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Minimal information for studies of extracellular vesicles (MISEV2023): from basic to advanced approaches

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POSITION PAPER

Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches

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Abstract

Extracellular vesicles (EVs), through their complex cargo, can reflect the state of their cell of origin and change the functions and phenotypes of other cells. These features indicate strong biomarker and therapeutic potential and have generated broad interest, as evidenced by the steady year-on-year increase in the numbers of scientific

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‡ISEV Board 2020-2022.
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‖Original section drafting contribution.

publications about EVs. Important advances have been made in EV metrology and in understanding and applying EV biology. However, hurdles remain to realising the potential of EVs in domains ranging from basic biology to clinical applications due to challenges in EV nomenclature, separation from non-vesicular extracellular particles, characterisation and functional studies. To address the challenges and opportunities in this rapidly evolving field, the International Society for Extracellular Vesicles (ISEV) updates its 'Minimal Information for Studies of Extracellular Vesicles', which was first published in 2014 and then in 2018 as MISEV2014 and MISEV2018, respectively. The goal of the current document, MISEV2023, is to provide researchers with an updated snapshot of available approaches and their advantages and limitations for production, separation and characterisation of EVs from multiple sources, including cell culture, body fluids and solid tissues. In addition to presenting the latest state of the art in basic principles of EV research, this document also covers advanced techniques and approaches that are currently expanding the boundaries of the field. MISEV2023 also includes new sections on EV release and uptake and a brief discussion of in vivo approaches to study EVs. Compiling feedback from ISEV expert task forces and more than 1000 researchers, this document conveys the current state of EV research to facilitate robust scientific discoveries and move the field forward even more rapidly.

KEYWORDS
ectosomes, exosomes, extracellular vesicles, extracellular particles, guidelines, microparticles, microvesicles, minimal information requirements, MISEV, reproducibility, rigor, standardisation

1 | AN INTRODUCTION TO ISEV AND MISEV

1.1 | Extracellular vesicles and MISEV

Extracellular vesicles (EVs) serve diverse and important roles in most biological systems, arising in part from their compositional complexity. EVs are lipid bilayer membrane-delimited, nano- to micro-sized particles that appear to be released by all cell types. The molecular and structural heterogeneity of EVs mean that many discoveries remain to be made in fundamental biology and development of biomarker and therapeutic applications, yet this same complexity also poses challenges at every stage of EV studies. From definition and categorisation to separation, characterisation, engineering and clinical applications, the 'Minimum Information for Studies of Extracellular Vesicles' (MISEV) aims to help all practitioners of EV research and application to follow best practices for each specific question and indication.

Now in its third iteration, MISEV2023, as a field consensus document seeks to provide recommendations and guidance on EV-related studies that encourage enhanced research design and reporting of experimental details, building on the criteria and guidelines set out in the previous two iterations. MISEV is produced by the International Society for Extracellular Vesicles (ISEV) (https://www.isev.org). Founded in 2011 with the mission to enhance EV research globally, ISEV is the leading professional society for scientists and clinicians involved in the study and use of extracellular vesicles. ISEV engages a diverse group of researchers across the world through its annual meeting, thematic workshops and other meetings (in-person and virtual), peer-reviewed journals, online learning platforms, and partnerships with other societies. ISEV is thus uniquely positioned to shepherd the development and dissemination of expert consensus on best-practice guidelines and scientific considerations.

MISEV2014 (Lotvall et al., 2014) was the first EV position paper produced by ISEV and designed to give robustness to EV analysis. MISEV2018 (Thery et al., 2018) gave a more in-depth and critical assessment of the approaches and methods used to move the field forward, much of which still holds today. MISEV2018 also includes suggested experimental approaches to address some of the remaining challenges and to provide robust EV characterisation. The earlier MISEV recommendations remain largely or entirely valid, and MISEV2023 should be read in the context of the previous documents.

Like the iterations before it, MISEV2023 provides succinct recommendations and guidance for EV researchers, with refinement of points raised in MISEV2018 and addition of recommendations and guidance for newer areas of development. MISEV2023 broadly covers the nomenclature, pre-processing variables, separation and characterisation of EVs, as well as in vitro and in vivo analysis of EV release, uptake and functions.

In addition to previous MISEV guidelines (Lotvall et al., 2014; Thery et al., 2018), ISEV has prompted and coordinated development and dissemination of expert consensus on best-practice guidelines and scientific considerations including inter-society

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TABLE 1 Journal of Extracellular Vesicles: ISEV position papers and statements.

Title	Year	Ref.
Standardization of sample collection, isolation and analysis methods in extracellular vesicle research	2013	(Witwer et al., 2013)
ISEV position paper: extracellular vesicle RNA analysis and bioinformatics	2013	(Hill et al., 2013)
Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles	2014	(Lotvall et al., 2014)
Applying extracellular vesicles-based therapeutics in clinical trials—an ISEV position paper	2015	(Lener et al., 2015)
Obstacles and opportunities in the functional analysis of extracellular vesicle RNA—an ISEV position paper	2017	(Mateescu et al., 2017)
Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines	2018	(Thery et al., 2018)
Biological membranes in EV biogenesis, stability, uptake, and cargo transfer: an ISEV position paper arising from the ISEV membranes and EVs workshop	2019	(Russell et al., 2019)
MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments	2020	(Welsh, Van Der Pol, Arkesteijn, et al., 2020)
Urinary extracellular vesicles: A position paper by the Urine Task Force of the International Society for Extracellular Vesicles	2021	(Erdbrügger et al., 2021)

position papers (Welsh, Van Der Pol, Arkesteijn, et al., 2020), and focused recommendations of topic-specific experts (Erdbrügger et al., 2021; Hill et al., 2013; Lener et al., 2015; Mateescu et al., 2017; Russell et al., 2019; Verweij et al., 2021; Witwer et al., 2013) (Table 1). More recently, the ISEV Rigor and Standardisation Subcommittee oversees appointment and activities of thematic task forces and special interest groups on specific sources of EVs and other EV-related topics. ISEV also recommends adoption of other reporting and atlas tools, such as the 'Minimum Information for the Publication of Quantitative Real-Time PCR Experiments' (MIQE) for real-time reverse transcriptase-quantitative polymerase chain reaction (qPCR) analyses (Bustin et al., 2009) and EV-TRACK (EV-TRACK Consortium et al., 2017; Roux et al., 2020). Overall, the activities and recommendations of ISEV share the aim of increasing rigor, reproducibility and transparency in EV research. The goal of this MISEV document is to help practitioners in all areas of EV research and application to implement or develop best practices for each individual EV source, type, research question or application.

1.2 | What MISEV IS and IS NOT

Since MISEV2018 appeared, there has been much discussion of what the guidelines mean and how they should or should not be applied. Informed by that discussion, what MISEV IS, and IS NOT, is summarised below.

