



Cite this: *Nanoscale*, 2018, **10**, 881

Technical challenges of working with extracellular vesicles

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Extracellular Vesicles (EVs) are gaining interest as central players in liquid biopsies, with potential applications in diagnosis, prognosis and therapeutic guidance in most pathological conditions. These nano-sized particles transmit signals determined by their protein, lipid, nucleic acid and sugar content, and the unique molecular pattern of EVs dictates the type of signal to be transmitted to recipient cells. However, their small sizes and the limited quantities that can usually be obtained from patient-derived samples pose a number of challenges to their isolation, study and characterization. These challenges and some possible options to overcome them are discussed in this review.

Received 9th November 2017,

Accepted 8th December 2017

DOI: 10.1039/c7nr08360b

rsc.li/nanoscale

1 Introduction

Extracellular Vesicles (EVs) are involved in the transmission of biological signals between populations of prokaryotic and eukaryotic cells. As central mediators of intercellular communication, EVs are involved in many cellular processes, such

as compensating for stress conditions, triggering physiological responses that contribute to the maintenance of cellular integrity, organismal homeostasis^{1–3} and regulating a range of biological activities. Their potential applications for diagnosis and guiding therapeutics, as well as determining prognosis of pathological conditions has allowed the field of EV-studies to grow steadily in recent years.

The term EVs, coined by the International Society for Extracellular Vesicles (ISEV, <http://www.isev.org>), categorizes vesicles based on their biogenesis or release pathway, and include exosomes (50–100 nm in diameter), originated from the endocytic pathway and with buoyant densities of 1.11–1.19 g mL⁻¹,⁴ shedding microparticles/microvesicles (100–1000 nm) released directly from the plasma membrane,⁵ apoptotic blebs (50 nm–2 μm; produced as a consequence of indiscriminate apoptotic disintegration),⁶ large oncosomes (1–10 μm)⁷ and other miscellaneous EV subsets.⁸ As the sizes of the different EVs-subsets overlap, as well as their cargo, several groups have now started characterizing the composition of EV-subtypes. Recent papers claim successful subclassification of EVs based on general surface proteomic profiling,^{9,10} or on transcriptional profiles of individual EV populations.^{11,12} EV subtypes have been isolated by a number of means, including recovery at different centrifugation forces, different filters, at slightly different positions in density gradients, *via* immuno-isolation by different surface molecules, chromatography or by flow cytometric sorting.

At present, functions of EVs have not been fully elucidated. However, they appear to be able to modulate host–pathogen

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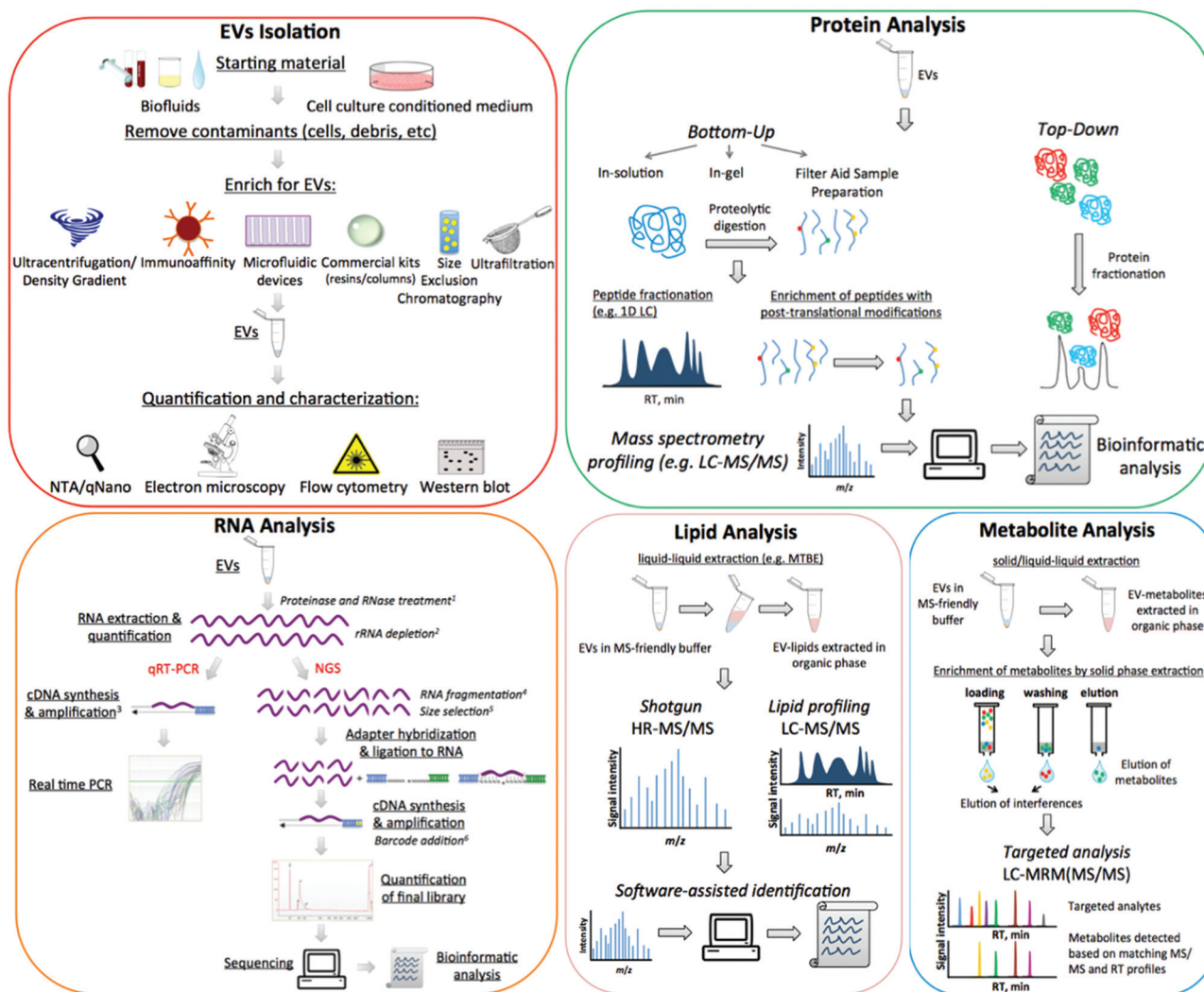


Fig. 1 Scheme of EVs isolation, quantification and characterization, including the analysis of EVs molecular content. The superscript numbers on the figures indicate the need of each step as follows: 1 – to ensure intravesicular origin of RNAs; 2 – to remove abundant ribosomal RNAs that may be present as fragments in EVs, and therefore not visible by Bioanalyzer analysis; 3 – cDNA synthesis may be performed with transcript-specific or general primers; cDNA pre-amplification before real time PCR is optional; 4 – to break down long RNA molecules; 5 – to separate small RNAs (<200 nt) from long RNAs (>200 nt); 6 – to tag molecules from different samples and enable multiplex analysis.

interactions¹³ and to contribute to several pathological conditions such as infectious and inflammatory diseases, neurological disorders and cancer. EVs are particularly important in clinical settings, largely because they contain a wealth of biomarkers that can be used to monitor clinical status, chemotherapy-response,¹⁴ disease progression,¹⁵ and many relevant and diverse clinical conditions. The composition of EVs is not random and each EV-cargo delivers specific molecular messages. Indeed these nanosized membrane vesicles transmit signals by proteins, lipids, nucleic acids, and sugars, and the unique molecular pattern of this package dictates the type of extracellular signal to be transmitted to recipient cells.¹ Despite the promise of revealing informative markers of medical interest, working with these small particles poses many technical challenges. In addition to the concerns over data analysis, there are further uncertainties over protocol

standardisation and how to define the pre-analytical and analytical variables that impact upon outcome measures.¹⁶ The aim of this review is to outline the most relevant challenges related to working with EVs, specifically discussing the advantages and disadvantages of different approaches and suggesting alternative routes to better overcome frequent issues. A detailed scheme of EV purification and characterization methods is presented in Fig. 1.

2 Pre-analytical factors

2.1 EVs derived from biofluids

EVs have been isolated from diverse biofluids including blood,¹⁷ urine,¹⁸ saliva,¹⁹ breast milk,²⁰ cerebrospinal²¹ and ascitic fluids,²² gastric juice,²³ bile,²⁴ sputum,²⁵ bronchoalveo-



constant. In another study, a 7-fold increase in the number of phosphatidylserine-exposing EVs was found in EVs isolated from plasma samples that had undergone a single freeze–thaw cycle, suggesting changes in the vesicle membrane phospholipids, whereas in urine-derived EVs the same effect was not observed.⁴⁷ Kalra *et al.* spiked EVs isolated from a colorectal cancer cell line into plasma samples and measured TSG101 protein expression after 10, 30 and 90 days in storage at 4 °C, –20 °C or –80 °C, as well as PKH67 dye labeling and uptake by cells (–20 °C for 30 days).⁴⁸ They found that all storage conditions yielded EVs with detectable TSG101 and capable of being transferred into cells.

Although several studies have investigated whether storage impacts specific characteristics of EVs, there is a lack of large-scale analysis of EVs cargo changes that may be caused by storage. To the best of our knowledge, no study has evaluated transcriptomic, proteomic and lipidomics alterations that may be induced upon EVs storage. Until more in-depth “omics” studies are performed, in addition to functional assays, the understanding of the true stability of EVs remains incomplete. In this sense, we recommend EVs to be evaluated as soon as possible after their isolation, especially for functional studies, avoiding repeated freezing and thawing cycles.

3 EV isolation protocols

There are several EV isolation and characterization techniques available, and each has its own set of pre-analytical factors that may influence yields. The ISEV has issued several position papers highlighting the importance of standardization of sample collection and EV isolation and characterization methods, which summarize all the detailed information that should be recorded and provided in publications.^{40,49,50}

Launched recently, the EV-TRACK database (<http://evtrack.org>) is an excellent initiative in the EV-research field to encourage standardization of vesicle isolation and characterization methods.⁵¹ This initiative was created by an international consortium of 92 researchers from 12 countries, who evaluated and scored the experimental parameters of 1226 recently published EV-related articles. The EV-METRIC is a summary score of the article’s adherence to and detailed reporting nine experimental parameters. The fact that the average EV-METRIC across all biofluid studies is only 20% indicates how the heterogeneous EV-field is currently deficient in methodological reporting. The online database is searchable, allowing easy identification and comparison of EV-related articles. Another interesting feature is that the authors can annotate their study’s database entries, adding experimental details that may have been overlooked during publication. Hopefully, EV-TRACK will contribute to better transparency and reporting in EVs publications, allowing improved interpretation and reproducibility of experiments.

3.1 Ultracentrifugation (UC)

The selection of an EV-isolation method largely depends on the source of sample. For less complex samples obtained from

cell cultures, it is beneficial to use differential UC with no preliminary steps, an approach that has long been considered the most efficient EV isolation technique. New isolation methods appear often, usually involving column enrichment or precipitation. However, the most popular primary isolation methods still in use are based on UC.⁵² On the other hand, isolation of EVs derived from complex biological fluids may benefit from the use of multiple methods to remove specific components (*e.g.* platelets, lipoproteins or protein complexes) before UC,⁵³ or even alternative isolation methods, involving column enrichment, sequential filtering or precipitation. However, as a means of first EV-isolation step, UC is a cost-efficient and widely accepted way to isolate EVs.⁵²

The isolation of EVs through differential UC relies on several centrifugation steps with increased centrifugal force to produce sequential pellets of cells and cell-debris (300–1000g), microvesicles (10 000–20 000g) and exosomes (100 000g). Some have described the use of serial ultrafiltration through 0.22 and 0.45 μm filters before pelleting⁵⁴ and variation of the centrifugation forces have been implemented in different protocols.

Advantages of UC include low cost – as ultracentrifuges are readily available in many labs – and the capacity to spin a wide range of volumes from a few millilitres up to >100 mL. However, there are a number of problems associated with isolating EVs by UC. One of the major caveats is the co-purification of non EV-associated proteins, particularly protein aggregates and lipoproteins,⁵⁵ which may be interpreted as integral or enriched EV components in downstream analyses. Ultrafiltration can be coupled with UC to reduce EV-protein aggregates.⁵⁶ Attention should be given to centrifugation forces applied to EVs. Nordin *et al.*, have shown some degree of EV disruption after UC for 70 min at 120 000g.⁵⁶ Therefore, when the recovery of intact EVs using UC is an aim, we recommend the use of a maximum of 100 000g.

