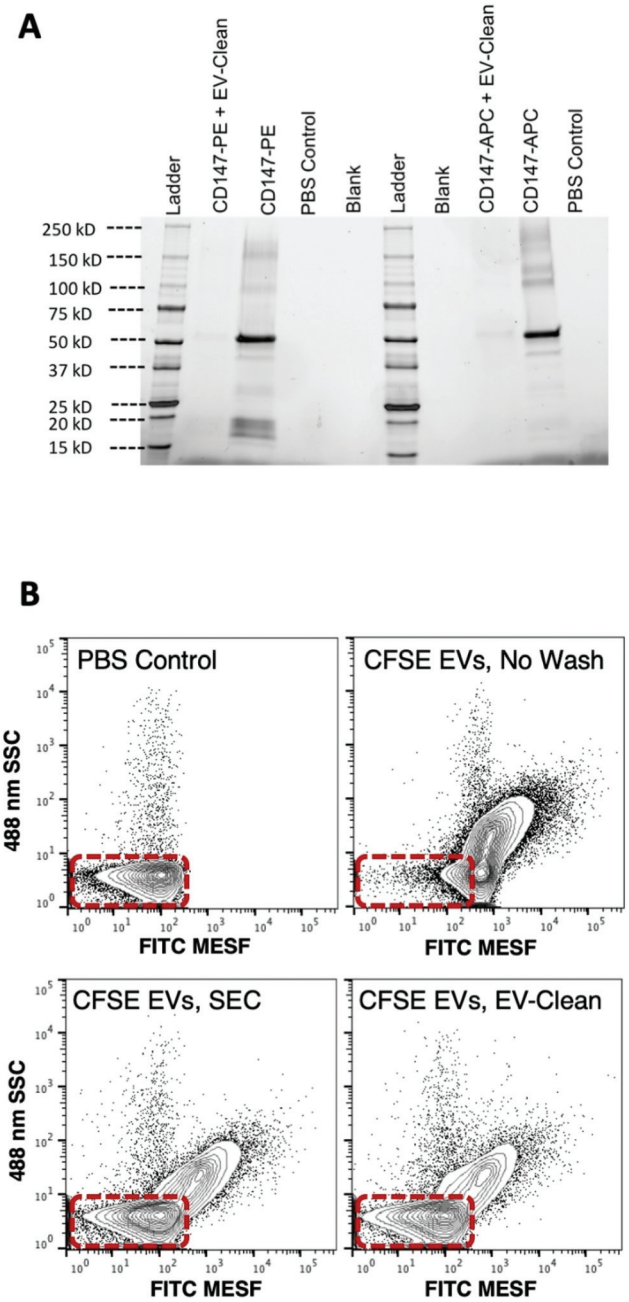

Fig. 4 Fluorescent label removal. The ability of EV-Clean methodology to remove fluorescent labels was tested using 1 μg of IgG antibodies conjugated to large fluorophores; phycoerythrin (PE) and allophycocyanin (APC), Panel A. A comparison of EV detectability from background signal was investigated, Panel B. DC2.4 EVs were detected using flow cytometry with samples stained with 20 μM of CFDA-SE with no purification (C-top right), stained and the purified used size exclusion chromatography (C-bottom left), and stained and purified using EV-Clean methodology (C-bottom right). A buffer only control to represent the true background noise of the instrument is also shown (C-top left). It can be seen that without removal of residual CFSE the system background noise increases from ~50 FITC MESF to 437 FITC MESF, reducing the signal to noise ratio and detectability of stained EVs.