MISEV IS:

1. An introduction to EV research.
2. A set of recommendations that are meant to increase rigor, reproducibility, and transparency during EV study design, execution and reporting.
3. A tool to assist reviewers and editors, using their own expert knowledge, in assessing the strengths and weaknesses of EV-related proposals, funding applications, abstracts and manuscripts.
4. A non-exhaustive set of examples of various useful EV techniques and platforms.
5. A rigor and standardisation framework that supports innovative EV research and applications and parties ranging from product developers to regulators.
6. An indication of current, broad consensus in the EV field as well as some areas of uncertainty and growth.
7. Relevant to translational and clinical research and applications, including production and initial evaluation of therapeutic EVs.
8. Applicable to all sorts of EV research and applications, not just those involving mammalian EVs. Although examples provided in MISEV may be specific to mammalian EVs, the basic principles are most likely applicable to all EV sources. These include

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informative nomenclature, definition of sources, description of separation/concentration techniques, and characterisation of EVs, properly controlled functional studies, and comprehensive reporting.

By contrast, MISEV IS NOT:

1. A one-size-fits-all blueprint, a comprehensive checklist of 'dos and don'ts', or a substitute for careful expert judgement. There is no technique or platform that is absolutely required or prohibited by MISEV. Similarly, MISEV does not mandate use of any particular marker or markers, enriched or depleted. Chosen techniques and targets should be fit for purpose, appropriate for the experimental system, contributing to overall MISEV compliance, and properly reported. Importantly, no research group has access to all techniques and platforms.
2. A barrier to innovation. When introducing a new technique or new application of EVs, it is possible that some aspects of the approach do not fit perfectly into the existing MISEV framework, or more likely, into a reviewer's interpretation of it. See above on absolute mandates and invoke the exceptions if you must. MISEV should not stifle innovation, but rather inform how innovative or new techniques are presented and validated.
3. A means to prevent publication or funding of a particular project. Just as MISEV should not stifle innovation, it should not be used to prevent research from being shared with the community. For example, an 'exosome' or 'ectosome' study that does not prove biogenesis can be presented instead as about EVs, or an 'EV' report without full characterisation as a broader extracellular particle study. Proper controls might be needed to prove the contribution of EVs to an effect, but if they cannot be done, it might suffice to acknowledge the caveats.
4. A comprehensive collection of citations, each of which entirely embodies the recommendations of MISEV. The MISEV document is not a literature review or compendium. Only a small percentage of the EV literature is cited here, and each citation is made for a specific purpose. Citation in MISEV does not imply endorsement of a report, author team, journal or publisher by ISEV, nor does it suggest primacy or perfection of the cited study. Some cited studies may contain aspects that are inconsistent with MISEV recommendations. Also, many excellent studies are not cited in this document.

In summary, the spirit of MISEV is embodied in just a handful of questions:

1. What terms do you use, and what do they mean?
2. From what/where did you obtain your EVs?
3. How did you separate, concentrate, characterise and store them?
4. How confidently can you attribute a function or biomarker to EVs versus other components?
5. Have you shared data and reported methods in sufficient detail to enable others to replicate or reproduce your results?

1.3 | How to use MISEV2023

MISEV2023 is intended to aid any and all EV researchers: from those just starting their EV journey to more established investigators who wish to understand the current state of the art and/or cutting-edge problems faced by the EV community. However, the result is a large document that may require some help to navigate.

Nomenclature (Section 2) is applicable to all EV studies. Clear and consistent language will help to ensure that results are understandable and comparable.

For those who are newer to EV research, we consider Sections 3–5 to be vital, covering minimum considerations for sample collection/processing, EV separation methods and EV characterisation, respectively.

Sections 6–9 provide further technique-specific guidance for EV characterisation, approaches to modulate EV release and uptake, EV functional studies, and the EV analysis in vivo. These sections provide the reader with up-to-date information to support informed decisions, but, for the most part, do not give specific recommendations.

The information and guidelines presented in MISEV2023 thus promote rigor, reproducibility and transparency in EV science, with the goal to ensure that conclusions are supported by the experiments performed and the information reported.

> Consensus: 89.3% (891) of MISEV2023 survey respondents agreed "completely," and 10.7% (107) agreed "mostly" with Section 1: An introduction to ISEV and MISEV. No respondents disagreed ("mostly" or "completely"), and no respondents stated that they had no opinion and/or expertise.

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2 | NOMENCLATURE

2.1 | EV definition and EV subtypes

Definition: The term 'extracellular vesicles' (EVs) refers to particles that are released from cells, are delimited by a lipid bilayer, and cannot replicate on their own (i.e., do not contain a functional nucleus). The current definition of EV is retained from MISEV2018, except that the 2018 use of the word 'naturally' (as in 'naturally released') has been removed to avoid unintended exclusion of engineered EVs or EVs produced under various cell culture conditions. In general, ISEV recommends use of the generic term 'EV' and operational extensions of this term instead of inconsistently defined and sometimes misleading terms such as 'exosomes' and 'ectosomes' that are associated with biogenesis pathways that are difficult to establish.

Regarding 'operational terms' that can be added as a prefix to 'EV' (Table 2), their use continues to be encouraged *with caution* if one or more EV subtypes are separated on the basis of characteristics such as size, density, molecular composition or cellular origin. We urge careful and clear definition of these operational terms. For example, terms such as 'small' and 'large' have been commonly used to denote EV populations over the last few years, usually after presumed size-based populations of EVs have been separated with methods such as filtration or differential ultracentrifugation (differential UC, dUC). However, although 'small' might generally refer to EVs <200 nm in diameter, there is no strict consensus on upper and lower size cut-offs, and it has also become clear that many separation methods, such as dUC, yield EV populations with overlapping size profiles. Thus, while such terminology may still be used, researchers should be aware of its limitations and strive to define terms as clearly as possible.

As mentioned above, terms related to presumed biogenesis pathways should be used only with caution and strong evidence. The term 'exosome' refers to EVs from internal compartments of the cell that are released via the multivesicular body (MVB), while the term 'ectosome' (a.k.a., microvesicle, microparticle) refers to EVs from the cell surface. Numerous specialised terms have also been used to denote EVs that arise during specific cellular processes such as cell migration ('migrasomes') or programmed cell death ('apoptotic bodies'). In some cases, biogenesis or release of specific EV subtypes may be inhibited or stimulated by pharmacological or genetic intervention (see also 7.1). Unfortunately, most EV separation techniques do not enrich for EVs produced by different mechanisms, and definitive characterisation of biogenesis-based subtypes is also difficult, with no universal molecular markers of ectosomes, exosomes or other EV subtypes. Therefore, ISEV discourages the use of biogenesis-based terms unless such an EV population is specifically separated and characterised. Of note, 'sEV' (for small EV) and 'exosome' are not synonymous: small EV populations include both small ectosomes and exosomes. For the reasons above, most of the existing 'exosome' and 'ectosome/microvesicle' literature refers to a broad population of EVs, and not to EVs that are released via specific biogenesis pathways. Some EV-like particles may not fully meet the definition of EVs as given above. For example, if a cell is extruded, the resulting particles have not been strictly 'released' from the cell.