3.2 Density gradients (DG)

An approach that is commonly used to overcome the problem of co-purification is to follow UC with further purification of EVs in a sucrose DG which makes use of EV density for better separation (26). EVs of a particular size and origin have a particular floatation density, usually in the range of 1.08–1.22 g mL^{-1} .⁵⁷ Several protocols have been described,⁵⁸ the majority of which involve resuspending an EV-enriched pellet following UC, overlaying a sucrose gradient in various buffers which may also contain deuterium oxide, performing UC again and collecting the appropriate fractions which are enriched in EVs. Differential UC shows the presence of larger vesicles by laser particle size measurement, and protein concentration studies show that DG UC yields higher protein concentration and more target exosome proteins.⁵⁹

Unfortunately, differential UC can be a lengthy process with the most time taken for the UC itself. For clinical grade data or highly enriched samples it is recommended to use multiple centrifugation speeds that will considerably lengthen the process, reducing its applicability for clinical settings.⁶⁰



Whereas sucrose gradient UC is relatively time consuming, it undoubtedly results in the isolation of purer EV samples than UC alone.⁵⁹ However, due to fractionation a relatively high starting concentration of EVs is required.⁶¹ Besides being able to isolate purer EVs, it should be noted that contaminants that have the same density of EVs, such as some viruses,⁶² plasma-derived high- and low-density lipoproteins^{63,64} and uromodulin and albumin⁶⁵ from urine, are likely to contaminate the EVs isolated by DGs. It has been shown that the similar floatation densities of HIV virus particles and EVs make them indistinguishable by conventional gradient separation.⁶⁶ Presumably, the co-purification of EVs and virus particles could be considered a universal problem due to common viral contaminations of cell cultures which go undetected. Cantin *et al.* found that it was possible to separate HIV particles and EVs using commercially available OptiPrep DG solution.⁶⁶ This employs a gradient of iodixanol rather than sucrose, and has been used for isolating fractions of pure EVs for proteomic analysis from conditioned cell culture medium⁶⁷ and plasma.⁴⁸

3.3 Immunoaffinity

An approach to improve the purity of the EVs population is to use immunoisolation, whereby EVs preparations are incubated with antibody-coated latex¹⁷ or magnetic beads,⁶⁸ allowing their separation based on the expression of markers of interest. This technique has the potential to specifically pull-down EVs with a particular surface marker while excluding contaminating particles or other EVs populations. A study comparing immunoisolation with DG separation and UC for the isolation of human colon cancer-derived EVs showed that immunoisolation produced the highest EVs yield as measured by quantitative protein mass spectrometry.⁶¹

As immunoisolation is not compatible with large-volume samples, samples with low EV content must be concentrated prior to incubation with antibody-coated beads. Therefore, immunoisolation is commonly used as an additional purification step following conventional EVs isolation from large sample volumes using UC and possibly DG,¹⁷ making this a lengthy procedure. When small volumes of starting material contain sufficient EVs to isolate and analyse (as with serum), immunoisolation can be used to isolate EVs directly.⁶⁹ Importantly, when isolating EVs from serum, it is essential to pre-coat the beads with a molecule such as sulfobetaine to reduce the non-specific adsorption of abundant serum proteins. This single-step EV isolation technique may prove to be a valuable diagnostic tool for identifying disease markers in EVs.^{69,70}

While one of the key advantages of immunoisolation is its specificity based on the chosen antibody, this approach may also be limiting since it is likely to isolate merely a sub-population of EVs. There is still poor understanding of which EVs markers are present on different EVs subpopulations, and what the subcellular and cellular origins of these EVs are. For example, there are no accepted, exclusive markers to distinguish between exosomes and microvesicles despite their

distinct mechanisms of biogenesis.⁷¹ In a diagnostic setting, the best case scenario would be to have a cell-type specific surface markers which are differentially regulated during disease and released in EVs as recently reported.⁶⁸

While immunoisolation has been successfully used for analysis of EVs by mass spectrometry⁶¹ and RNA sequencing,⁷² the use of immunoisolated EVs in functional studies is more difficult due to the challenge of dissociating high yields of active EVs from the beads. Chaotropic agents such as NaCl have been successfully used to elute EVs from beads where EVs have been shown to retain at least some functionality.⁷³ However, in spite of successful elution of EVs using mild conditions, small changes in EVs size and surface structure can occur.^{74,75} Thus, care must be taken when interpreting results from functional analyses using immunoisolated EVs.

3.4 Microfluidics

Microfluidics technologies can be used to isolate EVs populations of interest. Here, molecules enriched in the EVs membrane, such as specific lipids and proteins, can be used to isolate them.⁶⁹ Many groups have already described the use of customised on-chip devices to isolate EVs, partly because this approach enables the use of low input sample. Another important point is the feasibility of EVs evaluation directly from bodily fluids (without the need for prior extraction steps), which can be of particular interest in the diagnostic setting.⁷⁶ Combining microfluidics with previously described immunoaffinity has resulted in an immunoaffinity microfluidic device based on CD63, an abundant tetraspanin present in the EVs membrane.⁷⁷ More recently, the Exochip, another on-chip device allows not only EVs isolation based on CD63 immunoaffinity, but also their quantification by fluorescent dye staining.⁷⁸

EV features, such as electric properties, shape, size and density, can also be exploited in the development of customised chips.⁷⁹ As a note of caution we must state that it remains to be demonstrated if antibody-isolation approaches somehow modify the cargo or the functionality of EVs and if their biological activity is dependent on the simultaneous signalling given by a more diverse EVs set, that can not be isolated by specific antibodies.

3.5 Size exclusion chromatography (SEC)

EVs with different sizes can be separated using Size Exclusion Chromatography (SEC), a technique that has been more and more used currently, allowing the recovery of pure vesicles with increased functionality, purity and integrity. Advantages of SEC include the reduction of EVs-aggregation during the isolation procedure,⁵⁶ the efficient separation of EV from soluble proteins and capability of isolating pure, intact and biologically active EVs.⁸⁰ SEC removes 99% of the soluble plasma proteins and >95% of HDL from the purest fraction of EVs, does not induce aggregation of EVs, and retains the integrity and biological activity of EVs.^{56,80–82} Contaminants such as von Willebrand factor and LDL are unexpected based on particle size, but these can still be found possibly complexed



to EVs.^{63,83} SEC allows the EVs environment to be changed, such as from plasma to buffer, with no or minimal detrimental effects on EVs themselves. Also, it is fast, 10 to 20 minutes per sample, and relatively inexpensive,⁸⁴ which makes it more applicable in a clinical setting. A disadvantage of SEC is the dilution of EVs sample, which often requires second step *i.e.* re-concentration of EVs by ultrafiltration.^{56,80,81,83–86} Compared with DC, EVs isolated by SEC have a high yield of biophysically intact EVs although at the expense of dilution.^{56,86} A single-step plasma EVs isolation using SEC has been published, based on the use of qEV SEC columns, an efficient system to isolate EVs from plasma proteins.^{60,84} Also, instead of pelleting EVs by UC, as used by some, the application of protein concentrating devices allows the fast concentration of EV fractions.^{60,84} This provides an efficient means of isolating and concentrating EVs from human plasma, while avoiding some negative issues related to UC as previously mentioned. SEC and ultrafiltration can be coupled to provide higher EVs purity and good recovery rates, while still preserving their biophysical and functional properties.⁵⁶ Ultrafiltration of cultured media results in higher recovery of EVs after DG purification, and is a faster alternative to UC, whereas the SEC outperforms precipitation of EVs isolated from human plasma.⁶⁰

3.6 Ultrafiltration (UF)

Filtration-based EVs isolation methods can be used independently or in conjunction with other techniques such as UC. Advantages of filtration-based methods are the ability to use variable sample input volumes, simplicity and low cost. Davies *et al.* developed a microfluidic filtration system for isolation of EVs directly from unprocessed whole blood⁵⁵ which allowed the study of very limited amounts of blood from melanoma-bearing mice. After being isolated from a few microliters of blood by this pressure-based device, the EVs were shown to retain their intact morphology and to express Melan A mRNA, a melanoma tumor marker, as well as other classic EV-markers (as evidenced by Western blot of CD9, CD63 and CD81). Interestingly, a direct comparison with EVs isolated by UC was performed, and TEM analysis showed that UC caused many EVs to aggregate and to deform, which did not occur in the filtration-derived EVs, even under pressure.⁵⁵ A three-step sequential filtration-based protocol allowed the isolation of EVs from larger volumes, such as 150 mL of cell culture conditioned medium.⁸⁷ The authors conclude that sequential filtration with a 100 nm cut off final filtering step enriches for exosome-sized vesicles (81% compared to 23% for UC), suggesting that UC may lead to co-purification of larger vesicles and/or protein aggregates. UF is more appropriate with volumes in excess of 400 mL due to the higher flow rate, and that EVs loss is only observed with the first 50–100 mL of cultured media.⁶⁰ Whereas it is far more time efficient than centrifugation methods, taking only 20 minutes to concentrate over 150 mL of sample compared to 2 rounds of UC for 90 minutes each.⁶⁰ Importantly, UF can have a recovery of up to 80% and may concentrate EVs up to 240-fold.⁶⁰ This implies

that UF-based methods are effective to concentrate EVs. However, UF has some limitations such as it may result into non-specific binding of EVs to membranes^{35,56} and thus may present some loss of EV yield owing to trapping in filter pores.

3.7 General aspects of EVs isolation methods

Coincident with the EV-TRACK initiative, several groups have compared methodologies to improve the efficiency of the isolation and characterization of EVs. Even being considered gold standard for purification, differential centrifugation can provide technical difficulties to process large volumes of conditioned medium and it has been suggested that repeated UC steps can damage vesicles and reduce yield, impacting proteomic and RNA studies.⁵⁶ In this sense, the group of Dr Andreas Moeller (QIMR Berghofer Medical Research Institute, Australia) compared UC to UF for the isolation of exosomes from cell culture conditioned medium and concluded that the later provided faster and better recovery.⁶⁰ They then further compared four alternative methodologies (two precipitation-based methods: ExoQuick and Exo-spin; one SEC-based method: Izon qEV columns; and one DG purification-based method: Opti-Prep), concluding that precipitation-based methods led to higher particle yield but less purity (measured by particle to protein ratio), while qEV columns provided the purest preparations, including also plasma-derived exosomes.

A recent work has compared four exosome isolation protocols (single-step UC) and density-gradient UC-based protocols using iodixanol (Optiprep) and two commercially available precipitation-solution based protocols EXO-Quick (EQ) and total exosome isolation (TEI).⁸⁸ The results showed Optiprep to be the method of choice in terms of removing EV-associated protein/RNA complexes contaminants, allowing the recovery of exosome-specific proteins and RNA.⁸⁸ This was reinforced in a clinical study on plasma exosomes where Optiprep gradient centrifugation was the single method capable of removing contaminating plasma proteins.⁴⁸ Regarding the quality of the RNA, Van Deun *et al.* found several differences of RNA profiles between the methods, and strongly recommend to validate isolation methods prior to consider exosome-specific content, functions and biomarkers.

Moreover the group of Dr Pieter Vader (University Medical Center Utrecht, The Netherlands) and Samir El Andaloussi (Karolinska Institutet, Stockholm, Sweden), provided evidence that SEC-isolated EVs better retain their functional activities, as compared to UC-isolated EVs.⁸⁹ They investigated differences in functionality of cardiomyocyte progenitor cell derived EVs isolated using UC and SEC, concluding that SEC-isolated EVs were more efficient in stimulating the migration of endothelial cells, having EV-induced ERK1/2 phosphorylation as a measurement to compare the UC-EVs and SEC-EVs functionality.

Reinforcing the importance of UF the group of Dr An Hendrix (Ghent University, Belgium) compared five commonly used filters for their efficiency to recover exosomes from clinical samples.³⁵ Regenerated cellulose membrane filters, with



pores of 10 kDa, showed higher efficiency. They also compared colorimetric and fluorimetric kits to measure EV protein content, and found the Qubit fluorometric assay to be the most sensitive (considering the lower BSA standard of 200 $\mu\text{g mL}^{-1}$) and the more consistent in terms of quantification among technical and biological replicates. This group also suggested the Optiprep (Sigma Aldrich) to be the best method to isolate EVs and proposed a protocol to remove Optiprep leftovers from EVs. Other efforts have been done by Dr Marca Wauben's group (Utrecht University, The Netherlands) who developed a fluorescence-based flow cytometric high-throughput method for quantitative and qualitative analysis of nano-sized cell-derived membrane vesicles (50–100 nm) which cannot be visualized by conventional scatter-based analysis.^{90,91}

In order to ensure how pure are the EVs isolated by any of the possible protocols, the ratio of EVs and non-EV components can be assessed. One of the most used methods is the determination of the number of EVs per microgram of measured proteins in the isolated EVs. EVs purified using sucrose DG from conditioned cell culture media provided highly purified EVs: 3.3×10^{10} particles per μg protein. However, when isolated from biofluids EVs are much more contaminated by proteins giving ratios around 6.5×10^9 particles per μg protein for fresh serum and 1.1×10^9 particles per μg protein for fresh urine.⁹² This calculation should be interpreted with caution as the ratio depends on the source of the vesicles as well as the method of extraction.⁸⁸

4 Characterization of the isolated EVs

After isolation, EV populations need to be characterized for intended downstream applications, for which there is a variety of techniques available. This section will discuss some of the available options in light of the instrumental parameters of each technique.

4.1 Electron microscopy (EM)

The use of electron microscopy (EM) has considerably pushed the field of EVs and enabled the discovery of apoptotic vesicles, microvesicles and exosomes. The nanometer-scale resolving power of transmission electron microscopes has, however, its drawbacks, which include lengthy sample preparation, lack of multi-parametric phenotyping, and low throughput capacity. These characteristics make large EM studies rather challenging, with the amount of starting EVs material being particularly critical. Yet, morphological characterization using parameters such lipid and protein compositions, cellular origin, size, density and morphology⁴ is a key step for classification of isolated EVs. Thus, efforts to mitigate the challenges in the use of electron microscopy to study EVs are most welcome in the field.

One of these common challenges relates to the amount of available material for transmission EM preparation, microtomy and visualisation. Some tissues and body fluids release

an abundant amount of vesicles that are easily harvested by differential centrifugation or affinity purification chromatography. However, most often than not, isolated EVs are few and far between; and sample preparation itself can at times cause further loss of material. In an attempt to overcome this issue, recently there have been informal discussions, in conferences and focused meetings, about the use of proteinaceous material (e.g. matrigel, bovine serum albumin, or other inert protein) to 'encapsulate' EVs in a rich protein surrounding that is extensively cross-linked during fixation. This is because the protein around EVs, when cross-linked together, will form a firm matrix around the vesicles, protecting them from falling apart. Alternatively, the use of inert polysaccharides (e.g. agarose, agar, methylcellulose) to embed isolated EVs in a small piece of gel prior to EM processing has also been reported by individual laboratories with success.

A routine EM method that has been used with great success for the study of EVs is that of negatively-stained whole mount preparation.⁴ Here EVs are adsorbed onto a filmed metal grid, chemically fixed and negatively stained prior to observation using a transmission electron microscope. Whole mounts are extremely useful for morphological analysis (e.g. EV size, shape, density), and can be combined with immunolabelling techniques. This method is particularly useful for the identification and localisation of immunological epitopes on the external surface of EVs, whereas EV-internal epitopes are better visualised by positive staining methods.