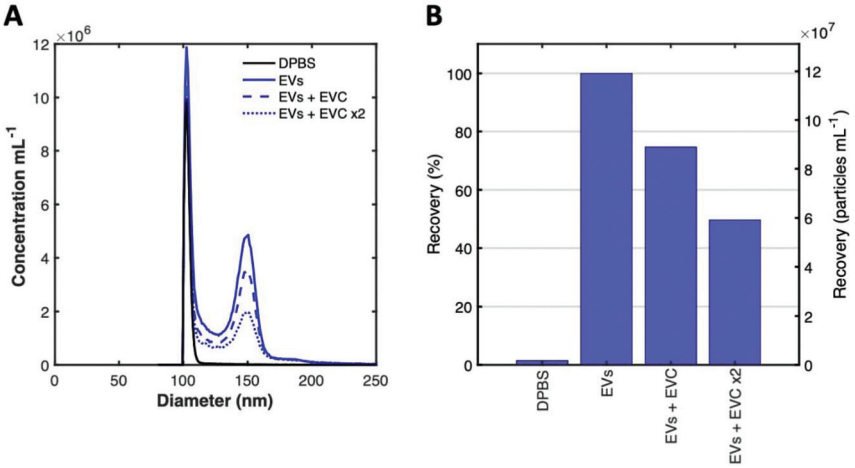

Fig. 5 Effect of EV-clean on EV recovery. The influence of EV-clean methodology on detectable EV recovery was assessed using flow cytometry (A). The percentage recovery of EVs gated from 115–200 nm using Capto Core 700 and incubated once or twice for 30 minutes was assessed (B).

Future development of the EV-Clean methodology in sample-processing, -labeling, and -washing workflows for heterogenous samples, such as plasma, may also benefit from combination with HTS-compatible methods which deplete large and abundant macromolecular complexes that confound measurements of EV concentration and size. In the basic format described above, this method enables removal of unbound labels from an HTS-compatible plate of a 96 samples in a small fraction of the time what is currently required with non-HTS-compatible approaches, such as size exclusion chromatography or ultracentrifugation. Parallel processing in a HTS format is also expected to improve sample processing metrics of consistency across large numbers of samples.

Author contributions

Conceptualization and methodology, JAW, JCJ; formal analysis, JAW, AI, IG, JCJ; investigation, JAW, BK, JK, TT, JS, DA and JCJ; data curation, JAW, BK, JK, TT, JS, DA, JCJ; writing – original draft preparation, JAW, JCJ; writing – review & editing, JAW, BK, JK, TT, KM, JS, DA, KA, KC, JB, AI, IG, JCJ; visualization, JAW; supervision, JCJ; project administration, JCJ; funding acquisition, KA, KC, JCJ.

Conflicts of interest

JAW, KM, JAB, JCJ are inventors on NIH patents and patent appications related to extracellular vesicles.

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References

1 C. Thery, K. W. Witwer, E. Aikawa, M. J. Alcaraz, J. D. Anderson, R. Andriantsitohaina, A. Antoniou, T. Arab, F. Archer, G. K. Atkin-Smith, D. C. Ayre, J. M. Bach, D. Bachurski, H. Baharvand, L. Balaj, S. Baldacchino, N. N. Bauer, A. A. Baxter, M. Bebawy, C. Beckham, A. Bedina Zavec, A. Benmoussa, A. C. Berardi, P. Bergese, E. Bielska, C. Blenkiron, S. Bobis-Wozowicz, E. Boilard, W. Boireau, A. Bongiovanni, F. E. Borras, S. Bosch, C. M. Boulanger, X. Breakefield, A. M. Breglio, M. A. Brennan, D. R. Brigstock, A. Brisson, M. L. Broekman, J. F. Bromberg, P. Bryl-Gorecka, S. Buch, A. H. Buck, D. Burger, S. Busatto, D. Buschmann, B. Bussolati, E. I. Buzas, J. B. Byrd, G. Camussi, D. R. Carter, S. Caruso, L. W. Chamley, Y. T. Chang, C. Chen, S. Chen, L. Cheng, A. R. Chin, A. Clayton, S. P. Clerici, A. Cocks, E. Cocucci, R. J. Coffey, A. Cordeiro-da-Silva, Y. Couch, F. A. Coumans, B. Coyle, R. Crescitelli, M. F. Criado, C. D'Souza-Schorey, S. Das, A. Datta Chaudhuri, P. de Candia, E. F. De Santana, O. De Wever, H. A. Del Portillo, T. Demaret, S. Deville, A. Devitt, B. Dhondt, D. Di Vizio, L. C. Dieterich, V. Dolo, A. P. Dominguez Rubio, M. Dominici, M. R. Dourado, T. A. Driedonks, F. V. Duarte, H. M. Duncan, R. M. Eichenberger, K. Ekstrom, S. El Andaloussi, C. Elie-Caille, U. Erdbrugger, J. M. Falcon-Perez, F. Fatima, J. E. Fish, M. Flores-Bellver, A. Forsonits, A. Frelet-Barrand, F. Fricke, G. Fuhrmann, S. Gabrielsson, A. Gamez-Valero, C. Gardiner, K. Gartner, R. Gaudin, Y. S. Gho, B. Giebel, C. Gilbert, M. Gimona, I. Giusti, D. C. Goberdhan, A. Gorgens, S. M. Gorski, D. W. Greening, J. C. Gross, A. Gualerzi, G. N. Gupta, D. Gustafson, A. Handberg,