2.2 | EV mimetics

A term such as 'EV mimetics' (EVMs) can be used to denote EV-like particles that are produced through direct disruption of cells, by de novo synthesis from molecular components, or by fusion of native EVs with, for example, liposomes. Whatever nomenclature is used for such particles, it will ideally indicate the general production process, differentiate the particles from native EVs, and not claim resemblance to EVs from a specific biogenesis pathway. That is, avoid 'exosome-like vesicles' and similar terms that incorrectly imply specific biogenesis-related properties. Some examples of possible terms, but without strict endorsement, are artificial cell-derived vesicles (ACDVs) for vesicles from extruded cells and synthetic vesicles (SVs) for EV mimetics that are synthesised de novo from molecular components or made as hybrid entities, for example, fusions between liposomes and native EVs (Table 2).

2.3 | How to approach non-vesicular extracellular particles (NVEPs)

There is a growing awareness of a wide diversity of non-vesicular extracellular particles that often co-separate with EVs, and the ISEV community specifically requested guidance in the run-up to MISEV2023 on how to handle and name these particles. Since ISEV is a society of EV experts, we cannot presume to establish a nomenclature for other types of extracellular particles, such as lipoprotein particles (LPPs), ribonucleoprotein particles (RNPs), viruses, or various newly proposed particle types like exomeres and supermeres. Nevertheless, how EVs relate to other particles—and how they can be separated from them and characterised along with them in complex mixtures—is of great relevance to the EV field. Therefore, MISEV2023 provides the following nomenclature proposals while recognising that other terms may be required for increased clarity (Figure 1, Table 2).

Extracellular particles (EPs) is the preferred overarching term for cell-derived multimolecular assemblies in the nanometre to micron size range, including both EVs and non-vesicular entities:

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TABLE 2 Quick-reference card on EV nomenclature and related terms.

Term	Definition	Usage
Extracellular vesicles (EVs)	Particles that are released from cells, are delimited by a lipid bilayer, and cannot replicate on their own.	Recommended
Non-vesicular extracellular particles (NVEPs)	Multimolecular assemblies that are released from cells and do not have a lipid bilayer (non-vesicular extracellular particle fraction).	Recommended
Extracellular particles (EPs)	Umbrella term for all particles outside the cell, including EVs and NVEPs.	Recommended
EV mimetic	EV-like particles that are produced through direct artificial manipulation. This term is preferred over 'exosome-like vesicles' and similar terms that imply specific biogenesis-related properties.	Recommended
Artificial cell-derived vesicles (ACDVs)	EV mimetics that are produced in the laboratory under conditions of induced cell disruption, such as extrusion.	Recommended
Synthetic vesicles (SVs)	EV mimetics that are synthesized de novo from molecular components or made as hybrid entities, e.g., fusions between liposomes and native EVs.	Recommended
Small EVs (operational term)	Based on the diameter of the separated particles, small EVs are often described as <200 nm in diameter. However, measured diameter is related to the specific characterization method.	Recommended, but caution required
Large EVs (operational term)	Based on the diameter of the separated particles, large EVs are often described as >200 nm in diameter. However, measured diameter is related to the specific characterization method.	Recommended, but caution required
Other 'operational terms'	Physical characteristics: e.g., diameter: small extracellular vesicles (sEVs), large EVs (lEVs), density: low, medium, high (defined ranges). Biochemical composition: e.g., contains a specific (macro)molecule, such as a protein. Cellular origin and/or conditions under which EVs were generated: terms that highlight specific aspects of biogenesis such as molecular mechanisms, energy-dependence (or lack thereof) and functional state of the parent cell related to stress or death.	Recommended, but caution required
Exosome	Biogenesis-related term indicating origin from the endosomal system. Unless subcellular origin can be demonstrated, it is likely that a broad population of EVs is being studied, not exosomes specifically. Exosomes represent a subtype of small EVs: the diameter of intraluminal vesicles of endosomes is generally smaller than 200 nm.	Discouraged unless subcellular origin can be demonstrated
Ectosome	Biogenesis-related term indicating origin from the plasma membrane. Unless subcellular origin can be demonstrated it is likely that a broad population of EVs is being studied, not ectosomes specifically. Ectosomes can have a wide range of sizes, including sizes similar to those of exosomes.	Discouraged unless subcellular origin can be demonstrated
Microvesicle	Biogenesis-related term indicating origin from the plasma membrane. However, historically, the term has often been used to designate large EVs or all EVs, whatever their subcellular origin. This term can therefore lead to confusion.	Discouraged
Exosome-like vesicles	As 'exosome' is a biogenesis-related term indicating origin from the endosomal system, this and similar terms are discouraged for synthesized EV mimetics.	Discouraged

Non-vesicular extracellular particles (NVEPs) are all non-EV particles made from cell-derived components of one or more molecular classes (e.g., proteins, nucleic acids); lipids, if present, do not form a delimiting bilayer membrane. NVEPs and EVs may have overlapping physicochemical properties, and NVEPs may greatly outnumber EVs in biological matrices. As a result, most EP separation methods result in NVEP/EV co-isolation. Similarly, many EP characterisation methods do not identify EVs specifically. NVEPs that are smaller than EVs may not be detected by some EV characterisation methods, thus their quantity in an EP preparation may remain unknown. Therefore, when EVs and NVEPs cannot be fully distinguished from each other, the term 'EP' may be appropriate, or the use of 'EV preparation' or 'EV-containing preparation'.

Table 2 is a quick-reference card of recommended nomenclature.

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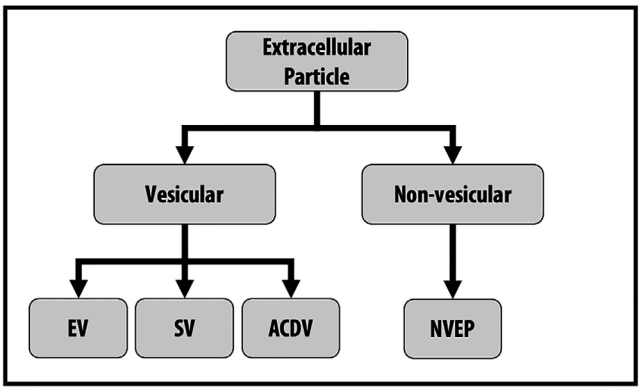

FIGURE 1 Hierarchy of EP nomenclature. Extracellular particles include vesicular and non-vesicular particles. This figure presents several distinctions that can be made between classes of EPs, as well as examples of possible nomenclature. EP: extracellular particle; EV: extracellular vesicle; SV: synthetic vesicle; ACDV: artificial cell-derived vesicle; NVEP: non-vesicular extracellular particle. See also Section 2 and Table 2.