Nevertheless, the visualisation of EVs by transmission electron microscopy, coupled or not with immunolocalisation techniques, suffers mostly from two major challenges: lack of contrast and preservation (of vesicle morphology and/or molecular epitopes). This is particularly critical when considering the potential of EVs as biomarkers in medical applications. Some whole-mount preparations yield cup-shaped exosomes, which has been suggested to be an artefact caused by sample dehydration.⁹³ Indeed, the preparation steps of chemical fixation, dehydration, observation under vacuum, and electron beam radiation damage could all interfere with an important feature of exosomes, that of size.^{94,95}

Cryo-electron microscopy (cryo-EM) comes at hand here, and hydrated exosomes studied by cryo-EM have been reported as close-to-spherical nanoparticles.^{96,97} As fixation tends to be physical (cryogenic) rather than chemical, cryo-EM is thought to preserve EVs closer to their native state,⁹⁸ in particular in terms of their morphology and immunological epitopes used in the identification of vesicle biomarkers. An interesting feature described in cryo-EM of exosomes from prion-infected cells was the presence of a second, internal membrane-bound region of denser core and, in many cases, of hexagonal shape.⁹⁷ Vesicles derived from human plasma and studied by the same method also show a variety of forms and sizes, suggesting multiple membrane profiles in EVs. Elongated vesicles, empty or not, were also observed, and so were EVs surrounded by smaller spherical structures.⁹⁹ Yet, because most cryo-EM procedures do not include the use of heavy metals, contrast of the material could be compromised if not for



various optical and diffraction techniques – the work of colleagues such as Alain Brisson from the Institut de Chimie & Biologie des Membranes & des Nano-objets (CBMN, Bordeaux, France) in the development of suitable imaging analysis methods, and of diagnostic assays using EVs, holds much promise here.

4.2 Nanoparticle tracking analysis (NTA)

NTA is a light scattering technique that makes use of both laser light scattering and the Brownian motion of particles to obtain the particle size distribution and concentration. This approach takes into consideration the particles' speed in a medium of known temperature and viscosity and allows the calculation of its hydrodynamic diameter. The first studies of EVs using NTA were published in 2011.¹⁰⁰ NTA is currently the most popular quantitative method of EV analysis.⁵² This is because few other high-throughput techniques have the appropriate resolution for single EV particle analysis. Whilst NTA allows relatively high throughput, and claims to have resolution down to small diameters, its ability to size and determine concentration accurately is however debatable.¹⁰¹ Moreover, when performing NTA measurements, before dilution of samples, attention should be given towards the lack of particulate matter in suspension buffers (usually phosphate buffered saline) to avoid artificial inflation of EV counts. The addition of a detergent such as Triton X to NTA preparations allows for confirmation of EV-lipid moieties. Its fluorescent mode is also capable of providing specific results for labelled particles. In a recent application of fluorescent NTA, it was shown the use of miRNA-specific molecular beacons encapsulated in cationic lipoplex nanoparticles that fuse non-specifically with negatively charged EVs and this allowed the precise quantification of EVs carrying an specific miRNA and how many copies of this miRNA could be found in these EVs.¹⁰² This paves the way to future therapeutic applications that rely in the identification/quantification of specific markers.

4.3 Resistive pulse sensing (RPS)

RPS utilises the Coulter principle to determine the absolute diameter and distribution of particles in a suspension in the range of ~50–10 000 nm.^{101,103} RPS utilised in the EV field is generally carried out using the qNano (Izon Science Ltd, Christchurch, New Zealand). The qNano consists of two fluid cells separated by a non-conductive membrane. An electric current is passed through a single pore in the membrane and, as particles pass through this pore, a transient attenuation of signal occurs that is approximately proportional to the particle volume. This system is calibrated using beads of a known diameter and concentration.¹⁰⁴ Sample volumes in the qNano can be as low as 10 μ L. By applying pressure differences between the fluidic cells, pressure-driven flow overcomes the flow produced by diffusion, electrophoresis and electro-osmosis. Disadvantages of this technique include: multiple pore sizes are required to measure the full EV size range; pores are prone to clogging; little phenotypic information regarding the EV's

derivation is gained; identifying EVs from similar sized contaminants is not possible.

4.4 Flow cytometry (FCM)

Although mostly used for cellular analysis, FCM is currently one of the most popular techniques used to study EVs, in particular microvesicles.¹⁰⁵ Flow cytometers that have been developed for dedicated EV analysis, referred to as dedicated FCM (dFCM), have been shown to be capable of resolving particles consistent with biological vesicles to <30 nm. dFCM are usually conventional flow cytometers that have been customised by a laboratory, although more recently they have also become commercially available.^{106–108} A persistent concern in FCM is the ability to reliably distinguish between EVs that carry a specific protein marker from those that do not, and thus to accurately measure the proportion of EVs of a certain type. Quantitative and qualitative analysis of EVs populations present within a sample are needed. Although being a prerequisite for a correct data interpretation, the use of proper controls that robustly validate the test samples and establish background levels are generally missing.¹⁰⁹ Fluorescently-stained polystyrene microspheres have been used as a standardisation method for EV analysis, yet their high refractive index compared to EVs means that they cannot reliably be used to directly approximate the size of EVs using FCM scatter parameters. Furthermore, due to FCM collecting light at different angles, scatter resolution cannot be compared using polystyrene microspheres without the use of refractive index normalisation using laser scatter physics modelling.¹¹⁰ Fluorescent sensitivity can be quantified using microspheres that have a known quantity of molecules of equivalent soluble fluorophore (MESF) that are recommended for standardising EV analysis. Reviews dedicated to EV standardisation using FCM can be found elsewhere.¹⁰⁵

5 Characterization of EV content

5.1 Characterization of EVs content: RNA

5.1.1 RNA purification. Several sources of bias due to differences in experimental methodologies may explain discrepancies between results of EV-studies, including EV-RNA related analysis. The two main RNA purification approaches are silica membrane column-based kits and/or organic extraction and precipitation-based kits. One of the earliest studies that systemically compared methods evaluated seven different extraction protocols and concluded that there were major differences in yield, purity and size distribution of the RNAs extracted by the kits.¹¹¹ Nowadays there is a plethora of commercial kits available from many different companies, specifically tailored for EV isolation and subsequent RNA extraction. Each method has its own peculiarities and attention must be paid when comparing results from different studies, taking into account the EVs and RNA isolation strategies employed. Another important source of bias relates to the possible co-purification of non-vesicular nucleic acids bound to the exter-



nal surface of EVs. To avoid this and isolate only intra-vesicular RNAs protected by the lipid membrane, ISEV recommends before RNA extraction performing a proteinase treatment first to eliminate protein complexes that may also protect RNAs from enzymatic degradation, followed by RNase treatment.¹¹² However, residual RNase activity may also damage RNA upon extraction of the luminal contents. Consequently careful consideration of the aim of the study should be first taken into account prior to RNase treatments.

The major drawback of working with EV-derived RNA, especially in the case of those isolated from biofluids, is the poor RNA yield, which is often below the detection limit of current quantification techniques such as fluorimetry (Qubit) and capillary electrophoresis (Bioanalyzer). One possible way of overcoming this limitation is normalizing all samples by the initial biofluid input volume used for extraction, and vacuum-concentrating all the extracted RNA for downstream analysis. Suggestions to improve EV-RNA yield include diluting biological fluid prior to extraction¹¹³ and also the observation that more starting material is not necessarily advantageous due to potential over-loading of sample in column based extraction methods.¹¹⁴ Despite the fact that more EVs are present in serum, plasma is more commonly studied due to the absence of EVs released by platelets during the clotting response.¹¹⁵

5.1.2 Microarray analysis. Microarray technology enabled the seminal discovery in 2007/2008 of RNAs contained in vesicles derived from mast cell lines¹¹⁶ and glioblastoma primary cells as well as patient serum samples.¹¹⁷ These studies used gene expression arrays and first identified thousands of transcripts in EVs, including miRNAs and mRNAs.^{116,117} The mRNA molecules were shown to be functional, leading to protein production in the recipient cells upon transfer of the EV-cargo.¹¹⁷

5.1.3 Quantitative reverse transcriptase-PCR (qRT-PCR) and PCR arrays. Similarly, several studies used quantitative real time PCR-based arrays to profile the RNA content of EVs.^{118–121} This is by far the most widely used method to evaluate and quantify the miRNA content of EVs, due to the low requirements of starting RNA (as little as 1 ng total RNA, using a pre-amplification protocol), the ability to evaluate hundreds of miRNAs of interest and the straightforward analysis of the data that requires simple calculations and no bioinformatics. As most studies that evaluate the RNA-content of EVs aim to identify biomarkers, qRT-PCR is a good, low-cost and reliable tool. The major drawback of this approach is the need defining *a priori* the list of possible markers, as their evaluation depends on the probes/primers available in the selected platform.

During the qRT-PCR procedure *per se*, one of the most important factors is the amount of starting molecules. When direct quantification of EVs cannot be performed, the normalization of the input to volume of sample input is recommended.¹²² Moreover, in an attempt to enhance the amount of amplifiable molecules, pre-amplification methods can be used,¹²³ with no apparent bias. Despite the qRT-PCR approach used (specific probes or non-specific DNA dyes),

results can be strongly biased according to the selected normalization method. A major issue is the absence of known endogenous controls to be used for qRT-PCR. In this sense, the Sample and Assay Standards Working Group of the Extracellular RNA Communication Consortium (ERCC), recently suggested the use of spike-in controls to properly normalize the RNA populations⁴³ an approach that has been successfully used.¹²⁴

5.1.4 Next generation sequencing (NGS). Recently, the RNA content of EVs from diverse sources has been characterised by next generation sequencing (NGS), allowing a more comprehensive analysis of the EV-RNA repertoire.^{125–128} The predominant view is that EVs are enriched in small non-coding RNAs, such as miRNAs, tRNAs and miscellaneous structural RNAs such as Y-RNAs, vault RNAs and SRP-RNAs.¹²⁹ This may be due to the fact that the majority of studies have focused on the small RNA fraction of the transcriptome, by performing either a gel- or bead-based size selection step that limits the analysis to small RNAs (<200 nucleotides).^{129–132} In this way, researchers have bypassed the issue of sequencing abundant rRNAs, but have consequently missed out on the identification of mRNAs and long RNAs that offer great potential as biomarkers. One study that performed whole transcriptome analysis of EVs derived from breast cancer cell lines found 97% of reads were derived from rRNA and argued that EVs are enriched in rRNA molecules that are fragmented and therefore do not appear as peaks on Bioanalyzer analysis.¹³³ Likewise, in another study with EVs derived from epidermoid and hepatocellular carcinoma cell lines, the authors found 92% rRNA, but as they chose not to perform any size selection step they only analysed RNAs greater than 50 nt, which therefore precluded the identification of miRNA molecules and some other small RNAs.¹³⁴ In another recent study with EVs isolated from human pleural effusion and plasma, mRNA amplification was performed using oligo dT primers, restricting the analysis to the poly-adenylated fraction of transcriptome.¹³⁵ An unbiased characterization of the complete vesicular transcriptome is currently lacking, mainly due to methodological constraints that require different library construction protocols for small or long RNA analysis wherein size selections limit the analysis to specific transcript fractions. The limited RNA yield that is obtainable from EVs, particularly those derived from biofluids, oftentimes prohibits the construction of more than one library per sample, not to mention the high costs involved. The depletion of rRNA before sequencing has not been possible also due to limited RNA yield, as the majority of commercial kits require at least 100 ng of RNA input, or has been attempted but was not successful to the fact that fragmented rRNA hinders probe-based capture.¹³³

Crescitelli *et al.* compared the RNA profiles of apoptotic bodies, microvesicles and exosomes isolated by UC from the conditioned medium of three different cell lines.¹¹ However, authors only undertook a Bioanalyzer analysis, which limits the results to RNA size profiles. This could be misleading as typical 28S and 18S rRNA peaks will not be visible if the rRNAs are present as fragments inside EVs, which has been reported



tion and high mass accuracy of survey scan, thereby providing the required accurate mass measurement of the lipid molecular weight. However, partial or complete overlap in the elemental compositions between lipids of different classes imposes a great challenge for their confident identification.¹⁸⁶

Lipid content can be analysed from EV pellets reconstituted in aqueous environment after their UC, or from highly concentrated EVs size exclusion chromatography fractions. Typically, the lipid amount used for analysis is expressed relative to protein quantity, and may vary from 100 μg protein equivalent or more.^{187,188} EV lipids are most commonly extracted using Bligh and Dyer¹⁸⁹ and Folch¹⁹⁰ liquid-liquid extraction, where lipids are enriched in the lower organic layer of chloroform/methanol/water phase. However, both methods carry a risk of contamination by protein from the upper aqueous phase during the collection of organic phase, which may have deleterious effects to the downstream nLC/LC-MS/MS analysis. Additionally, due to the hydrophobic nature of chloroform, both methods may discriminate extraction of partially polar lipids and additionally challenge their detection. Recently methyl-*tert*-butyl-ether (MTBE) extraction of lipids was demonstrated to extract almost all lipid classes from highly complex biological samples with equivalent or better recoveries than the “gold standard” Folch extraction.¹⁹¹ To date, lipidomic profiling of EVs derived from different cells,^{187,192} species¹⁹³ and biological tissues^{194,195} have been published, together with recent reviews on lipidomics of EVs.¹⁷⁶ Since EVs are cell-derived particles, their lipid composition is related to its parent cell. Eventhough, a direct comparison of the lipid profile between exosomes and plasma membrane of the releasing cells allowed the identification of specific lipid classes that appear to be enriched in the exosomal fraction. Membranes of typical EVs are mainly enriched in fully saturated glycerophosphatidylcholines, glycerophosphatidylethanolamines and glycerophosphatidylserines, that are responsible for membrane rigidity and integrity, and sphingomyelin, cholesterol lipids and ganglioside GM3.¹⁹⁶ A study characterized more than 280 lipid species from metastatic prostate cancer cell-line derived exosomes. Exosomes were enriched in glycosphingolipids, sphingomyelin, cholesterol, and phosphatidylserine indicating a particular lipid sorting in the exosome membrane.¹⁹⁷ Although lipid content of EVs attracts increasing attention, not much is known about their biological activity. Sphingomyelin is reported to mediate antigenic activity of tumour-derived EVs both *in vivo* and *in vitro*.⁷⁴ Lysophosphatidylcholines in EVs derived from mature dendritic cells appear to act *via* G-protein coupled receptors and trigger lymphocyte chemotaxis.¹⁹⁸

5.4 Characterization of EVs content: metabolites

During EV formation, small metabolites such as sugars, amino acids, nucleotides, different enzymatic cofactors, lipid metabolites and other redox regulatory molecules can be packed into EVs. Sub-nM concentrations of metabolites and low sample amounts impose a great challenge for the metabolomics of EVs, which may be one of the reasons why this type of analysis is only sporadically reported.^{199–201} Metabolites comprise a

broad array of small molecular weight analytes, derived as intermediates and products of enzymatically-driven cellular processes. Due to their smaller size, metabolites are characterized by their lower hydrophobicity compared to lipids. Thus, they are usually enriched *via* extraction from vesicle pellets with methanol or methanol/water. However, co-extraction of highly abundant lipids may suppress ionization of metabolites of interest and greatly challenge their detection even with the most sensitive targeted mass spectrometric detection. Thus, metabolites of interest are further enriched using solid phase extraction (SPE) cartridges. The choice of SPE chemistry depends solely on the analyte chemical nature and eluting condition. The most common conditions include loading of sample in 10% aqueous methanol (pH 2), hexane washing of hydrophobic lipids, and elution of metabolites in methanolic ethylacetate or butylacetate.^{199,201} Eluates are further vacuum dried and can be stored at $-20\text{ }^{\circ}\text{C}$, or analysed immediately by mass spectrometry.