R. A. Haraszti, P. Harrison, H. Hegyesi, A. Hendrix, A. F. Hill, F. H. Hochberg, K. F. Hoffmann, B. Holder, H. Holthofer, B. Hosseinkhani, G. Hu, Y. Huang, V. Huber, S. Hunt, A. G. Ibrahim, T. Ikezu, J. M. Inal, M. Isin, A. Ivanova, H. K. Jackson, S. Jacobsen, S. M. Jay, M. Jayachandran, G. Jenster, L. Jiang, S. M. Johnson, J. C. Jones, A. Jong, T. Jovanovic-Talisman, S. Jung, R. Kalluri, S. I. Kano, S. Kaur, Y. Kawamura, E. T. Keller, D. Khamari, E. Khomyakova, A. Khvorova, P. Kierulf, K. P. Kim, T. Kislinger, M. Klingeborn, D. J. Klinke 2nd, M. Kornek, M. M. Kosanovic, A. F. Kovacs, E. M. Kramer-Albers, S. Krasemann, M. Krause, I. V. Kurochkin, G. D. Kusuma, S. Kuypers, S. Laitinen, S. M. Langevin, L. R. Languino, J. Lannigan, C. Lasser, L. C. Laurent, G. Lavieu, E. Lazaro-Ibanez, S. Le Lay, M. S. Lee, Y. X. F. Lee, D. S. Lemos, M. Lenassi, A. Leszczynska, I. T. Li, K. Liao, S. F. Libregts, E. Ligeti, R. Lim, S. K. Lim, A. Line, K. Linnemannstons, A. Llorente, C. A. Lombard, M. J. Lorenowicz, A. M. Lorincz, J. Lotvall, J. Lovett, M. C. Lowry, X. Loyer, Q. Lu, B. Lukomska, T. R. Lunavat, S. L. Maas, H. Malhi, A. Marcilla, J. Mariani, J. Mariscal, E. S. Martens-Uzunova, L. Martin-Jaular, M. C. Martinez, V. R. Martins, M. Mathieu, S. Mathivanan, M. Maugeri, L. K. McGinnis, M. J. McVey, D. G. Meckes Jr., K. L. Meehan, I. Mertens, V. R. Minciacchi, A. Moller, M. Moller Jorgensen, A. Morales-Kastresana, J. Morhayim, F. Mullier, M. Muraca, L. Musante, V. Mussack, D. C. Muth, K. H. Myburgh, T. Najrana, M. Nawaz, I. Nazarenko, P. Nejsum, C. Neri, T. Neri, R. Nieuwland, L. Nimrichter, J. P. Nolan, E. N. Nolte-'t Hoen, N. Noren Hooten, L. O'Driscoll, T. O'Grady, A. O'Loghlen, T. Ochiya, M. Olivier, A. Ortiz, L. A. Ortiz, X. Osteikoetxea, O. Ostergaard, M. Ostrowski, J. Park, D. M. Pegtel, H. Peinado, F. Perut, M. W. Pfaffl, D. G. Phinney, B. C. Pieters, R. C. Pink, D. S. Pisetsky, E. Pogge