Recommendations:

- **'Extracellular vesicles'** is the term for particles that are delimited by a lipid bilayer and cannot replicate on their own (vesicular component of extracellular particles).
- Operational terms are encouraged, but with caution, as these can be influenced by separation methods.
- Biogenesis terms are discouraged unless subcellular origin can be demonstrated for the specific EV source and condition. With few exceptions, a broad population of EVs is studied, not ectosomes or exosomes specifically.
- **'Extracellular particles'** is the overarching term for cell-derived multimolecular assemblies in the nanometer to micron size range, including both vesicular and non-vesicular entities.
- **'Non-vesicular extracellular particles'** is an accurate term for cell-derived multimolecular assemblies that are non-vesicular in nature (i.e., the non-vesicular fraction of extracellular particles).

> Consensus: 79.5% (793) of MISEV2023 survey respondents agreed "completely," and 19.9% (199) agreed "mostly" with Section 2: Nomenclature. 0.4% (4) "mostly" disagreed, and 0.2% (2) stated that they had no opinion and/or expertise. No respondents disagreed "completely."

3 | COLLECTION AND PRE-PROCESSING: PRE-ANALYTICAL VARIABLES THROUGH TO STORAGE

An array of factors in sample collection, pre-processing (i.e., before specific EV separation/concentration steps), and storage of EV-containing sources and their derivatives may affect EVs quantitatively and qualitatively. Some considerations related to these factors are common between many EV source materials, such as how to maximise (and measure) the quality of starting material; reporting all relevant donor characteristics for biofluid/solid tissue samples; measures of the quantity and quality of the source material as the baseline for the data collected during EV characterisation; and standardising and reporting pre-processing variables. In contrast, other recommendations may be specific to the starting source, such as approaches to remove source-specific contaminants/co-isolates and to confirm their removal.

3.1 | Common recommendations

- Describe the source of EV-containing materials. For materials from human and non-human animal donors, report relevant donor characteristics, including but not limited to age, biological sex, substance exposures (medications, substance use) and disease.
- Report the quantity (e.g., sample volume, mass) and quality of source material.
- Provide all methodologic details of sample collection.
- Consider how pre-separation storage may influence the EVs that are eventually separated. Where relevant, avoid repeated freeze-thaw cycles or assess effects of freeze-thaw.

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- Report all storage parameters pre- and post-EV separation (including use of preservatives or cryoprotectants, temperature, time, freezing procedure, storage vessel, number of freeze-thaw cycles and thawing method).
- Remove cells from all EV source materials as early as possible in pre-processing. Cell disruption can form particles resembling native EVs, and post-collection cellular processes like activation and death can alter EV composition and function.
- Assess and report the degree of depletion of cells and source-specific, common EV co-isolates during pre-processing and, later, after EV separation/concentration.
- Implement quality control measures throughout the sample collection, pre-processing and EV separation.
- If samples must be pooled to obtain sufficient EVs for study, report the number of individual samples in a pool, the donor demographics contributing to the pool, the quantity (e.g., volume) of each individual sample and final quantity. Where possible, follow up with individual samples.
- In studies that seek to determine if EVs or EV cargo can serve as biomarkers of a disease or condition, also test whether non-enriched materials, for example, NVEPs or whole biofluid, may have similar associations.
- For those EV sources for which ISEV has a Task Force (isev.org/taskforces), we recommend that researchers keep themselves updated and informed on outputs of that Task Force. See also the next sections with some specific recommendations.

3.2 | Cell culture-conditioned medium

All types of cells cultured in vitro release EVs and other factors into their culture medium, thus creating cell culture-conditioned medium [CCM; Shekari et al., 2023]. This includes eukaryotic cells from multi- and unicellular organisms and prokaryotic cells including gram-positive and -negative bacteria and *Mycobacteria*. Most recommendations in this section apply to CCM from all cell types; additional and more specific details on bacterial EVs are provided in Section 3.3.

Cell culture parameters for both eukaryotic and prokaryotic cells include the producing cells (e.g., name, viability, passage number and seeding and harvest density); medium components (e.g., basal medium, complex additives such as serum, nutrients, micronutrients, antibiotics/mycotics and any other additives); culture conditions, including 2D/3D/suspension culture, temperature, pH, gas concentrations and any physical stimuli; duration of conditioning; harvesting approaches; and any detected contaminations or infections. Cell culture conditions directly and indirectly affect EV yield, composition, and function. Culture media components can contain EVs or may be taken up by cells and repackaged into EVs (Lehrich et al., 2021; Palviainen et al., 2019). Complex supplements such as blood serum [e.g., foetal bovine/calf serum (FBS/FCS)] and platelet lysate (PL) are often used in mammalian cell culture, but they are rich in EVs, NVEPs, and various, often undefined entities, including DNA fragments and micronutrients (Arigony et al., 2013; Lehrich et al., 2021). Depleting EVs from these supplements can be difficult to accomplish and verify (Erdbrügger et al., 2021; Lehrich et al., 2018), and depletion of complex supplements, for example, by ultracentrifugation, may depend on degree of dilution. Commercial 'EV-free' products should also not be assumed to be devoid of EVs without verification. Use of both EV-depleted medium and 'defined' (serum/PL-free) media may alter cell physiology and EV production (Lehrich et al., 2021). Since viable and dying cells may release different subtypes of EVs (Crescitelli et al., 2013; Shlomovitz et al., 2021), and since EVs produced by only a few percent of dying cells may outnumber EVs generated by healthy cells, the proportion of live and dying cells in a culture affects proportions of EV subtypes and EV quantity. Unwanted microbial contamination (common: *Mycoplasma*), should be checked and reported. These microbes affect many characteristics of producing cells (Zhang et al., 2000); they or their constituents may be repackaged into EVs of the host culture (Yang et al., 2012); and some may also release their own EVs (Gaurivaud et al., 2018).

Recommendations:

- CCM recommendations made in MISEV2018 (Thery et al., 2018) are still relevant. These include, but are not limited to, reporting medium composition and preparation; characteristics of producing cells including identity, seeding and harvest density and viability at harvest; culture conditions including vessel/system, surface coating (if any), temperature, and gas concentrations; physical or chemical stimulants/treatments, if any; frequency, intervals and method of CCM harvest; and any CCM storage before EV separation. If cells are from a primary source, rather than an established cell line, report harvesting and pre-culturing conditions such as enzymatic digestion.
- If serum, PL or other complex additives are used, report the source and the percent of the total medium. If EV depletion of such additives is done, report method and degree of depletion (including dilution, which may be necessary prior to depletion methods involving centrifugation) using the same methods used to characterise released EVs. Vendors of EV-depleted supplements are also encouraged to report method and degree of EV depletion.
- Non-conditioned medium controls should be processed and characterised to assess the contribution of the medium itself to putative EV measurements.