Although information of the metabolome of EVs is limited, all published studies to date support the significance of EVs as carriers of important metabolome fingerprints which can be used for defining specific changes in cellular homeostasis. Increasing evidence suggests that, during defined stages of the acute inflammatory process, neutrophil-derived EVs carry specific enzymatically-derived polyunsaturated fatty acid (PUFA) metabolites such as 4-, 7-, 14- and 17-hydroxy-docosahexaenoic acid, 5-, 12-, 15-hydroxy-arachidonic acid, 12-, 15- and 18-hydroxy-eicosapentaenoic acid and others.^{199,201} These metabolites are of particular importance because they serve as direct precursors of anti-inflammatory and pro-resolving lipid mediators,²⁰² such as resolvins, maresins, protectins and lipoxins. Prostaglandin (PG) rich vesicles trigger PG-dependent intracellular pathways in the host cells.²⁰³ Thus, depending on the metabolome cargo, EVs in target cells can induce formation of pro-inflammatory, or anti-inflammatory and pro-resolving lipid mediators, and thereby influence inflammatory outcomes and tissue homeostasis.

Metabolomics is an emerging technique with focus on identification and quantification of a wide variety of small molecules which may be indicative of physiological status in health and disease. Metabolites are produced *in vivo*, usually *via* enzymatically-driven processes; thus their circulating levels are very low, and often out of the reach even for the most sensitive detection techniques. Thus, analysis of EV-metabolome relies on a careful and well-designed sample enrichment, and sensitive MS-detection. Recently some authors²⁰⁰ for the first time described methodology for the LC-MS/MS profiling of blood plasma EVs, and EVs derived from cultured cells. Coenzyme Q10, ubiquinone 9,25-hydroxy-hexadecydrovitamin D3, malonyl-CoA, deoxyvitamin D3 and others, are only some of identified metabolites that belong to the wide range of metabolite classes. However, MS-profiling often does not offer the sensitivity necessary for detection of very low abundance metabolites. Targeted mass spectrometry (*e.g.* multiple reaction monitoring, MRM) provides necessary sensitivity and specificity, however it discriminates detection and identifi-



cation of those metabolites that are not “targeted” by designed acquisition methods, and cannot be used for wide-band metabolome profiling.²⁰⁴

5.4.1 Bioinformatic tools used in lipidomics and metabolomics. Comprehensive analysis of large lipidomics and metabolomics data-sets is a challenging and time consuming task. Although significant improvement in the field has been made over the past few years, identification of lipids and metabolites is still not as straightforward as protein identification.²⁰⁵ The LIPID MAPS (<http://www.lipidmaps.org>) is a free of charge online platform for the analysis of MS data based on their accurate mass measurement.²⁰⁶ Recently, LIPID MAPS have implemented a search tool that uses precursor or product ion lists and matches them with thousands of different lipids with *in silico*-generated structures or those present in the database. The Human Metabolome Database (<http://www.hmdb.ca>) is a freely available database that accounts for 42 003 metabolites found in the human body and supports analysis of MS, MS/MS and GC-MS data uploaded in a form of peak lists. LipidXplorer-assisted analysis of large shotgun lipidomics data sets is used routinely for lipid identification, independently of any resource of reference mass spectra, and solely based on assumed molecular fragmentation pathways.²⁰⁷ The software and MFQL library are free for download (https://wiki.mpi-cbg.de/lipidx/Main_Page) and can be used regardless of the mass spectrometer type. It is important to note that the mass resolution and accuracy influence strongly unequivocal lipid identification. Lipid View (ABSciex) and Progenesis QI for small molecules (Waters) are commercially available platforms. Both softwares can use MS and MS/MS that they match against publicly available and in-house databases.

Although many different MS platforms for lipid analyses are available nowadays, analysis of EV-bound metabolites and lipids remains a significant challenge. Very low amount of sample still imposes the greatest challenge for EVs wide-band profiling. To date, both analyses have already advanced our knowledge on biomolecular composition of EVs and helped us better define their structure-to-function relationship. Nevertheless, further advances in lipidomics and metabolomics are necessary to allow application of MS for the analysis of EVs derived from various different sources and biological tissues. The main technical pitfalls of working with EVs and potential ways to avoid them are summarized in Table 1.

6 *In vivo* studies of EV-function

The ability to discriminate vesicle populations of interest from the general EV pool in tissue compartments and bodily fluids remains technically challenging. The cellular origins of EVs, their route of administration, their concentration and their mode of targeting significantly impacts on EV bio-distribution.²²⁴ However, non-traditional routes of EV administration, such as orally administered EVs have shown uptake *via* the intestinal mucosal surface and subsequent accumulation in the liver and spleen.²²⁵ Irrespective of administration route,

a key outcome measure is biodistribution, to trace where exogenously applied EVs go after they have been introduced into an animal.

Fluorescent labelling of cellular proteins have revolutionized *in vivo* studies of cells. However, similar protein marker tracing of EV through EV-associated proteins like CD63-GFP (green fluorescent protein) have been shown to be an effective detection approach. A fraction of EVs is CD63-positive and further work is required to establish the cellular processing of CD63 on EV populations. Numerous protein markers (CD63, TSG101, Alix and heat shock proteins) are widely reported as associated to EVs but their overall contribution to the total EV population remains contentious. Therefore, utilising these proteins as imaging or labelling moieties requires caution. The nanometer-size scale of EVs presents challenges in studies that require spatiotemporal resolution. In addition, exosomes may be as small as 50 nm in size and tagging them with a 5 nm GFP molecule may significantly affect both their biodistribution, kinetics and their functional effects.

Secondary labelling of isolated EV using lipophilic dyes (DiR (1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide, D12731, Invitrogen, Life Technologies, PKH26/67 Sigma-Aldrich or Dil Stain (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate ('DiI'; DiIC18(3))) Thermo)) have shown distribution to the spleen, liver, lungs, pancreas, kidneys and gastrointestinal tract. These lipophilic methods are limited in scope and require the harvest of tissues of interest for analysis, or the use of highly sensitive cameras. Lipophilic dyes may persist in organs and tissues after EV degradation or may associate with other lipids. Injected liposomes that model the EV-population are rapidly cleared from the circulation by the spleen and liver <1 hour after injection, suggesting differences for EV/liposomes clearance based on lipid profiles;²²⁶ whereas Gaussia luciferase combined with metabolic biotinylation greatly enhances the spatiotemporal resolution of EV tracing *in vivo*.²²⁷

Whilst labelling and re-applying EVs *in vivo* enables the investigation of distribution, there is still a paucity of knowledge regarding the downstream effects of these EVs, or even whether the numbers injected are physiologically relevant. Indeed, the rapid generation time of both species, combined with an easily manipulated genome, makes them ideal for studying both the role of EVs in development, as well as the mechanistic underpinnings of their release. It is even possible to study the effect of EVs on stereotypical behaviours in invertebrates; for example Wang *et al.*, 2014²²⁸ have shown that ciliary EVs are responsible for the tail-chasing behaviour seen in some worm species.

However, detailed investigations utilising vertebrates are needed to further test the biodistribution of EVs and ascertain the optimal route of administration for target organs or tissues. Rodents, which are traditionally used in preclinical research, provide rapid breeding cycle, complete genome sequence and some physiological similarities to humans. Two key biodistribution studies in rodents were carried out by Xandra Breakefield's and Samir El Andaloussi's group, both





Table 1 Potential technical pitfalls, recommendations and remaining challenges according to different techniques of EVs isolation and characterization

	Sample type/technique	Potential pitfalls	Recommendations	Remaining challenges
Pre-analytical factors	Cell culture conditioned medium	Presence of contaminating bovine vesicles and proteins from FBS leading to misinterpretation of EV quantification/characterisation Use of EV-depleted FBS ²⁰⁹ or serum starvation ²¹⁰ may stress cells leading to alterations in EVs concentration and content	The use of EV-depleted FBS is recommended ²⁰⁸ In the case of complete serum removal, cells should be assessed for stress induction that could alter EVs characteristics. Autophagy, apoptosis and necrosis should be monitored	Much needed standardisation of protocols by the EV-community; current lack of knowledge regarding serum starvation stress over optimal duration of EVs production, concentration and content
	Blood	Artificial inflation of EVs counts <i>ex vivo</i> as a result of platelet activation due to blood collection, blood transportation and excessive delay in plasma separation Changes in EVs counts/cargo observed are not related to particular disease/phenotype being investigated, but are rather due to other clinical and environmental factors that influence EVs counts such as age, gender, circadian-clock, medications, fasting state, hormonal status, presence of infection, <i>etc.</i> ; abundant proteins may hamper the identification of bona fide EV protein cargo	Follow guidelines such as those from the International Society on Thrombosis and Haemostasis, including citrated tubes for blood collection, and adequate separation of platelet-free plasma using two rounds of centrifugations immediately after blood collection. Abundant proteins (such as albumin and immunoglobulins) should be depleted Compare with a matched control group without disease and try to normalize as much as possible the differences between individuals within a group, controlling for all factors cited	Difficulty applying rigorous blood collection and plasma separation/storage in a large-scale clinical setting Unmet need for technological advances that allow disease-specific EVs to be isolated, quantified and characterized separately from all other normal-cell-derived EVs
	Urine	Membrane-bound proteins could be affected by proteases in the urine; EVs may remain attached to the collection tubes	Urine should be collected in tubes containing protease-inhibitors; containers should be vortexed to avoid EVs being adhered to tube wall; abundant proteins (such as uromodulin) should be depleted	Handling of large volume samples and the need to concentrate urine for the adequate isolation of EVs challenges its use in a clinical setting
EV isolation protocols	Ultracentrifugation (UC)	Variability in sedimentation efficiencies, no absolute separation of EVs subpopulations. High centrifugal forces may lead to EVs aggregation and disruption of larger EVs; lower centrifugal forces reduce recovery of smaller EVs Co-sedimentation of protein, lipoprotein and RNA aggregates as well as viral particles	Low-angle fixed-angle rotors provide better separation efficiency ²¹¹ and reducing bio-fluid viscosity prior to centrifugation improves recovery; ^{113,212} protocols need to be adjusted according to the targeted EV-subtypes Combine ultracentrifugation with density gradients to further purify samples ⁶⁶	There is a lack of methods to allow the precise assessment of EV damage and the recovery of the full range of EVs
	Density gradients (DG)	Sample fractionation leads to EVs being divided into numerous sub-samples	Ensure higher initial EV counts due to substantial dilution of EVs among the sub-samples	Ultracentrifugation steps as well as the preparation of the gradient are time consuming; the process is laborious and difficult to be implemented in a clinical setting
	Immunoaffinity	Concentrated EV samples are required in small volumes Markers must be pre-selected by the user	Spin columns, precipitation and other methods can be used to reduce sample volume Characterise markers specific to EVs in the system of interest, it has however been demonstrated that markers vary between EVs of interest ²¹³	Clearer definition of EV subpopulations and their characteristic markers. Less aggressive dissociation of EVs from beads to avoid affecting functionality. Minimizing antibody contamination and buffers' interference



Table 1 (Contd.)

Sample type/technique	Potential pitfalls	Recommendations	Remaining challenges	
Commercial kits	Saturation of beads with abundant, non-EV proteins found in biofluids. Elution of EVs from beads might damage the structure of vesicles. Captured vesicles might not retain functionality after elution. Eluted antibodies or antibody remnants and harsh elution buffers might interfere with downstream analysis	Coat beads to minimise non-specific adsorption of abundant proteins from serum samples ²¹⁴	The unknown biochemical basis of many kits restricts our ability to fully understand EV populations extracted and the non-vesicular contaminants	
	Contaminating non-vesicular molecules (such as protein, protein aggregates, lipids and free nucleic acids) may occur in all isolation methods, but may be more predominant here due to precipitation process	Evaluate the more prevalent non-vesicular contaminants of the sample under investigation and test kit to establish optimal protocol for experimental aim		
	Potential interference by elution buffers in downstream applications	Include controls in functional tests checking the effects of kit elution buffers on cells receiving EVs		
Size exclusion chromatography (SEC)	Inability to concentrate diluted EV sample, which, often requires second step <i>i.e.</i> re-concentration of EVs by ultrafiltration ^{56,80,81,83–86}	To rapidly concentrate EV fractions the application of protein concentrating devices is recommended	Concentrating devices need to be standardized in order to allow results to be comparable across studies	
Ultrafiltration (UF)	Non-specific binding of EVs to column membranes leading to reduced yield owing to trapping in filter pores ^{35,56}	Ultrafiltration coupled with SEC is recommended for enhanced EV purity, preserving their properties ^{56,60}	Forcing vesicles through filters might damage larger EVs whereas the use of less force, through gravity or by using less pressure makes the process longer and less adequate in some cases ⁴⁰	
Characterisation of isolated EVs	Electron microscopy (EM)	Dehydration and fixation of EVs during processing may disrupt membranes leading to artefacts Inability to readily discern heterogeneous EV populations	Use cryo-EM in combination with immunogold labelling, where samples are not dehydrated ²¹⁵ Use of alternative super-resolution microscopy techniques <i>i.e.</i> PALM ²¹⁶ or STORM ²¹⁷	Development of new methods both quantifying and characterising EVs. No currently available EM approaches for routine analysis of EVs in a clinical scenario
	Nanoparticle tracking analysis (NTA)/resistive pulse sensing (RPS)	Resuspension buffers may contain particles misinterpreted as vesicles Accuracy in reporting size and concentration is debatable	Include controls to ensure that EV suspension buffers analysed in order to establish a control/background EVs or their contents can be GFP labelled to ensure proper analysis. ¹⁰² Bead calibration should be used ²¹⁸	Polystyrene bead refractive index not identical to that of EVs. Current issues in discerning clustered from non-clustered vesicles, especially in samples of higher EV-concentration (NTA). Establishment of lower detection limit for RPS
	Flow cytometry (FCM)	Distinction between single particle positive and negative EV samples Scatter resolution	Use reciprocal dilution to ensure single particle detection or use multicolour and multiplex approaches to resolve individual vesicles ²¹⁹ Use scatter beads of known size and refractive index. Use fluorescence beads with known molecules of equivalent soluble fluorophore (MESF) units ²²⁰	High refractive index of commonly used reference beads compared to EVs
Characterisation of EV contents	RNA purification	Low RNA yields DNA contamination in EV RNA samples	Normalize all samples by the initial biofluid input volume used for extraction, and vacuum-concentrate extracted RNA for downstream analysis DNase treatment of RNA samples should be performed prior to downstream analysis	Difficulty in eliminating residual RNase that would reduce the available luminal EVs RNA
		Non-vesicular RNA complexed with proteins may be bound to the outside of the EV membrane	Use proteinase followed by RNase treatment of intact vesicles to eliminate protein complexes protecting non-vesicular	



Table 1 (Contd.)