von Strandmann, I. Polakovicova, I. K. Poon, B. H. Powell, I. Prada, L. Pulliam, P. Quesenberry, A. Radeghieri, R. L. Raffai, S. Raimondo, J. Rak, M. I. Ramirez, G. Raposo, M. S. Rayyan, N. Regev-Rudzki, F. L. Ricklefs, P. D. Robbins, D. D. Roberts, S. C. Rodrigues, E. Rohde, S. Rome, K. M. Rouschop, A. Rughetti, A. E. Russell, P. Saa, S. Sahoo, E. Salas-Huenuleo, C. Sanchez, J. A. Saugstad, M. J. Saul, R. M. Schiffelers, R. Schneider, T. H. Schoyen, A. Scott, E. Shahaj, S. Sharma, O. Shatnyeva, F. Shekari, G. V. Shelke, A. K. Shetty, K. Shiba, P. R. Siljander, A. M. Silva, A. Skowronek, O. L. Snyder 2nd, R. P. Soares, B. W. Sodar, C. Soekmadji, J. Sotillo, P. D. Stahl, W. Stoorvogel, S. L. Stott, E. F. Strasser, S. Swift, H. Tahara, M. Tewari, K. Timms, S. Tiwari, R. Tixeira, M. Tkach, W. S. Toh, R. Tomasini, A. C. Torrecilhas, J. P. Tosar, V. Toxavidis, L. Urbanelli, P. Vader, B. W. van Balkom, S. G. van der Grein, J. Van Deun, M. J. van Herwijnen, K. Van Keuren-Jensen, G. van Niel, M. E. van Royen, A. J. van Wijnen, M. H. Vasconcelos, I. J. Vechetti Jr., T. D. Veit, L. J. Vella, E. Velot, F. J. Verweij, B. Vestad, J. L. Vinas, T. Visnovitz, K. V. Vukman, J. Wahlgren, D. C. Watson, M. H. Wauben, A. Weaver, J. P. Webber, V. Weber, A. M. Wehman, D. J. Weiss, J. A. Welsh, S. Wendt, A. M. Wheelock, Z. Wiener, L. Witte, J. Wolfram, A. Xagorari, P. Xander, J. Xu, X. Yan, M. Yanez-Mo, H. Yin, Y. Yuana, V. Zappulli, J. Zarubova, V. Zekas, J. Y. Zhang, Z. Zhao, L. Zheng, A. R. Zheutlin, A. M. Zickler, P. Zimmermann, A. M. Zivkovic, D. Zocco and E. K. Zuba-Surma, Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines, *J. Extracell. Vesicles*, 2018, **7**(1), 1535750.