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3.3 | Bacteria

The diversity of bacteria, bacterial EVs and source material characteristics makes it difficult to issue universal recommendations on sample type, pre-processing, separation, collection and characterisation. Bacterial EVs arise from outer and inner membranes of gram-negative bacteria and cytoplasmic membranes of gram-positive bacteria through blebbing and lytic biogenesis pathways (Toyofuku et al., 2023). Different species, strains (Bitto, Cheng, et al., 2021; Bitto, Zavan, et al. 2021; McMillan & Kuehn, 2023; Zavan et al., 2023), and growth conditions (Hong et al., 2019; Keenan & Allardyce, 2000) affect EV heterogeneity on multiple levels, including function (Turner et al., 2018). Bacterial EVs can be harvested from mono- or polymicrobial culture in vitro, in vivo/ex vivo sources such as body fluids or faeces, and environmental samples ranging from soil to seawater. Despite this diversity, some recommendations are possible.

For most bacterial species, studies of the influence of culture conditions on the yield and composition of bacterial EVs are in their infancy, but most considerations for culture-derived eukaryotic EVs also apply to bacterial EVs (Bose et al., 2020; Brown et al., 2015). These include effects of media composition, oxygenation/aeration and culture format (for bacteria: standing, shaking, roller bottle, bioreactor, planktonic cell or biofilm) and growth phase (Bitto, Cheng, et al., 2021; Bitto, Zavan, et al. 2021; Kuehn & Kesty, 2005; Mehanny et al., 2022; Zavan et al., 2019). Thus, culture details should be reported.

Following sample collection, as for eukaryotic EVs, all methodologic details of separation/concentration should be reported. Non-specific methods like precipitation and ultracentrifugation may co-isolate and/or aggregate unwanted non-EV materials. For bacteria, these may include pili, flagellae, phage, and protein, lipoprotein, and nucleoprotein complexes. Filtration and chromatography methods are gentler alternatives (Bitto & Kaparakis-Liaskos, 2022; Liangsupree et al., 2021). In density gradient ultracentrifugation, densities of EV-rich fractions should be determined for each bacterium and growth condition, with clear reporting of fractions (Bitto & Kaparakis-Liaskos, 2022; Singorenko et al., 2017). Consider that different separation methods may enrich or deplete subtypes of bacterial EVs.

Detailed characterisation of bacterial EV preparations beyond core measurements of size distribution and macromolecular content is limited by the availability of validated, commercially available affinity reagents to bacterial markers for only a limited number of species. In many cases, markers of co-isolating materials require further definition. Lipopolysaccharide [LPS, gram-negative bacteria, Tulkens et al., 2020], lipotechoic acid [LTA, gram-positive bacteria, Champagne-Jorgensen et al., 2021] and mycobacterial lipids (Prados-Rosales et al., 2011) are universal markers for these broad classes of bacterial EVs. LPS and LTA have the advantage of commercially available antibodies. However, LPS can be present in NVEPs including LPS micelles and complexes with LPS binding protein that may be present in in vivo samples (Page et al., 2022), so appropriate controls should be included. Finally, for functional assays, normalisation methods for bacterial EV input should be accurately reported, e.g., different protein assay types can return different values (Bitto, Zavan, et al., 2021).

Recommendations:

- In addition to other culture parameters, report bacterial growth phase at harvest.
- Limit storage prior to EV separation/concentration, especially if samples are left unfiltered.
- When obtaining bacterial EVs from in vivo and environmental sources, consider that host EVs or EVs from non-target species are likely present.
- LPS and LTA are broad markers of gram-negative and -positive bacteria, respectively, with well-characterised, commercially-available affinity reagents. In many species, specific markers of EVs and non-EV materials remain unavailable.
- Non-vesicular co-isolates of bacterial EVs may include pili, flagellae, phage and protein, lipoprotein and nucleoprotein complexes.

3.4 | Blood

Blood is the most studied biofluid in EV research, and most studies involve human blood. Previous MISEV guidelines, ISEV position papers and other publications (Clayton et al., 2019; Coumans et al., 2017; Thery et al., 2018; Witwer et al., 2013) highlighted the importance of standardisation and reporting of (i) donor variables, for example, age, biological sex, circadian rhythm, diet, exercise level and medication, and (ii) pre-analytical processing variables such as blood collection, preparation, handling, storage, anticoagulants, centrifugation protocols and handling time (Buntsma et al., 2022; Dhondt et al., 2020, 2023; Lacroix et al., 2012; López-Guerrero et al., 2023; Gyorgy et al., 2014; Palviainen et al., 2020), which remain valid. Here, we focus on the complexity of blood, which contains cells, lipoproteins, proteins and other factors that may be retained in EV preparations and confound downstream analysis. The degree to which blood samples are processed and EVs are separated from common co-isolates depends on the study aim and the downstream analysis. The MIBlood-EV was developed by the ISEV Blood Task Force to enable scientists

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to report the traceability of blood-derived samples used for EV studies (Lucien et al., 2023). The MIBlood-EV is divided into categories of: (a) general study information, (b) blood collection, processing, storage, (c) qualitative and quantitative evaluation of haemolysis, platelets and lipoproteins, three major confounding factors in blood EV research.

Blood cells account for about 45% of the blood volume, so removal of cells before any cell-disruptive processing such as freeze/thawing (which forms EV-like cell fragments) and avoidance of cell activation (and thus release of EVs post-collection) is particularly important. Red blood cells are dense and thus relatively easy to separate from EVs by low-speed centrifugation. However, red blood cells may lyse ('haemolysis') during blood collection and processing, releasing internal contents such as haemoglobin, which turns the plasma or serum a reddish instead of yellow colour. Most other blood cells can also be efficiently removed by centrifugation. In contrast, 1–3 μm platelets are derived from megakaryocytes, highly abundant in blood, and overlap in size range and/or density with EVs. The presence of even a few platelets may affect downstream EV analysis, and activated platelets will release large numbers of EVs. Although various centrifugation protocols are used to deplete platelets from plasma and serum (Bracht et al., 2023; Karimi et al., 2022), these protocols incompletely separate platelets from EVs, and extent of platelet depletion is typically unreported. Additional depletion of residual platelets from plasma and serum can be achieved by filtration (Bettin et al., 2022; Bracht et al., 2023).