Sample type/technique	Potential pitfalls	Recommendations	Remaining challenges
RNA analysis by qRT-PCR	Low RNA yield, especially from clinical samples, may hamper the quantification and analysis of less abundant RNA populations	extracellular RNAs from enzymatic degradation ²²¹ Pre-amplification can enhance the amount of amplifiable molecules with no apparent bias. ¹²³ Use spike-in controls to properly normalize the RNA populations. ¹²⁴ If possible, use multiple endogenous controls based on EV-transcriptomic data, or use absolute quantification (by digital PCR, for example) for validation	The technique is intrinsically limited to a very specific set of pre-selected RNAs, not allowing a broader view of the EV-transcriptome
RNA analysis by NGS	Library preparation issues: adapter dimers, ligation and size-selection bias	Ribosomal RNA (rRNA) depletion may be unsuitable due to low starting input of RNA and presence of fragmented rRNA. Extensive fragmentation of RNA may be used to generate total transcriptome library and analyse small and long RNAs simultaneously. ¹²⁸ Reduce adapter dimers using specific kits ⁵⁰ or use fragmented RNA to minimize artefactual adapter dimer formation and preclude size selection ¹²⁸	Fragmentation of rRNA species hinders efficient rRNA removal. ¹³³ Excessive RNA fragmentation can lead to difficulties in properly mapping the short reads. Comprehensive EV-transcriptional databases are lacking, especially from NGS data derived from clinical samples
Protein analysis	Co-purification of protein, protein aggregates and lipoprotein contaminants	Remove abundant proteins such as immunoglobulins by combining ultracentrifugation and protein G agarose bead incubation. ²²² especially when working with EVs derived from biofluids	The depletion of contaminant proteins requires a significant mass which is not always available from clinical samples. Proteomics methods need to be developed to accommodate the analysis of reduced mass of protein
	Heterogeneity of protein markers on different EV populations, for example presence of CD63, HSP70, flotillin-1, and actin present in both exosomes and microvesicles Contamination of samples with cellular components	Use a range of markers or non-marker-specific methods to distinguish differences between cells and EVs such as silver staining or SYPRO Ruby staining. ¹⁶⁴ Non-EVs proteins, such as calnexin, GM130, cytochrome C and histones, should be used as negative controls to ensure purity of isolation ¹⁵⁸	Identification of protein markers capable of differentiating specific EVs subtypes
	Protein degradation during vesicle manipulation	Use of protease and phosphatase inhibitors is highly recommended during EV protein extraction	Protease treatment needs to be standardized according to the biofluid-origin of the EVs under study
Lipid analysis	Contamination by proteins during extraction Difficulty in extracting and representing all lipid classes	Liquid-liquid extraction is preferred method for EV lipid analysis. ^{189,190} Use of internal standards is recommended to ensure high mass accuracy and confident identification. If possible use LC to ensure separation of lipids prior to sensitive MS/MS. MTBE extraction was shown to extract all lipid classes with better recovery than the Folch method ¹⁹¹	In liquid-liquid extraction protein contamination from the aqueous phase and bias towards extraction of partially polar lipids may occur. Public databases of lipids are still far from complete
Metabolome analysis	Low metabolite yields and reproducibility (LC/MS)	Enrich sample using solid phase extraction prior to MS. ^{199,201} Major lipid and protein species should be removed in this process to ensure sensitive MS analysis	Development of better bioinformatic analysis pipelines for metabolites. Public databases of metabolites are still far from complete
	Cell culture metabolite contamination	Use of a cell-free medium control ²²³	

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors acknowledge the support received from the British Council/Newton Fund (203855854), Fundação de Amparo à Pesquisa do Estado de São Paulo (2012/24574-3; 2015/50257-3; 2014/26897-0; 2014/06863-3), Associação Beneficente Alzira Denise Hertzog Silva (ABADHS), Conselho Nacional de Desenvolvimento Científico e Tecnológico (441878/2014-8; 202077/2015-2), Brazil; Ricerca Finalizzata (Convenzione no. 172/GR-2011-02350301) from the Ministero della Salute, Italy; Science without Borders/CAPES, Brazil (BEX 7057/15-6). MIR and ED-N are research fellows from CNPq, Brazil.

References

- 1 M. Yáñez-Mó, P. R.-M. Siljander, Z. Andreu, A. B. Zavec, F. E. Borràs, E. I. Buzas, K. Buzas, E. Casal, F. Cappello, J. Carvalho, E. Colás, A. Cordeiro-da Silva, S. Fais, J. M. Falcon-Perez, I. M. Ghobrial, B. Giebel, M. Gimona, M. Graner, I. Gursel, M. Gursel, N. H. H. Heegaard, A. Hendrix, P. Kierulf, K. Kokubun, M. Kosanovic, V. Kralj-Iglic, E.-M. Krämer-Albers, S. Laitinen, C. Lässer, T. Lener, E. Ligeti, A. Linē, G. Lipps, A. Llorente, J. Lötvall, M. Manček-Keber, A. Marcilla, M. Mittelbrunn, I. Nazarenko, E. N. M. Nolte-t Hoen, T. A. Nyman, L. O'Driscoll, M. Olivan, C. Oliveira, É. Pállinger, H. A. Del Portillo, J. Reventós, M. Rigau, E. Rohde, M. Sammar, F. Sánchez-Madrid, N. Santarém, K. Schallmoser, M. S. Ostendorf, W. Stoorvogel, R. Stukelj, S. G. Van der Grein, M. H. Vasconcelos, M. H. M. Wauben and O. De Wever, *J. Extracell. Vesicles*, 2015, **4**, 27066.
- 2 G. Desdín-Micó and M. Mittelbrunn, *Cell Adhes. Migr.*, 2017, **11**, 127–134.
- 3 T. Takeuchi, M. Suzuki, N. Fujikake, H. A. Popiel, H. Kikuchi, S. Futaki, K. Wada and Y. Nagai, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, E2497–E2506.
- 4 C. Théry, S. Amigorena, G. Raposo and A. Clayton, *Curr Protoc cell Biol*, 2006, ch. 3, pp. 3.22.1–3.22.29.
- 5 H. F. Heijnen, A. E. Schiel, R. Fijnheer, H. J. Geuze and J. J. Sixma, *Blood*, 1999, **94**, 3791–3799.
- 6 G. K. Atkin-Smith, R. Tixeira, S. Paone, S. Mathivanan, C. Collins, M. Liem, K. J. Goodall, K. S. Ravichandran, M. D. Hulett and I. K. H. Poon, *Nat. Commun.*, 2015, **6**, 7439.
- 7 D. Di Vizio, J. Kim, M. H. Hager, M. Morello, W. Yang, C. J. Lafargue, L. D. True, M. A. Rubin, R. M. Adam, R. Beroukhi, F. Demichelis and M. R. Freeman, *Cancer Res.*, 2009, **69**, 5601–5609.
- 8 M. Nawaz, G. Camussi, H. Valadi, I. Nazarenko, K. Ekström, X. Wang, S. Principe, N. Shah, N. M. Ashraf, F. Fatima, L. Neder and T. Kislinger, *Nat. Rev. Urol.*, 2014, **11**, 688–701.
- 9 J. Kowal, G. Arras, M. Colombo, M. Jouve, J. P. Morath, B. Primdal-Bengtson, F. Dingli, D. Loew, M. Tkach and C. Théry, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, E968–E977.
- 10 G. Kibria, E. K. Ramos, K. E. Lee, S. Bedoyan, S. Huang, R. Samaeekia, J. J. Athman, C. V. Harding, J. Lötvall, L. Harris, C. L. Thompson and H. Liu, *Sci. Rep.*, 2016, **6**, 36502.
- 11 R. Crescitelli, C. Lässer, T. G. Szabo, A. Kittel, M. Eldh, I. Dianzani, E. I. Buzás and J. Lötvall, *J. Extracell. Vesicles*, 2013, **2**, 1–10.
- 12 T. R. Lunavat, L. Cheng, D.-K. Kim, J. Bhadury, S. C. Jang, C. Lässer, R. A. Sharples, M. D. López, J. Nilsson, Y. S. Ghossein, A. F. Hill and J. Lötvall, *RNA Biol.*, 2015, **12**, 810–823.
- 13 J. S. Schorey and S. Bhatnagar, *Traffic*, 2008, **9**, 871–881.
- 14 S. Yu, H. Cao, B. Shen and J. Feng, *Oncotarget*, 2015, **6**, 37151–37168.
- 15 A. Hoshino, B. Costa-Silva, T.-L. Shen, G. Rodrigues, A. Hashimoto, M. Tesic Mark, H. Molina, S. Kohsaka, A. Di Giannatale, S. Ceder, S. Singh, C. Williams, N. Soplop, K. Uryu, L. Pharmed, T. King, L. Bojmar, A. E. Davies, Y. Ararso, T. Zhang, H. Zhang, J. Hernandez, J. M. Weiss, V. D. Dumont-Cole, K. Kramer, L. H. Wexler, A. Narendran, G. K. Schwartz, J. H. Healey, P. Sandstrom, K. J. Labori, E. H. Kure, P. M. Grandgenett, M. A. Hollingsworth, M. de Sousa, S. Kaur, M. Jain, K. Mallya, S. K. Batra, W. R. Jarnagin, M. S. Brady, O. Fodstad, V. Muller, K. Pantel, A. J. Minn, M. J. Bissell, B. A. Garcia, Y. Kang, V. K. Rajasekhar, C. M. Ghajar, I. Matei, H. Peinado, J. Bromberg and D. Lyden, *Nature*, 2015, **527**, 329–335.
- 16 E. M. Mora, S. Álvarez-Cubela and E. Oltra, *Int. J. Mol. Sci.*, 2016, **17**, 13.
- 17 M.-P. Caby, D. Lankar, C. Vincendeau-Scherrer, G. Raposo and C. Bonnerot, *Int. Immunol.*, 2005, **17**, 879–887.
- 18 O. E. Bryzgunova, M. M. Zaripov, T. E. Skvortsova, E. A. Lekchnov, A. E. Grigor'eva, I. A. Zaporozhchenko, E. S. Morozkin, E. I. Ryabchikova, Y. B. Yurchenko, V. E. Voitsitskiy and P. P. Laktionov, *PLoS One*, 2016, **11**, 1–17.
- 19 Y. Ogawa, M. Kanai-Azuma, Y. Akimoto, H. Kawakami and R. Yanoshita, *Biol. Pharm. Bull.*, 2008, **31**, 1059–1062.
- 20 C. Lässer, V. S. Alikhani, K. Ekström, M. Eldh, P. T. Paredes, A. Bossios, M. Sjöstrand, S. Gabrielsson, J. Lötvall and H. Valadi, *J. Transl. Med.*, 2011, **9**, 9.
- 21 J. C. Akers, V. Ramakrishnan, R. Kim, S. Phillips, V. Kaimal, Y. Mao, W. Hua, I. Yang, C.-C. Fu, J. Nolan, I. Nakano, Y. Yang, M. Beaulieu, B. S. Carter and C. C. Chen, *J. Neuro-Oncol.*, 2015, **123**, 205–216.
- 22 R. Cappellesso, A. Tinazzi, T. Giurici, F. Simonato, V. Guzzardo, L. Ventura, M. Crescenzi, S. Chiarelli and A. Fassina, *Cancer Cytopathol.*, 2014, **122**, 685–693.