2 J. Lotvall, A. F. Hill, F. Hochberg, E. I. Buzas, D. Di Vizio, C. Gardiner, Y. S. Gho, I. V. Kurochkin, S. Mathivanan, P. Quesenberry, S. Sahoo, H. Tahara, M. H. Wauben, K. W. Witwer and C. Thery, Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles, *J. Extracell. Vesicles*, 2014, **3**, 26913.

3 E. van der Pol, F. A. Coumans, A. E. Grootemaat, C. Gardiner, I. L. Sargent, P. Harrison, A. Sturk, T. G. van Leeuwen and R. Nieuwland, Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing, *J. Thromb. Haemost.*, 2014, **12**(7), 1182–1192.

4 Y. Tian, M. Gong, Y. Hu, H. Liu, W. Zhang, M. Zhang, X. Hu, D. Aubert, S. Zhu, L. Wu and X. Yan, Quality and efficiency assessment of six extracellular vesicle isolation methods by nano-flow cytometry, *J. Extracell. Vesicles*, 2020, **9**(1), 1697028.

5 K. M. Lennon, D. L. Wakefield, A. L. Maddox, M. S. Brehove, A. N. Willner, K. Garcia-Mansfield, B. Meechoovet, R. Reiman, E. Hutchins, M. M. Miller, A. Goel, P. Pirrotte, K. Van Keuren-Jensen and T. Jovanovic-Talisman, Single molecule characterization of individual extracellular vesicles from pancreatic cancer, *J. Extracell. Vesicles*, 2019, **8**(1), 1685634.

6 Y. Tian, L. Ma, M. Gong, G. Su, S. Zhu, W. Zhang, S. Wang, Z. Li, C. Chen, L. Li, L. Wu and X. Yan, Protein Profiling and Sizing of Extracellular Vesicles from Colorectal Cancer Patients via Flow Cytometry, *ACS Nano*, 2018, **12**(1), 671–680.

7 C. Gardiner, D. Di Vizio, S. Sahoo, C. Thery, K. W. Witwer, M. Wauben and A. F. Hill, Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey, *J. Extracell. Vesicles*, 2016, **5**, 32945.

8 E.-T. Consortium, J. Van Deun, P. Mestdagh, P. Agostinis, O. Akay, S. Anand, J. Anckaert, Z. A. Martinez, T. Baetens, E. Beghein, L. Bertier, G. Berx, J. Boere, S. Boukouris, M. Bremer, D. Buschmann, J. B. Byrd, C. Casert, L. Cheng, A. Cmoch, D. Daveloose, E. De Smedt, S. Demirsoy, V. Depoorter, B. Dhondt, T. A. Driedonks, A. Dudek, A. Elsharawy, I. Floris, A. D. Foers, K. Gartner, A. D. Garg, E. Geeurickx, J. Gettemans, F. Ghazavi, B. Giebel,

T. G. Kormelink, G. Hancock, H. Helsmoortel, A. F. Hill, V. Hyenne, H. Kalra, D. Kim, J. Kowal, S. Kraemer, P. Leidinger, C. Leonelli, Y. Liang, L. Lippens, S. Liu, A. Lo Cicero, S. Martin, S. Mathivanan, P. Mathiyalagan, T. Matusek, G. Milani, M. Monguio-Tortajada, L. M. Mus, D. C. Muth, A. Nemeth, E. N. Nolte-'t Hoen, L. O'Driscoll, R. Palmulli, M. W. Pfaffl, B. Primdal-Bengtson, E. Romano, Q. Rousseau, S. Sahoo, N. Sampaio, M. Samuel, B. Scicluna, B. Soen, A. Steels, J. V. Swinnen, M. Takatalo, S. Thaminy, C. Thery, J. Tulkens, I. Van Audenhove, S. van der Grein, A. Van Goethem, M. J. van Herwijnen, G. Van Niel, N. Van Roy, A. R. Van Vliet, N. Vandamme, S. Vanhauwaert, G. Vergauwen, F. Verweij, A. Wallaert, M. Wauben, K. W. Witwer, M. I. Zonneveld, O. De Wever, J. Vandesompele and A. Hendrix, EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research, *Nat. Methods*, 2017, **14**(3), 228–232.

9 K. W. Witwer, E. I. Buzas, L. T. Bemis, A. Bora, C. Lasser, J. Lotvall, E. N. Nolte-'t Hoen, M. G. Piper, S. Sivaraman, J. Skog, C. Thery, M. H. Wauben and F. Hochberg, Standardization of sample collection, isolation and analysis methods in extracellular vesicle research, *J. Extracell. Vesicles*, 2013, **2**, 20360.