Lipoproteins are another main confounding class of NVEPs, including high-density, low-density, intermediate-density, and very low-density lipoproteins (HDL, LDL, IDL, VLDL) as well as larger chylomicrons. They overlap in size (all but HDL), density (HDL), and/or molecular composition with blood EVs, and some lipoprotein subtypes outnumber blood EVs by orders of magnitude (Johnsen et al., 2019; Simonsen, 2017). Because neither density- nor size-based separation can separate all lipoproteins from EVs, a combination of methods that exploit different physical and biochemical properties (here reported in Chapter 4) is recommended when more pure EV populations are required (Karimi et al., 2018; Ter-Ovanesyan et al., 2023; Van Deun et al., 2020; Vergauwen et al., 2021; Zhang, Borg, et al., 2020).

Blood also contains high concentrations of free, 'soluble' proteins such as serum albumin, immunoglobulins and fibrinogen, as well as protein and ribonucleoprotein (RNP) aggregates, that may co-isolate with EVs and affect downstream analysis. These proteins are generally smaller and denser than EVs, allowing separation from EVs by size exclusion chromatography (SEC), density gradient centrifugation, or combinations thereof.

Of note, the surface of EVs, especially in complex environments such as blood, is covered with a biomolecular corona of various molecules and particles [(Palviainen et al., 2020; Tóth et al., 2021; Wolf et al., 2022; Yerneni et al., 2022) and see also Section 4.7]. Hence, some blood proteins and lipoproteins, previously defined as contaminants of the EV preparation, may be truly associated with EVs and remain even after the EVs have been rigorously but gently separated from blood.

Recommendations:

- Effects of donor characteristics on blood and EV properties are better studied for blood than for many other EV sources and are thus especially important to consider and report. For example, large lipoprotein particles such as chylomicrons have elevated concentrations after dietary intake, so to minimise their influence, collect blood from overnight-fasted donors.
- When blood is collected by venipuncture, use the largest feasible needle gauge to minimise platelet activation and haemolysis. To minimise bacterial and skin cell contamination and to avoid tissue factor-mediated platelet activation, it may also be a good practice to discard a small volume of the blood draw (e.g., for human blood draws, the first 2–3 mL).
- Select blood collection tubes/anticoagulants that are compatible with downstream analyses.
- Following collection, minimise platelet activation and EV release by avoiding excessive agitation and low temperatures and processing to plasma or serum as quickly as possible.
- Use a plasma or serum preparation protocol that efficiently removes platelets but not EVs. If centrifugation is used, draw supernatant from the top down with a pipette, leaving a specified amount of plasma or serum on top of the pellet to avoid disturbing the pellet and releasing platelets.
- Major contaminants/co-isolates of blood EVs are platelets, lipoproteins, haemolysis products, and a host of soluble/aggregated proteins including RNPs. Determine and report relative enrichment of EVs over whichever of these materials is important in a given study.
- Complete the MIBlood-EV reporting tool and attach it as supplementary material for any manuscript with research using blood specimens. The completed document should also be added to the MIBlood-EV shared folder (details at: https://www.isev.org/rigor-standardization)

3.5 | Urine

Urine is the second most-analysed biofluid after blood and can be obtained non-invasively, serially and in large quantities. Urinary EV (uEVs) and their contents are promising biomarkers and bio-regulators in health and disease of the kidney, the urogenital tract and possibly other organs and systems (Burger et al., 2014; Carreras-Planella et al., 2021; Erdbrügger et al., 2021; Morikawa et al., 2019; Ramirez-Garrastacho et al., 2022). Challenges in uEV studies arise from the diverse cellular origin of uEVs and the

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dynamic composition of urine, which varies by fluid intake, time of collection, diet, exercise, age, biological sex, medication and health and disease status. Please refer to previous, specific recommendations of the Urine Task Force of ISEV for all stages of uEV research: a position paper (Erdbrügger et al., 2021) and a 'Quick Reference Card' (van Royen et al., 2023).

Here, we focus on considerations specific for urine as an EV source. For urine collection and storage, many biobanked urine samples have not been processed to remove cells prior to storage, so uEV-specific biobanks or new collections may be needed. For any urine sample, urine proteins are the most common co-isolates/contaminants of uEV preparations (Dhondt et al., 2020). Protein abundance in urine spans five orders of magnitude. Amongst the highest-abundance urinary proteins (Tamm-Horsfall protein (THP), albumin and 20 other serum-filtered proteins) THP can not only co-isolate with uEVs, reducing uEV purity, but also polymerise into lattice-like networks that trap uEVs, reducing uEV yield. THP can be depolymerised and reduced by changing urine ionic strength or pH or by treating with reducing reagents (Correll et al., 2022; Liu, Cauvi, et al., 2018; Pisitkun et al., 2004). Removal of THP may be needed for downstream characterisation procedures such as mass spectrometry, but it is less necessary for other approaches (e.g., single-EV analysis by immunolabelling).

uEV studies in particular require careful normalisation approaches because of the magnitude of inter- and intra-individual variation in urine concentrations (i.e., of solutes in the urine; specific gravity), resulting from changes in the external environment, water and salt homeostasis, and circadian patterns. Because uEV levels may vary with urine concentration, normalisation between samples is necessary to counterbalance data variance. Unfortunately, there is no consensus method or marker(s) accounting for excretion rate and uEV processing that can be used for the robust normalisation of uEV quantity and/or content. Currently, normalisation for excretion rate is done based on absolute (total protein, uEV number, uEV biomarker) or relative (time collection, relative to urinary creatinine, osmolality) measures. In studies of organ-specific uEVs, organ-specific markers can be used; for example, prostate-specific antigen (PSA) concentrations can account for the proportion of prostate fluid in urine.

Recommendations:

- Follow previously published ISEV recommendations (Erdbrügger et al., 2021; van Royen et al., 2023).
- Perform uEV research using cell-free urine and cell-free urine biobanks.
- Where appropriate, report methodology and outcome of uEV co-isolate/contaminant depletion (THP, albumin and other serum-filtered proteins).
- For normalisation, collect data both on uEVs and non-EV urine parameters (e.g., creatinine, PSA or others as applicable) to estimate absolute or relative excretion rates.