- 23 H.-I. Choi, J.-P. Choi, J. Seo, B. J. Kim, M. Rho, J. K. Han and J. G. Kim, *Exp. Mol. Med.*, 2017, **49**, e330.
- 24 L. Li, K. B. Piontek, V. Kumbhari, M. Ishida and F. M. Selaru, *J. Visualized Exp.*, 2016, 7–9.
- 25 C. Porro, S. Lepore, T. Trotta, S. Castellani, L. Ratclif, A. Battagliano, S. Di Gioia, M. C. Martínez, M. Conese and A. B. Maffione, *Respir. Res.*, 2010, **11**, 94.
- 26 C. J. E. Wahlund, A. Eklund, J. Grunewald and S. Gabrielsson, *Front. Cell Dev. Biol.*, 2017, **5**, 39.
- 27 M. N. Madison, J. L. Welch and C. M. Okeoma, *Bio-Protoc.*, 2017, **7**, e2216.
- 28 A. E. Grigor'eva, S. N. Tamkovich, A. V. Eremina, A. E. Tupikin, M. R. Kabilov, V. V. Chernykh, V. V. Vlassov, P. P. Laktionov and E. I. Ryabchikova, *Biomed. Khim.*, 2016, **62**, 99–106.
- 29 N. S. Barteneva, E. Fasler-Kan, M. Bernimoulin, J. N. H. Stern, E. D. Ponomarev, L. Duckett and I. A. Vorobjev, *BMC Cell Biol.*, 2013, **14**, 23.
- 30 C. C. Pritchard, E. Kroh, B. Wood, J. D. Arroyo, K. J. Dougherty, M. M. Miyaji, J. F. Tait and M. Tewari, *Cancer Prev. Res.*, 2012, **5**, 492–497.
- 31 B. Fendl, R. Weiss, M. B. Fischer, A. Spittler and V. Weber, *Biochem. Biophys. Res. Commun.*, 2016, **478**, 168–173.
- 32 L. Wisgrill, C. Lamm, J. Hartmann, F. Preißing, K. Dragosits, A. Bee, L. Hell, J. Thaler, C. Ay, I. Pabinger, A. Berger and A. Spittler, *Cytometry, Part A*, 2016, **89**, 663–672.
- 33 R. Lacroix, C. Judicone, P. Poncelet, S. Robert, L. Arnaud, J. Sampol and F. Dignat-George, *J. Thromb. Haemostasis*, 2012, **10**, 437–446.
- 34 T. Meninger, G. Lerman, N. Regev-Rudzki, D. Gold, I. Z. Ben-Dov, Y. Sidi, D. Avni and E. Schwartz, *J. Infect. Dis.*, 2017, **215**, 378–386.
- 35 G. Vergauwen, B. Dhondt, J. Van Deun, E. De Smedt, G. Berx, E. Timmerman, K. Gevaert, I. Miinalainen, V. Cocquyt, G. Braems, R. Van den Broecke, H. Denys, O. De Wever and A. Hendrix, *Sci. Rep.*, 2017, **7**, 2704.
- 36 L.-G. Liang, M.-Q. Kong, S. Zhou, Y.-F. Sheng, P. Wang, T. Yu, F. Inci, W. P. Kuo, L.-J. Li, U. Demirci and S. Wang, *Sci. Rep.*, 2017, **7**, 46224.
- 37 K. W. Pedersen, B. Kierulf and A. Neurauter, *Methods Mol. Biol.*, 2017, **1660**, 65–87.
- 38 S. I. Gilani, U. D. Anderson, M. Jayachandran, T. L. Weissgerber, L. Zand, W. M. White, N. Milic, M. L. G. Suarez, R. R. Vallapureddy, Å. Nääv, L. Erlandsson, J. C. Lieske, J. P. Grande, K. A. Nath, S. R. Hansson and V. D. Garovic, *J. Am. Soc. Nephrol.*, 2017, **28**, 3363–3372.
- 39 M. I. Zonneveld, A. R. Brisson, M. J. C. van Herwijnen, S. Tan, C. H. A. van de Lest, F. A. Redegeld, J. Garssen, M. H. M. Wauben and E. N. Nolte-'t Hoen, *J. Extracell. Vesicles*, 2014, **3**, 1–12.
- 40 K. W. Witwer, E. I. Buzás, L. T. Bemis, A. Bora, C. Lässer, J. Lötvall, E. N. Nolte-'t Hoen, M. G. Piper, S. Sivaraman, J. Skog, C. Théry, M. H. Wauben and F. Hochberg, *J. Extracell. Vesicles*, 2013, **2**, 20360.
- 41 Z. Wei, A. O. Batagov, D. R. F. Carter and A. M. Krichevsky, *Sci. Rep.*, 2016, **6**, 31175.
- 42 G. V. Shelke, C. Lässer, Y. S. Gho and J. Lötvall, *J. Extracell. Vesicles*, 2014, **3**, 1–8.
- 43 L. C. Laurent, A. B. Abdel-Mageed, P. D. Adelson, J. Arango, L. Balaj, X. Breakefield, E. Carlson, B. S. Carter, B. Majem, C. C. Chen, E. Cocucci, K. Danielson, A. Courtright, S. Das, Z. Y. Abd Elmageed, D. Enderle, A. Ezrin, M. Ferrer, J. Freedman, D. Galas, R. Gandhi, M. J. Huentelman, K. Van Keuren-Jensen, Y. Kalani, Y. Kim, A. M. Krichevsky, C. Lai, M. Lal-Nag, C. D. Laurent, T. Leonardo, F. Li, I. Malenica, D. Mondal, P. Nejad, T. Patel, R. L. Raffai, R. Rubio, J. Skog, R. Spetzler, J. Sun, K. Tanriverdi, K. Vickers, L. Wang, Y. Wang, Z. Wei, H. L. Weiner, D. Wong, I. K. Yan, A. Yeri and S. Gould, *J. Extracell. Vesicles*, 2015, **4**, 26533.
- 44 Z. Wei, A. O. Batagov, D. R. F. Carter and A. M. Krichevsky, *Sci. Rep.*, 2016, **6**, 31175.
- 45 C. Théry, M. Boussac, P. Véron, P. Ricciardi-Castagnoli, G. Raposo, J. Garin and S. Amigorena, *J. Immunol.*, 2001, **166**, 7309–7318.
- 46 Á. M. Lőrincz, C. I. Timár, K. A. Marosvári, D. S. Veres, L. Otrokocsi, Á. Kittel and E. Ligeti, *J. Extracell. Vesicles*, 2014, **3**, 25465.
- 47 Y. Yuana, A. N. Böing, A. E. Grootemaat, E. van der Pol, C. M. Hau, P. Cizmar, E. Buhr, A. Sturk and R. Nieuwland, *J. Extracell. Vesicles*, 2015, **4**, 29260.
- 48 H. Kalra, C. G. Adda, M. Liem, C.-S. Ang, A. Mechler, R. J. Simpson, M. D. Hulett and S. Mathivanan, *Proteomics*, 2013, **13**, 3354–3364.
- 49 J. Lötvall, A. F. Hill, F. Hochberg, E. I. Buzás, 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. Théry, *J. Extracell. Vesicles*, 2014, **3**, 26913.
- 50 B. Mateescu, E. J. K. Kowal, B. W. M. van Balkom, S. Bartel, S. N. Bhattacharyya, E. I. Buzás, A. H. Buck, P. de Candia, F. W. N. Chow, S. Das, T. A. P. Driedonks, L. Fernández-Messina, F. Haderk, A. F. Hill, J. C. Jones, K. R. Van Keuren-Jensen, C. P. Lai, C. Lässer, I. di Liegro, T. R. Lunavat, M. J. Lorenowicz, S. L. N. Maas, I. Mäger, M. Mittelbrunn, S. Momma, K. Mukherjee, M. Nawaz, D. M. Pegtel, M. W. Pfaffl, R. M. Schifferlers, H. Tahara, C. Théry, J. P. Tosar, M. H. M. Wauben, K. W. Witwer and E. N. M. Nolte-'t Hoen, *J. Extracell. Vesicles*, 2017, **6**, 1286095.
- 51 EV-TRACK Consortium, J. Van Deun, P. Mestdagh, P. Agostinis, Ö. 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. P. Driedonks, A. Dudek, A. Elsharawy, I. Floris, A. D. Foers, K. Gärtner, A. D. Garg, E. Geeurickx, J. Gettemans, F. Ghazavi, B. Giebel, T. G. Kormelink, G. Hancock, H. Helmoortel, 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. Matussek, G. Milani, M. Monguió-Tortajada, L. M. Mus, D. C. Muth, A. Németh, E. N. M. 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. Théry, 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, *Nat. Methods*, 2017, **14**, 228–232.
- 52 C. Gardiner, D. Di Vizio, S. Sahoo, C. Théry, K. W. Witwer, M. Wauben and A. F. Hill, *J. Extracell. Vesicles*, 2016, **5**, 32945.
- 53 Z. Andreu, E. Rivas, A. Sanguino-Pascual, A. Lamana, M. Marazuela, I. González-Alvaro, F. Sánchez-Madrid, H. de la Fuente and M. Yáñez-Mó, *J. Extracell. Vesicles*, 2016, **5**, 31655.
- 54 C. Théry, S. Amigorena, G. Raposo and A. Clayton, *Curr Protoc cell Biol*, 2006, ch. 3, unit 3.22.
- 55 R. T. Davies, J. Kim, S. C. Jang, E.-J. Choi, Y. S. Gho and J. Park, *Lab Chip*, 2012, **12**, 5202.
- 56 J. Z. Nordin, Y. Lee, P. Vader, I. Mäger, H. J. Johansson, W. Heusermann, O. P. B. Wiklander, M. Hällbrink, Y. Seow, J. J. Bultema, J. Gilthorpe, T. Davies, P. J. Fairchild, S. Gabrielsson, N. C. Meisner-Kober, J. Lehtiö, C. I. E. Smith, M. J. A. Wood and S. El Andaloussi, *Nanomedicine*, 2015, **11**, 879–883.
- 57 G. Raposo and W. Stoorvogel, *J. Cell Biol.*, 2013, **200**, 373–383.
- 58 H. G. Lamparski, A. Metha-Damani, J.-Y. Yao, S. Patel, D.-H. Hsu, C. Ruegg and J.-B. Le Pecq, *J. Immunol. Methods*, 2002, **270**, 211–226.
- 59 Z. Zhang, C. Wang, T. Li, Z. Liu and L. Li, *Oncol. Lett.*, 2014, **8**, 1701–1706.
- 60 R. J. Lobb, M. Becker, S. W. Wen, C. S. F. Wong, A. P. Wiegman, A. Leimgruber and A. Möller, *J. Extracell. Vesicles*, 2015, **4**, 27031.
- 61 B. J. Tauro, D. W. Greening, R. A. Mathias, H. Ji, S. Mathivanan, A. M. Scott and R. J. Simpson, *Methods*, 2012, **56**, 293–304.
- 62 E. Nolte-t Hoen, T. Cremer, R. C. Gallo and L. B. Margolis, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, 9155–9161.
- 63 B. W. Sódar, Á. Kittel, K. Pálóczi, K. V. Vukman, X. Osteikoetxea, K. Szabó-Taylor, A. Németh, B. Sperlágh, T. Baranyai, Z. Giricz, Z. Wiener, L. Turiák, L. Drahos, É. Pállinger, K. Vékey, P. Ferdinandy, A. Falus and E. I. Buzás, *Sci. Rep.*, 2016, **6**, 24316.
- 64 Y. Yuana, J. Levels, A. Grootemaat, A. Sturk and R. Nieuwland, *J. Extracell. Vesicles*, 2014, **3**, 23262.
- 65 A. Kamińska, M. Platt, J. Kasprzyk, B. Kuśnierz-Cabala, A. Gala-Błądzińska, O. Woźnicka, B. R. Jany, F. Krok, W. Piekoszewski, M. Kuźniewski and E. Ł. Stępień, *J. Diabetes Res.*, 2016, **2016**, 5741518.
- 66 R. Cantin, J. Diou, D. Bélanger, A. M. Tremblay and C. Gilbert, *J. Immunol. Methods*, 2008, **338**, 21–30.
- 67 S. Mathivanan, J. W. E. Lim, B. J. Tauro, H. Ji, R. L. Moritz and R. J. Simpson, *Mol. Cell. Proteomics*, 2010, **9**, 197–208.
- 68 K. Koga, K. Matsumoto, T. Akiyoshi, M. Kubo, N. Yamanaka, A. Tasaki, H. Nakashima, M. Nakamura, S. Kuroki, M. Tanaka and M. Katano, *Anticancer Res.*, 2005, **25**, 3703–3707.
- 69 A. Liga, A. D. B. Vliegthart, W. Oosthuyzen, J. W. Dear and M. Kersaudy-Kerhoas, *Lab Chip*, 2015, **15**, 2388–2394.
- 70 R. Xu, D. W. Greening, H.-J. Zhu, N. Takahashi and R. J. Simpson, *J. Clin. Invest.*, 2016, **126**, 1152–1162.
- 71 H. Kalra, C. G. Adda, M. Liem, C. S. Ang, A. Mechler, R. J. Simpson, M. D. Hulett and S. Mathivanan, *Proteomics*, 2013, **13**, 3354–3364.
- 72 C. E. Yoo, G. Kim, M. Kim, D. Park, H. J. Kang, M. Lee and N. Huh, *Anal. Biochem.*, 2012, **431**, 96–98.
- 73 L. Balaj, N. A. Atai, W. Chen, D. Mu, B. A. Tannous, X. O. Breakefield, J. Skog and C. A. Maguire, *Sci. Rep.*, 2015, **5**, 10266.
- 74 C. W. Kim, H. M. Lee, T. H. Lee, C. Kang, H. K. Kleinman and Y. S. Gho, *Cancer Res.*, 2002, **62**, 6312–6317.
- 75 A. Ghosh, M. Davey, I. C. Chute, S. G. Griffiths, S. Lewis, S. Chacko, D. Barnett, N. Crapoulet, S. Fournier, A. Joy, M. C. Caissie, A. D. Ferguson, M. Daigle, M. V. Meli, S. M. Lewis and R. J. Ouellette, *PLoS One*, 2014, **9**, e110443.
- 76 D. K. Jeppesen, M. L. Hvam, B. Primdahl-Bengtson, A. T. Boysen, B. Whitehead, L. Dyrskjöt, T. F. Orntoft, K. A. Howard and M. S. Ostfeld, *J. Extracell. Vesicles*, 2014, **3**, 25011.
- 77 C. Chen, J. Skog, C.-H. Hsu, R. T. Lessard, L. Balaj, T. Wurdinger, B. S. Carter, X. O. Breakefield, M. Toner and D. Irimia, *Lab Chip*, 2010, **10**, 505–511.
- 78 S. S. Kanwar, C. J. Dunlay, D. M. Simeone and S. Negrath, *Lab Chip*, 2014, **14**, 1891–1900.
- 79 D. Taller, K. Richards, Z. Slouka, S. Senapati, R. Hill, D. B. Go and H.-C. Chang, *Lab Chip*, 2015, **15**, 1656–1666.
- 80 C.-S. Hong, S. Funk, L. Muller, M. Boyiadzis and T. L. Whiteside, *J. Extracell. Vesicles*, 2016, **5**, 29289.
- 81 T. Baranyai, K. Herczeg, Z. Onódi, I. Voszka, K. Módos, N. Marton, G. Nagy, I. Mäger, M. J. Wood, S. El Andaloussi, Z. Pálincás, V. Kumar, P. Nagy, Á. Kittel, E. I. Buzás, P. Ferdinandy and Z. Giricz, *PLoS One*, 2015, **10**, e0145686.
- 82 F. A. W. Coumans, A. R. Brisson, E. I. Buzas, F. Dignat-George, E. E. E. Drees, S. El-Andaloussi, C. Emanuelli, A. Gasecka, A. Hendrix, A. F. Hill, R. Lacroix, Y. Lee, T. G. van Leeuwen, N. Mackman, I. Mäger, J. P. Nolan, E. van der Pol, D. M. Pegtel, S. Sahoo, P. R. M. Siljander, G. Sturk, O. de Wever and R. Nieuwland, *Circ. Res.*, 2017, **120**, 1632–1648.
- 83 B. W. Sódar, Á. Kovács, T. Visnovitz, É. Pállinger, K. Vékey, G. Pocsfalvi, L. Turiák and E. I. Buzás, *Expert Rev. Proteomics*, 2017, 1–18.