10 S. Jeurissen, G. Vergauwen, J. Van Deun, L. Lapeire, V. Depoorter, I. Miinalainen, R. Sormunen, R. Van den Broecke, G. Braems, V. Cocquyt, H. Denys and A. Hendrix, The isolation of morphologically intact and biologically active extracellular vesicles from the secretome of cancer-associated adipose tissue, *Cell Adh. Migr.*, 2017, **11**(2), 196–204.

11 K. Takov, D. M. Yellon and S. M. Davidson, Comparison of small extracellular vesicles isolated from plasma by ultracentrifugation or size-exclusion chromatography: yield, purity and functional potential, *J. Extracell. Vesicles*, 2019, **8**(1), 1560809.

12 K. W. Witwer, E. I. Buzas, L. T. Bemis, A. Bora, C. Lasser, J. Lotvall, E. N. Nolte-'t Hoen, M. G. Piper, S. Sivaraman, J. Skog, C. Thery, M. H. Wauben and F. Hochberg, Standardization of sample collection, isolation and analysis methods in extracellular vesicle research, *J. Extracell. Vesicles*, 2013, **2**(1), 20360.

13 D. Issadore, C. Min, M. Liong, J. Chung, R. Weissleder and H. Lee, Miniature magnetic resonance system for point-of-care diagnostics, *Lab Chip*, 2011, **11**(13), 2282–2287.

14 H. Shao, J. Chung, L. Balaj, A. Charest, D. D. Bigner, B. S. Carter, F. H. Hochberg, X. O. Breakefield, R. Weissleder and H. Lee, Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy, *Nat. Med.*, 2012, **18**(12), 1835–1840.

15 H. Shao, T. J. Yoon, M. Liong, R. Weissleder and H. Lee, Magnetic nanoparticles for biomedical NMR-based diagnostics, *Beilstein J. Nanotechnol.*, 2010, **1**, 142–154.

16 E. van der Pol, F. Coumans, Z. Varga, M. Krumrey and R. Nieuwland, Innovation in detection of microparticles and exosomes, *J. Thromb. Haemost.*, 2013, **11**(Suppl 1), 36–45.

17 E. van der Pol, L. de Rond, F. A. W. Coumans, E. L. Gool, A. N. Boing, A. Sturk, R. Nieuwland and T. G. van Leeuwen, Absolute sizing and label-free identification of extracellular vesicles by flow cytometry, *Nanomedicine*, 2018, **14**(3), 801–810.

18 E. van der Pol, A. G. Hoekstra, A. Sturk, C. Otto, T. G. van Leeuwen and R. Nieuwland, Optical and non-optical methods for detection and characterization of microparticles and exosomes, *J. Thromb. Haemost.*, 2010, **8**(12), 2596–2607.

19 A. Gasecka, R. Nieuwland, M. Budnik, F. Dignat-George, C. Eyileten, P. Harrison, R. Lacroix, A. Leroyer, G. Opolski, K. Pluta, E. van der Pol, M. Postula, P. Siljander, J. M. Siller-Matula and K. J. Filipiak, Ticagrelor attenuates the increase of extracellular vesicle concentrations in plasma after acute myocardial infarction compared to clopidogrel, *J. Thromb. Haemost.*, 2020, **18**(3), 609–623.

20 S. Zhu, L. Ma, S. Wang, C. Chen, W. Zhang, L. Yang, W. Hang, J. P. Nolan, L. Wu and X. Yan, Light-scattering detection below the level of single fluorescent molecules for high-resolution characterization of functional nanoparticles, *ACS Nano*, 2014, **8**(10), 10998–11006.

21 J. P. Nolan and J. C. Jones, Detection of platelet vesicles by flow cytometry, *Platelets*, 2017, **28**(3), 256–262.

22 E. J. van der Vlist, E. N. Nolte-'t Hoen, W. Stoorvogel, G. J. Arkesteijn and M. H. Wauben, Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry, *Nat. Protoc.*, 2012, **7**(7), 1311–1326.