3.6 | Cerebrospinal fluid

Cerebrospinal fluid (CSF) bathes the central nervous system (CNS) and contains biomarkers of CNS health and disease (Gaetani et al., 2019; Hühmer et al., 2006; Jack et al., 2018; Rao et al., 2013). Several CSF- specific factors must be considered in CSF EV studies. CSF is produced in the brain ventricles and circulates through the brain and spinal cord in a continuous flow (Czarniak et al., 2023). This flow establishes a rostro-caudal gradient, with lower levels of some brain proteins (e.g., S-100β, total or phosphorylated Tau), but higher levels of others (e.g., neurofilament, amyloid-β40 or β42) in the lumbar region relative to the brain (Jingami et al., 2019; Rostgaard et al., 2023). Hence, collection site (e.g., lumbar/spinal canal vs. brain) and volume may affect CSF composition (Cameron et al., 2019; Teunissen et al., 2009). Common confounders of CSF studies include residual cells and blood contamination, since protein concentrations in blood are 200−400 times greater than in CSF (You et al., 2005). Useful measurements of contaminants include cell counts (e.g., CSF samples that contain >500 erythrocytes/μL might be excluded (Teunissen et al., 2009) and protein assays for hemoglobin, catalase, peroxiredoxin, carbonic anhydrase I, apolipoprotein B-100, IgM, apolipoprotein B-100, fibrinogen or haptoglobin (Aasebø et al., 2014; You et al., 2005). Human donor characteristics reported to affect CSF biomarkers (Klener et al., 2014; Lewczuk et al., 2006; Mattsson et al., 2011) include sex (Li et al., 2017), ethnicity (Howell et al., 2017), disease-relevant genotypes (Li et al., 2017), medications (Riekse et al., 2006; Wong, 2007) and substance use (Liu et al., 2020; Wang et al., 2021). Age (Shah et al., 2011; Wong et al., 2000; Zhang et al., 2005) may be particularly important for cohort design and normalisation considerations, since human CSF protein concentrations are high in neonates, decline through childhood and increase from adolescence through adulthood (Howell et al., 2017; Shah et al., 2011; Zhang et al., 2005). For biomarkers that cycle with circadian rhythm, the time of day for collection is important (Lucey et al., 2017). However, these effects of pre-analytical variables may or may not affect EVs.

CSF EV studies are also challenged by very low concentration of EVs in CSF and the precious nature of CSF samples. Since CSF collection is relatively invasive, total CSF volume is limited for most patients, and sampling is usually done only for specific disease indications, the total number of samples and their volumes are small. For example, most established human CSF biorepositories are able to share 1.0 mL or less of each sample. As a result, high-yield separation approaches and high-sensitivity characterisation assays are especially needed for CSF EV studies (Krušić Alić et al., 2022; Sandau et al., 2020; Ter-Ovanesyan et al., 2021). Pooling

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samples from multiple donors may be an option to optimise new protocols or to perform omics characterisation, with or without follow-up with higher-sensitivity specific molecular assays for individual samples.

Recommendations:

- Report anatomic collection site and volume of CSF drawn because of possible influence of the rostral-caudal CSF gradient.
- Measure levels of specific co-isolates/contaminants, such as blood cells and blood proteins, and establish exclusion criteria where appropriate, for example, >500 erythrocytes/μL from biomarker studies.
- High-yield separations and high-sensitivity characterisation methods are especially important for studying CSF EVs, and sample pooling may be needed.

3.7 | Saliva

Healthy adult humans produce 500–1500 mL saliva per day, varying with pathological and physiological conditions (Chiappin et al., 2007). Saliva is non-invasively accessed, making it an attractive source of biomarkers, EV-associated or not, especially for oral and periodontal conditions (Nonaka & Wong, 2022; Ogawa et al., 2008). In saliva EV studies, common co-isolates include salivary components such as eukaryotic cells and subcellular structures, proteins such as enzymes and antibodies, electrolytes, food debris, bacterial cells and bacterial EVs (Aps & Martens, 2005; Chiappin et al., 2007; Han, Bartold, et al., 2021; Kaczor-Urbanowicz et al., 2019; Ngamchuea et al., 2017; Ogawa et al., 2008). The overall composition of saliva depends on the relative activity and contributions of the three major pairs of salivary glands—parotid, submandibular and sublingual—as well as 300–750 minor salivary glands (Aps & Martens, 2005; Khurshid et al., 2016), which may secrete different amounts of salivary enzymes and mucins.

Parameters to report in saliva studies are whether whole saliva or saliva from one type of gland only is collected; the method of saliva collection (Beale et al., 2016; Khurshid et al., 2016; Navazesh, 1993); salivation stimulus, if any (Gomar-Vercher et al., 2018). Recency of food and drink intake may have outsized effects on saliva quantity and quality and should be standardised if possible or assessed at collection. From studies of whole saliva, age (Xu et al., 2019), biological sex (Li-Hui et al., 2016), smoking (Rad et al., 2010), stress (Keremi et al., 2017), exercise (Ligtenberg et al., 2016), oral hygiene, medical conditions and medications, and mental health status (Aps & Martens, 2005; Bhattarai et al., 2018) may be associated with differences in one or more of viscosity, pH, concentrations of different proteins, and saliva flow rate. However, it is not known if these factors affect or are associated with the concentration and composition of saliva EVs, so additional studies are needed.

Recommendations:

- Report the source of saliva clearly (whole or from a specific gland), the method used for collection, and any stimulus used.
- Standardise allowed food and drink intake prior to collection or, at minimum, assess these factors at collection.

3.8 | Synovial fluid

Synovial fluid (SF) is a viscous fluid within the spaces of joints. SF EVs have potential as biomarkers and therapeutic agents for joint disorders (Boere et al., 2019) since SF is in direct contact with affected tissues (Michael et al., 2019). The viscosity of SF is due to large amounts of protein and the glycosaminoglycan hyaluronic acid (HA). This viscosity poses several hurdles to reproducible SF EV studies, for example, making it challenging to pellet cells/debris prior to freezing and hampering EV recovery. Indeed, most reported samples have been frozen and thawed before EV separation and characterisation, with inconsistent pre-freezing removal of cells and debris (Gao et al., 2020; Rüwald et al., 2020). Hyaluronidase treatment of SF is required for accurate detection of inflammatory cells and soluble mediators (Boere et al., 2019). Most research groups use hyaluronidase to decrease SF viscosity before EV separation, but others do not (Mustonen et al., 2021). SEC may outperform UC in removal of proteins such as albumin, fibronectin and apolipoprotein A-I (Foers et al., 2018). Donor characteristics that may associate with differences in SF variables and possibly EVs include biological sex (Kolhe et al., 2020) and disease identity and stage (Foers et al., 2020; Schioppo et al., 2021).

Recommendations:

- Consider the use of hyaluronidase to reduce viscosity and obtain homogenised synovial fluid before EV separation and characterisation.

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3.9 | Milk

Milk is a rich and complex source of nutritional and immunological components, which include cells, milk fat globules (MFGs), casein micelles, soluble molecules and EVs (Ballard & Morrow, 2013). EVs separated from milk of at least 16 different species have thus far been reported, chiefly human and bovine. To allow separation of relatively pure EVs, milk components that share EV characteristics such as density and size [MFGs and cellular debris (Busatto et al., 2019)] should be removed, for example, by centrifugation), and milk should be kept at body temperature for short-term storage (Zonneveld et al., 2014). Casein micelles, which overlap in size with EVs, are the biggest challenge, especially for milk of ruminant species. Casein micelles can be precipitated by pelleting after acidifying milk to pH 4.6 (Mukhopadhya, Santoro, Moran, et al., 2021; Rahman et al., 2019; Santoro et al., 2023; Somiya et al., 2018), aggregated by enzymatic treatment (Gao et al., 2019), or dissociated by sequestering calcium with EDTA (Gao et al., 2019) or sodium citrate (Benmoussa et al., 2020). Currently, there is no preferred method, but acidification and EDTA are used most often. Following pre-processing, cleared milk supernatant can be stored until EV separation. Methods such as UC, dgUC and SEC may be combined for higher purity, since single-step approaches will yield a low purity. Colloidal properties and acceptable storage times until processing may be different for raw, homogenised, pasteurised, ultra-high temperature-treated, and dried/powdered milk (Mukhopadhya, Santoro, & O'Driscoll, 2021). Furthermore, the effects of storage length and temperatures have yet to be comprehensively determined.