- E. Y. Yu, C. S. Higano and M. Tewari, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 14888–14893.
- 122 D. Enderle, A. Spiel, C. M. Coticchia, E. Berghoff, R. Mueller, M. Schlumpberger, M. Sprenger-Haussels, J. M. Shaffer, E. Lader, J. Skog and M. Noerholm, *PLoS One*, 2015, **10**, 1–19.
- 123 L. Moldovan, K. Batte, Y. Wang, J. Wisler and M. Piper, *Methods Mol. Biol.*, 2013, **1024**, 129–145.
- 124 M. J. Marzi, F. Montani, R. M. Carletti, F. Dezi, E. Dama, G. Bonizzi, M. T. Sandri, C. Rampinelli, M. Bellomi, P. Maisonneuve, L. Spaggiari, G. Veronesi, F. Bianchi, P. P. Di Fiore and F. Nicassio, *Clin. Chem.*, 2016, **62**, 743–754.
- 125 K. C. Miranda, D. T. Bond, J. Z. Levin, X. Adiconis, A. Sivachenko, C. Russ, D. Brown, C. Nusbaum and L. M. Russo, *PLoS One*, 2014, **9**, e96094.
- 126 L. Cheng, X. Sun, B. J. Scicluna, B. M. Coleman and A. F. Hill, *Kidney Int.*, 2014, **86**, 433–444.
- 127 A. Eirin, S. M. Riester, X.-Y. Zhu, H. Tang, J. M. Evans, D. O'Brien, A. J. van Wijnen and L. O. Lerman, *Gene*, 2014, **551**, 55–64.
- 128 M. G. Amorim, R. Valieris, R. D. Drummond, M. P. Pizzi, V. M. Freitas, R. Sinigaglia-Coimbra, G. A. Calin, R. Pasqualini, W. Arap, I. T. Silva, E. Dias-Neto and D. N. Nunes, *Sci. Rep.*, 2017, **7**, 14395.
- 129 E. N. M. Nolte't Hoen, H. P. J. Buermans, M. Waasdorp, W. Stoorvogel, M. H. M. Wauben and P. A. C. 't Hoen, *Nucleic Acids Res.*, 2012, **40**, 9272–9285.
- 130 X. Huang, T. Yuan, M. Tschannen, Z. Sun, H. Jacob, M. Du, M. Liang, R. L. Dittmar, Y. Liu, M. Liang, M. Kohli, S. N. Thibodeau, L. Boardman and L. Wang, *BMC Genomics*, 2013, **14**, 319.
- 131 J. E. Freedman, M. Gerstein, E. Mick, J. Rozowsky, D. Levy, R. Kitchen, S. Das, R. Shah, K. Danielson, L. Beaulieu, F. C. P. Navarro, Y. Wang, T. R. Galeev, A. Holman, R. Y. Kwong, V. Murthy, S. E. Tanriverdi, M. Koupenova-Zamor, E. Mikhalev and K. Tanriverdi, *Nat. Commun.*, 2016, **7**, 11106.
- 132 T. Yuan, X. Huang, M. Woodcock, M. Du, R. Dittmar, Y. Wang, S. Tsai, M. Kohli, L. Boardman, T. Patel and L. Wang, *Sci. Rep.*, 2016, **6**, 19413.
- 133 P. Jenjaroenpun, Y. Kremenska, V. M. Nair, M. Kremenskoy, B. Joseph and I. V. Kurochkin, *PeerJ*, 2013, **1**, e201.
- 134 F. A. Lefebvre, L. P. Benoit Bouvrette, L. Perras, A. Blanchet-Cohen, D. Garnier, J. Rak and É. Lécuyer, *Sci. Rep.*, 2016, **6**, 27680.
- 135 F. A. San Lucas, K. Allenson, V. Bernard, J. Castillo, D. U. Kim, K. Ellis, E. A. Ehli, G. E. Davies, J. L. Petersen, D. Li, R. Wolff, M. Katz, G. Varadhachary, I. Wistuba, A. Maitra and H. Alvarez, *Ann. Oncol.*, 2016, **27**, 635–641.
- 136 B. Mateescu, E. J. K. Kowal, B. W. M. van Balkom, S. Bartel, S. N. Bhattacharyya, E. I. Buzás, A. H. Buck, P. de Candia, F. W. N. Chow, S. Das, T. A. P. Driedonks, L. Fernández-Messina, F. Haderk, A. F. Hill, J. C. Jones, K. R. Van Keuren-Jensen, C. P. Lai, C. Lässer, I. di Liegro, T. R. Lunavat, M. J. Lorenowicz, S. L. N. Maas, I. Mäger, M. Mittelbrunn, S. Momma, K. Mukherjee, M. Nawaz, D. M. Pegtel, M. W. Pfaffl, R. M. Schiffelers, H. Tahara, C. Théry, J. P. Tosar, M. H. M. Wauben, K. W. Witwer and E. N. M. Nolte't Hoen, *J. Extracell. Vesicles*, 2017, **6**, 1286095.
- 137 M. Del Re, E. Biasco, S. Crucitta, L. Derosa, E. Rofi, C. Orlandini, M. Miccoli, L. Galli, A. Falcone, G. W. Jenster, R. H. van Schaik and R. Danesi, *Eur. Urol.*, 2017, **71**, 680–687.
- 138 W. W. Chen, L. Balaj, L. M. Liao, M. L. Samuels, S. K. Kotsopoulos, C. A. Maguire, L. Loguidice, H. Soto, M. Garrett, L. D. Zhu, S. Sivaraman, C. Chen, E. T. Wong, B. S. Carter, F. H. Hochberg, X. O. Breakefield and J. Skog, *Mol. Ther.–Nucleic Acids*, 2013, **2**, e109.
- 139 S. Andrews, FastQC: A quality control tool for high throughput sequence data.
- 140 H. Li and R. Durbin, *Bioinformatics*, 2009, **25**, 1754–1760.
- 141 B. Langmead and S. L. Salzberg, *Nat. Methods*, 2012, **9**, 357–359.
- 142 A. Dobin, C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson and T. R. Gingeras, *Bioinformatics*, 2013, **29**, 15–21.
- 143 Y. Liao, G. K. Smyth and W. Shi, *Bioinformatics*, 2014, **30**, 923–930.
- 144 S. Anders, P. T. Pyl and W. Huber, *Bioinformatics*, 2015, **31**, 166–169.
- 145 A. Mortazavi, B. A. Williams, K. McCue, L. Schaeffer and B. Wold, *Nat. Methods*, 2008, **5**, 621–628.
- 146 B. Li and C. N. Dewey, *BMC Bioinf.*, 2011, **12**, 323.
- 147 S. Anders and W. Huber, *Genome Biol.*, 2010, **11**, R106.
- 148 M. D. Robinson, D. J. McCarthy and G. K. Smyth, *Bioinformatics*, 2010, **26**, 139–140.
- 149 A. Subramanian, P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander and J. P. Mesirov, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 15545–15550.
- 150 R. C. Gentleman, V. J. Carey, D. M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A. J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J. Y. H. Yang and J. Zhang, *Genome Biol.*, 2004, **5**, R80.
- 151 D. M. Pegtel, L. Peferoen and S. Amor, *Philos. Trans. R. Soc. London, Ser. B*, 2014, **369**, 20130516–20130516.
- 152 M. Tkach and C. Théry, *Cell*, 2016, **164**, 1226–1232.
- 153 E.-Y. Lee, D.-S. Choi, K.-P. Kim and Y. S. Gho, *Mass Spectrom. Rev.*, 2008, **27**, 535–555.
- 154 L. Miguet, K. Pacaud, C. Felden, B. Hugel, M. C. Martinez, J.-M. Freyssinet, R. Herbrecht, N. Potier, A. van Dorsselaer and L. Mauvieux, *Proteomics*, 2006, **6**, 153–171.
- 155 T. Pisitkun, R.-F. Shen and M. A. Knepper, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 13368–13373.
- 156 C. Lässer, M. Eldh and J. Lötvall, *J. Visualized Exp.*, 2012, e3037.



- 157 Y. Yoshioka, Y. Konishi, N. Kosaka, T. Katsuda, T. Kato and T. Ochiya, *J. Extracell. Vesicles*, 2013, **2**, 20424.
- 158 J. Kowal, G. Arras, M. Colombo, M. Jouve, J. P. Morath, B. Primdal-Bengtson, F. Dingli, D. Loew, M. Tkach and C. Théry, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, E968–E977.
- 159 S. A. Melo, L. B. Luecke, C. Kahlert, A. F. Fernandez, S. T. Gammon, J. Kaye, V. S. Lebleu, E. A. Mittendorf, J. Weitz, N. Rahbari, C. Reissfelder, C. Pilarsky, M. F. Fraga, D. Piwnica-Worms and R. Kalluri, *Nature*, 2015, **523**, 177–182.
- 160 Y.-S. Kim, W.-H. Lee, E.-J. Choi, J.-P. Choi, Y. J. Heo, Y. S. Gho, Y.-K. Jee, Y.-M. Oh and Y.-K. Kim, *J. Immunol.*, 2015, **194**, 3361–3368.
- 161 M. Cao, J. Ning, C. I. Hernandez-Lara, O. Belzile, Q. Wang, S. K. Dutcher, Y. Liu and W. J. Snell, *eLife*, 2015, **4**, 5242.
- 162 J. Sotillo, M. Pearson, J. Potriquet, L. Becker, D. Pickering, J. Mulvenna and A. Loukas, *Int. J. Parasitol.*, 2016, **46**, 1–5.
- 163 I. Cestari, E. Ansa-Addo, P. Deolindo, J. M. Inal and M. I. Ramirez, *J. Immunol.*, 2012, **188**, 1942–1952.
- 164 W. Nakai, T. Yoshida, D. Diez, Y. Miyatake, T. Nishibu, N. Imawaka, K. Naruse, Y. Sadamura and R. Hanayama, *Sci. Rep.*, 2016, **6**, 33935.
- 165 B. Domon and R. Aebersold, *Nat. Biotechnol.*, 2010, **28**, 710–721.
- 166 D. A. Wolters, M. P. Washburn and J. R. Yates, *Anal. Chem.*, 2001, **73**, 5683–5690.
- 167 V. Lange, P. Picotti, B. Domon and R. Aebersold, *Mol. Syst. Biol.*, 2008, **4**, 222.
- 168 S. D. Sherrod, M. V. Myers, M. Li, J. S. Myers, K. L. Carpenter, B. Maclean, M. J. Maccoss, D. C. Liebler and A.-J. L. Ham, *J. Proteome Res.*, 2012, **11**, 3467–3479.
- 169 S. Gallien, E. Duriez, C. Crone, M. Kellmann, T. Moehring and B. Domon, *Mol. Cell. Proteomics*, 2012, **11**, 1709–1723.
- 170 A. C. Peterson, J. D. Russell, D. J. Bailey, M. S. Westphall and J. J. Coon, *Mol. Cell. Proteomics*, 2012, **11**, 1475–1488.
- 171 L. C. Gillet, P. Navarro, S. Tate, H. Röst, N. Selevsek, L. Reiter, R. Bonner and R. Aebersold, *Mol. Cell. Proteomics*, 2012, **11**, O111.016717.
- 172 J. D. Egerton, A. Kuehn, G. E. Merrihew, N. W. Bateman, B. X. MacLean, Y. S. Ting, J. D. Canterbury, D. M. Marsh, M. Kellmann, V. Zabrouskov, C. C. Wu and M. J. MacCoss, *Nat. Methods*, 2013, **10**, 744–746.
- 173 J. D. Venable, M.-Q. Dong, J. Wohlschlegel, A. Dillin and J. R. Yates, *Nat. Methods*, 2004, **1**, 39–45.
- 174 C.-C. Tsou, D. Avtonomov, B. Larsen, M. Tucholska, H. Choi, A.-C. Gingras and A. I. Nesvizhskii, *Nat. Methods*, 2015, **12**, 258–264, 7 p following 264.
- 175 A. Abramowicz, P. Widlak and M. Pietrowska, *Mol. Biosyst.*, 2016, **12**, 1407–1419.
- 176 S. Kreimer, A. M. Belov, I. Ghiran, S. K. Murthy, D. A. Frank and A. R. Ivanov, *J. Proteome Res.*, 2015, **14**, 2367–2384.
- 177 D.-S. Choi, D.-K. Kim, Y.-K. Kim and Y. S. Gho, *Mass Spectrom. Rev.*, 2015, **34**, 474–490.
- 178 O. N. Jensen, *Nat. Rev. Mol. Cell Biol.*, 2006, **7**, 391–403.
- 179 K. W. Moremen, M. Tiemeyer and A. V. Nairn, *Nat. Rev. Mol. Cell Biol.*, 2012, **13**, 448–462.
- 180 C. Looze, D. Yui, L. Leung, M. Ingham, M. Kaler, X. Yao, W. W. Wu, R.-F. Shen, M. P. Daniels and S. J. Levine, *Biochem. Biophys. Res. Commun.*, 2009, **378**, 433–438.
- 181 M. Gonzalez-Begne, B. Lu, X. Han, F. K. Hagen, A. R. Hand, J. E. Melvin and J. R. Yates, *J. Proteome Res.*, 2009, **8**, 1304–1314.
- 182 P. A. Gonzales, T. Pisitkun, J. D. Hoffert, D. Tchapyjnikov, R. A. Star, R. Kleta, N. S. Wang and M. A. Knepper, *J. Am. Soc. Nephrol.*, 2009, **20**, 363–379.
- 183 H. Huang, M. Haar Petersen, M. Ibañez-Vea, P. S. Lassen, M. R. Larsen and G. Palmisano, *Mol. Cell. Proteomics*, 2016, **15**, 3282–3296.
- 184 G. Palmisano, B. L. Parker, K. Engholm-Keller, S. E. Lendal, K. Kulej, M. Schulz, V. Schwammle, M. E. Graham, H. Saxtorph, S. J. Cordwell and M. R. Larsen, *Mol. Cell. Proteomics*, 2012, **11**, 1191–1202.
- 185 M. N. Melo-Braga, T. Verano-Braga, I. R. León, D. Antonacci, F. C. S. Nogueira, J. J. Thelen, M. R. Larsen and G. Palmisano, *Mol. Cell. Proteomics*, 2012, **11**, 945–956.
- 186 D. Schwudke, K. Schuhmann, R. Herzog, S. R. Bornstein and A. Shevchenko, *Cold Spring Harbor Perspect. Biol.*, 2011, **3**, a004614.
- 187 R. Wubbolts, R. S. Leckie, P. T. M. Veenhuizen, G. Schwarzmann, W. Möbius, J. Hoernschemeyer, J.-W. Slot, H. J. Geuze and W. Stoorvogel, *J. Biol. Chem.*, 2003, **278**, 10963–10972.
- 188 K. Laulagnier, C. Motta, S. Hamdi, S. Roy, F. Fauvelle, J.-F. Pageaux, T. Kobayashi, J.-P. Salles, B. Perret, C. Bonnerot and M. Record, *Biochem. J.*, 2004, **380**, 161–171.
- 189 E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.*, 1959, **37**, 911–917.
- 190 J. Folch, M. Lees and G. H. Sloane Stanley, *J. Biol. Chem.*, 1957, **226**, 497–509.
- 191 V. Matyash, G. Liebisch, T. V. Kurzchalia, A. Shevchenko and D. Schwudke, *J. Lipid Res.*, 2008, **49**, 1137–1146.
- 192 R. A. Haraszi, M. Didiot, E. Sapp, J. Leszyk, S. A. Shaffer, H. E. Rockwell, F. Gao, N. R. Narain, M. DiFiglia, M. A. Kiebish, N. Aronin and A. Khvorova, *J. Extracell. Vesicles*, 2016, **5**, 32570.
- 193 M. C. Vallejo, E. S. Nakayasu, A. L. Matsuo, T. J. P. Sobreira, L. V. G. Longo, L. Ganiko, I. C. Almeida and R. Puccia, *J. Proteome Res.*, 2012, **11**, 1676–1685.
- 194 P. Del Boccio, F. Raimondo, D. Pieragostino, L. Morosi, G. Cozzi, P. Sacchetta, F. Magni, M. Pitto and A. Urbani, *Electrophoresis*, 2012, **33**, 689–696.
- 195 X. Gallart-Palau, A. Serra, A. S. W. Wong, S. Sandin, M. K. P. Lai, C. P. Chen, O. L. Kon and S. K. Sze, *Sci. Rep.*, 2015, **5**, 14664.
- 196 D. S. Choi, D. K. Kim, Y. K. Kim and Y. S. Gho, *Proteomics*, 2013, **13**, 1554–1571.
- 197 A. Llorente, T. Skotland, T. Sylvänne, D. Kauhanen, T. Róg, A. Orłowski, I. Vattulainen, K. Ekroos and K. Sandvig, *Biochim. Biophys. Acta*, 2013, **1831**, 1302–1309.