23 E. N. Nolte-'t Hoen, E. J. van der Vlist, M. Aalberts, H. C. Mertens, B. J. Bosch, W. Bartelink, E. Mastrobattista, E. V. van Gaal, W. Stoorvogel, G. J. Arkesteijn and M. H. Wauben, Quantitative and qualitative flow cytometric analysis of nanosized cell-derived membrane vesicles, *Nanomedicine*, 2012, **8**(5), 712–720.

24 L. de Rond, E. van der Pol, C. M. Hau, Z. Varga, A. Sturk, T. G. van Leeuwen, R. Nieuwland and F. A. W. Coumans, Comparison of Generic Fluorescent Markers for Detection of Extracellular Vesicles by Flow Cytometry, *Clin. Chem.*, 2018, **64**(4), 680–689.

25 J. A. Welsh, E. Van Der Pol, G. J. A. Arkesteijn, M. Bremer, A. Brisson, F. Coumans, F. Dignat-George, E. Duggan, I. Ghiran, B. Giebel, A. Gorgens, A. Hendrix, R. Lacroix, J. Lannigan, S. Libregts, E. Lozano-Andres, A. Morales-Kastresana, S. Robert, L. De Rond, T. Tertel, J. Tigges, O. De Wever, X. Yan, R. Nieuwland, M. H. M. Wauben, J. P. Nolan and J. C. Jones, MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments, *J. Extracell. Vesicles*, 2020, **9**(1), 1713526.

26 C. P. Lai, E. Y. Kim, C. E. Badr, R. Weissleder, T. R. Mempel, B. A. Tannous and X. O. Breakefield, Visualization and tracking of tumour extracellular vesicle delivery and RNA translation using multiplexed reporters, *Nat. Commun.*, 2015, **6**, 7029.

27 R. Spannaus, C. Miller, D. Lindemann and J. Bodem, Purification of foamy viral particles, *Virology*, 2017, **506**, 28–33.

28 S. T. Mundle, M. Kishko, R. Groppo, J. DiNapoli, J. Hamberger, B. McNeil, H. Kleanthous, M. Parrington,

L. Zhang and S. F. Anderson, Core bead chromatography for preparation of highly pure, infectious respiratory syncytial virus in the negative purification mode, *Vaccine*, 2016, **34**(32), 3690–3696.

29 Y. F. Tseng, T. C. Weng, C. C. Lai, P. L. Chen, M. S. Lee and A. Y. Hu, A fast and efficient purification platform for cell-based influenza viruses by flow-through chromatography, *Vaccine*, 2018, **36**(22), 3146–3152.

30 T. Weigel, T. Solomaier, A. Peuker, T. Pathapati, M. W. Wolff and U. Reichl, A flow-through chromatography process for influenza A and B virus purification, *J. Virol. Methods*, 2014, **207**, 45–53.

31 A. Morales-Kastresana, B. Telford, T. A. Musich, K. McKinnon, C. Clayborne, Z. Braig, A. Rosner, T. Demberg, D. C. Watson, T. S. Karpova, G. J. Freeman, R. H. DeKruyff, G. N. Pavlakis, M. Terabe, M. Robert-Guroff, J. A. Berzofsky and J. C. Jones, Labeling Extracellular Vesicles for Nanoscale Flow Cytometry, *Sci. Rep.*, 2017, **7**(1), 1878.

32 J. A. Welsh, P. Horak, J. S. Wilkinson, V. J. Ford, J. C. Jones, D. Smith, J. A. Holloway and N. A. Englyst, FCMPASS Software Aids Extracellular Vesicle Light Scatter Standardization, *Cytometry, Part A*, 2020, **97**(6), 569–581.

33 J. A. Welsh, J. C. Jones and V. A. Tang, Fluorescence and Light Scatter Calibration Allow Comparisons of Small Particle Data in Standard Units across Different Flow Cytometry Platforms and Detector Settings, *Cytometry, Part A*, 2020, **97**(6), 592–601.