- 198 C. Subra, K. Laulagnier, B. Perret and M. Record, *Biochimie*, 2007, **89**, 205–212.
- 199 J. Dalli and C. N. Serhan, *Blood*, 2012, **120**, e60–e72.
- 200 T. Altadill, I. Campoy, L. Lanau, K. Gill, M. Rigau, A. Gil-Moreno, J. Reventos, S. Byers, E. Colas and A. K. Cheema, *PLoS One*, 2016, **11**, e0151339.
- 201 J. Dalli and C. Serhan, *Microbiol. Spectrum*, 2016, **4**, 37–54.
- 202 R. A. Colas, M. Shinohara, J. Dalli, N. Chiang and C. N. Serhan, *Am. J. Physiol.: Cell Physiol.*, 2014, **307**, C39–C54.
- 203 C. Subra, D. Grand, K. Laulagnier, A. Stella, G. Lambeau, M. Paillasse, P. De Medina, B. Monsarrat, B. Perret, S. Silvente-Poirot, M. Poirot and M. Record, *J. Lipid Res.*, 2010, **51**, 2105–2120.
- 204 D. W. Greening, R. Xu, S. K. Gopal, A. Rai and R. J. Simpson, *Expert Rev. Proteomics*, 2017, **14**, 69–95.
- 205 H. C. Köfeler, A. Fauland, G. N. Rechberger and M. Trötzlmüller, *Metabolites*, 2012, **2**, 19–38.
- 206 E. Fahy, D. Cotter, M. Sud and S. Subramaniam, *Biochim. Biophys. Acta*, 2011, **1811**, 637–647.
- 207 R. Herzog, D. Schwudke, K. Schuhmann, J. L. Sampaio, S. R. Bornstein, M. Schroeder and A. Shevchenko, *Genome Biol.*, 2011, **12**, R8.
- 208 J. Lötvall, A. F. Hill, F. Hochberg, E. I. Buzás, 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. Théry, *J. Extracell. Vesicles*, 2014, **3**, 26913.
- 209 G. V. Shelke, C. Lässer, Y. S. Gho and J. Lötvall, *J. Extracell. Vesicles*, 2014, **3**, 24783.
- 210 K. Aubertin, A. K. A. Silva, N. Luciani, A. Espinosa, A. Djemat, D. Charue, F. Gallet, O. Blanc-Brude and C. Wilhelm, *Sci. Rep.*, 2016, **6**, 35376.
- 211 F. Momen-Heravi, L. Balaj, S. Alian, P.-Y. Mantel, A. E. Halleck, A. J. Trachtenberg, C. E. Soria, S. Oquin, C. M. Bonebreak, E. Saracoglu, J. Skog and W. P. Kuo, *Biol. Chem.*, 2013, **394**, 1253–1262.
- 212 F. Momen-Heravi, L. Balaj, S. Alian, J. Tigges, V. Toxavidis, M. Ericsson, R. J. Distel, A. R. Ivanov, J. Skog and W. P. Kuo, *Front. Physiol.*, 2012, **3**, 354.
- 213 V. R. Minciacci, A. Zijlstra, M. A. Rubin and D. Di Vizio, *Prostate Cancer Prostatic Dis.*, 2017, **20**, 251–258.
- 214 G. Kim, C. E. Yoo, M. Kim, H. J. Kang, D. Park, M. Lee and N. Huh, *Bioconjugate Chem.*, 2012, **23**, 2114–2120.
- 215 R. Linares, S. Tan, C. Gounou and A. R. Brisson, *Methods Mol. Biol.*, 2017, **1545**, 43–54.
- 216 E. Betzig, G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych, J. S. Bonifacino, M. W. Davidson, J. Lippincott-Schwartz and H. F. Hess, *Science*, 2006, **313**, 1642–1645.
- 217 M. J. Rust, M. Bates and X. Zhuang, *Nat. Methods*, 2006, **3**, 793–795.
- 218 R. A. Dragovic, C. Gardiner, A. S. Brooks, D. S. Tannetta, D. J. P. Ferguson, P. Hole, B. Carr, C. W. G. Redman, A. L. Harris, P. J. Dobson, P. Harrison and I. L. Sargent, *Nanomedicine*, 2011, **7**, 780–788.
- 219 L. Löf, T. Ebai, L. Dubois, L. Wik, K. G. Ronquist, O. Nolander, E. Lundin, O. Söderberg, U. Landegren and M. Kamali-Moghaddam, *Sci. Rep.*, 2016, **6**, 34358.
- 220 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, *Sci. Rep.*, 2017, **7**, 1878.
- 221 A. F. Hill, D. M. Pegtel, U. Lambertz, T. Leonardi, L. O'Driscoll, S. Pluchino, D. Ter-Ovanesyan and E. N. M. Nolte-t Hoen, *J. Extracell. Vesicles*, 2013, **2**, 22859.
- 222 J. M. Street, P. E. Barran, C. L. Mackay, S. Weidt, C. Balmforth, T. S. Walsh, R. T. A. Chalmers, D. J. Webb and J. W. Dear, *J. Transl. Med.*, 2012, **10**, 5.
- 223 L. Palomo, E. Casal, F. Royo, D. Cabrera, S. Van-Liempd and J. M. Falcon-Perez, *Front. Immunol.*, 2014, **5**, 651.
- 224 O. P. B. Wiklander, J. Z. Nordin, A. O'Loughlin, Y. Gustafsson, G. Corso, I. Mäger, P. Vader, Y. Lee, H. Sork, Y. Seow, N. Heldring, L. Alvarez-Erviti, C. I. E. Smith, K. Le Blanc, P. Macchiarini, P. Jungebluth, M. J. A. Wood and S. El Andaloussi, *J. Extracell. Vesicles*, 2015, **4**, 26316.
- 225 M. L. Manca, C. Cencetti, P. Matricardi, I. Castangia, M. Zaru, O. D. Sales, A. Nacher, D. Valenti, A. M. Maccioni, A. M. Fadda and M. Manconi, *Int. J. Pharm.*, 2016, **511**, 198–204.
- 226 T. Smyth, M. Kullberg, N. Malik, P. Smith-Jones, M. W. Graner and T. J. Anchordoquy, *J. Controlled Release*, 2015, **199**, 145–155.
- 227 C. P. Lai, O. Mardini, M. Ericsson, S. Prabhakar, C. A. Maguire, J. W. Chen, B. A. Tannous and X. O. Breakefield, *ACS Nano*, 2014, **8**, 483–494.
- 228 J. Wang and M. M. Barr, *Cell Mol Neurobiol*, 2016, **36**, 449–457.
- 229 N. Tominaga, N. Kosaka, M. Ono, T. Katsuda, Y. Yoshioka, K. Tamura, J. Lötvall, H. Nakagama and T. Ochiya, *Nat. Commun.*, 2015, **6**, 6716.
- 230 A. Bronisz, Y. Wang, M. O. Nowicki, P. Peruzzi, K. Ansari, D. Ogawa, L. Balaj, G. De Rienzo, M. Mineo, I. Nakano, M. C. Ostrowski, F. Hochberg, R. Weissleder, S. E. Lawler, E. A. Chiocca and J. Godlewski, *Cancer Res.*, 2014, **74**, 738–750.
- 231 K. M. Danielson, J. Estanislau, J. Tigges, V. Toxavidis, V. Camacho, E. J. Felton, J. Khoory, S. Kreimer, A. R. Ivanov, P.-Y. Mantel, J. Jones, P. Akuthota, S. Das and I. Ghiran, *PLoS One*, 2016, **11**, e0144678.
- 232 E. Eitan, J. Green, M. Bodogai, N. A. Mode, R. Bæk, M. M. Jørgensen, D. W. Freeman, K. W. Witwer, A. B. Zonderman, A. Biragyn, M. P. Mattson, N. Noren Hooten and M. K. Evans, *Sci. Rep.*, 2017, **7**, 1342.
- 233 H. Zhou, P. S. T. Yuen, T. Pisitkun, P. A. Gonzales, H. Yasuda, J. W. Dear, P. Gross, M. A. Knepper and R. A. Star, *Kidney Int.*, 2006, **69**, 1471–1476.
- 234 L. Musante, M. Saraswat, E. Duriez, B. Byrne, A. Ravidà, B. Domon and H. Holthofer, *PLoS One*, 2012, **7**, e37279.



- 235 X. Gallart-Palau, A. Serra, A. S. W. Wong, S. Sandin, M. K. P. Lai, C. P. Chen, O. L. Kon and S. K. Sze, *Sci. Rep.*, 2015, **5**, 14664.
- 236 C. Coman, F. A. Solari, A. Hentschel, A. Sickmann, R. P. Zahedi and R. Ahrends, *Mol. Cell. Proteomics*, 2016, **15**, 1453–1466.
- 237 S. Di Palma, D. Stange, M. van de Wetering, H. Clevers, A. J. R. Heck and S. Mohammed, *J. Proteome Res.*, 2011, **10**, 3814–3819.
- 238 M. Maurer, A. C. Müller, C. Wagner, M. L. Huber, E. L. Rudashevskaya, S. N. Wagner and K. L. Bennett, *J. Proteome Res.*, 2013, **12**, 1040–1048.
- 239 L. Giusti, P. Iacconi, F. Ciregia, G. Giannaccini, G. L. Donatini, F. Basolo, P. Miccoli, A. Pinchera and A. Lucacchini, *J. Proteome Res.*, 2008, **7**, 4079–4088.
- 240 N. A. Kulak, G. Pichler, I. Paron, N. Nagaraj and M. Mann, *Nat. Methods*, 2014, **11**, 319–324.
- 241 C. Coman, F. A. Solari, A. Hentschel, A. Sickmann, R. P. Zahedi and R. Ahrends, *Mol. Cell. Proteomics*, 2016, **15**, 1453–1466.
- 242 J. Tisoncik-Go, D. J. Gasper, J. E. Kyle, A. J. Einfeld, C. Selinger, M. Hatta, J. Morrison, M. J. Korth, E. M. Zink, Y.-M. Kim, A. A. Schepmoes, C. D. Nicora, S. O. Purvine, K. K. Weitz, X. Peng, R. R. Green, S. C. Tilton, B.-J. Webb-Robertson, K. M. Waters, T. O. Metz, R. D. Smith, Y. Kawaoka, M. Suresh, L. Josset and M. G. Katze, *Cell Host Microbe*, 2016, **19**, 254–266.
- 243 R. A. Quinn, J. A. Navas-Molina, E. R. Hyde, S. J. Song, Y. Vázquez-Baeza, G. Humphrey, J. Gaffney, J. J. Minich, A. V. Melnik, J. Herschend, J. DeReus, A. Durant, R. J. Dutton, M. Khosroheidari, C. Green, R. da Silva, P. C. Dorrestein and R. Knight, *mSystems*, 2016, **1**, e00038–e00016.