Recommendations:

- Keep milk at body temperature for short-term storage prior to storage or EV separation.
- Common EV co-isolates include cells/components, milk fat globules, and casein micelles. These should be removed (and/or, in the case of micelles, disrupted), and their presence tracked through the EV separation process.

3.10 | Solid tissue

Cell-EV interactions in solid tissues may primarily involve EVs that are released near the site of action. It is thus important to study EVs in tissue. However, greatly complicating the study of tissue EVs is the interrelated diversity of tissue harvesting and storage methods, cellular and extracellular matrix composition, and physical properties. Despite these challenges, two basic approaches to tissue EV studies have been developed and applied mostly to brain or tumor tissues.

Tissues can be used for EV studies by keeping tissues/cells 'alive' in culture after harvesting or by harvesting EVs directly from tissue before or after storage. Some tissues can be cultured ex vivo over several days and culture medium harvested for EV separation (Lunavat et al., 2017; Jeurissen et al., 2017; Jingushi et al., 2018). EV preparations may include tissue EVs present in the original tissue, EVs released during culture (perhaps with different properties from the native EVs), and products of cell death in culture like apoptotic bodies (Carrel & Burrows, 1911). Keeping tissue under conditions as close to their in situ environment as possible may be very important, such as maintaining tissue hydrated prior to culturing and avoiding high oxygen concentrations, although limited evidence has been gathered for the influence of these factors on collected EVs. Alternatively, tissue is processed immediately after resection (Cianciaruso et al., 2019; Crescitelli et al., 2020; Crescitelli et al., 2021; Gallart-Palau et al., 2016; Huang et al., 2020; Jang et al., 2019; Jeppesen et al., 2019; Perez-Gonzalez et al., 2012; Steenbeek et al., 2018) or after prior storage, usually freezing (Hurwitz et al., 2018; Hurwitz et al., 2019; Huang et al., 2020; Perez-Gonzalez et al., 2012; Vella et al., 2017; Yelamanchili et al., 2015). A preliminary study found no major differences in EV composition in fresh versus frozen tissues (Shen et al., 2023). Tissues are typically divided into small pieces [using tissue homogenizers (Gallart-Palau et al., 2016; Hurwitz et al., 2018; Hurwitz et al., 2019; Yelamanchili et al., 2015), vortexing (Banigan et al., 2013) or slicing (Huang et al., 2020; Jeppesen et al., 2019; Polanco et al., 2016; Vella et al., 2017)], followed by enzymatic treatment to disrupt the extracellular matrix (ECM) (Jingushi et al., 2018). These methods result in different degrees of cell damage, potentially introducing EV-like artefacts.

Recommendations:

- For ex vivo culturing approaches, keep the tissue as close as possible to its 'native' conditions, including maintaining hydration and nutrition. Consider also the influence of cell death processes on the EV preparation.
- For separating EVs directly from tissue (without ex vivo culturing), establish or follow best practices for the specific tissue in harvesting (e.g., perfusion or not of an animal model to minimise effects of blood); storage (does freezing affect outcome?); physical and enzymatic tissue separation (if done) and influence of specific EV separation/concentration methods.

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- Tissue EV characterisation should focus in particular on tracing the presence of cellular components that may be expected to be depleted in EVs, since cells and cellular artefacts may be the key contaminants of tissue EV preparations.

3.11 | Other sources

Not all sources of EVs are covered above; only those for which ISEV recently had or currently has a Task Force. ISEV members are welcome to propose formation of new task forces where no ISEV task force yet exists. These, in turn, may help to inform best practice.

3.12 | Pre-separation and post-separation storage

Storage conditions of both pre-separation sources and post-separation EVs may also affect EV yields, contents, functionality, and the ratio of single particles and aggregates. For most EV sources, pre-processing is advisable prior to pre-separation storage to remove potentially interfering entities such as cells. However, stringent pre-processing is not always possible. Details of whatever steps are performed should be reported, and an explanation given if pre-processing cannot be done. Acceptable storage prior to EV separation varies by source. Storage conditions, including any additives [e.g., bactericidal agents (Lucas et al., 2021)], should be fully reported and the influence on EV quantity and quality investigated if not already known.

Following separation of EVs, EVs should be studied in as native a form as possible. However, for most studies, stored EVs are used. Here, several considerations apply. All storage vessels and their materials should be reported, as EVs can be lost by attaching to surfaces (Evtushenko et al., 2020). Separated EVs may be stable without freezing for some time, but this may vary by EV composition and source and of course information on storage of EVs from some matrices is more comprehensive to date compared to information on EVs from other matrices. Long-term storage is typically at −80°C, although other temperatures have been examined. For example, saliva EVs were reportedly stable at 4°C for up to 20 months, retaining membrane integrity and protein content (Kumeda et al., 2017). Urinary EVs have reportedly been stored at −20°C for up to four years (Barreiro et al., 2021). Lyophilisation of EVs is also possible (Trenkenschuh et al., 2022). There is conflicting evidence on the effects of freeze-thaw cycles on EV properties. A study of saliva EVs found minimal effects of freeze-thawing on membrane integrity (defined as dipeptidyl peptidase IV activity) (Kumeda et al., 2017). However, studies of various sources of EVs have reported particle concentration and other changes with freeze-thawing (Gelibter et al., 2022; Görgens et al., 2022). Cryoprotectants may reduce effects of freeze-thaw (Le Saux et al., 2020; Lőrincz Á et al., 2014); for example, supplementing phosphate buffered saline (PBS) with human albumin and trehalose (PBS-HAT) reportedly improved short- and long-term stability for EVs stored at −80°C and through several freeze-thaw cycles (Görgens et al., 2022). Since optimal storage conditions may vary by EV composition and source, the freezing method (e.g., snap-freezing in liquid nitrogen, gradual freezing), suspension buffer (including cryoprotectants and other additives), temperature, duration of storage until use, thawing method (speed, temperature) and number of freeze-thaw cycles should be reported. Freeze-thaw cycles should be minimised, for example, by a careful aliquoting strategy, and samples with different numbers of freeze cycles may not be directly comparable.

> Consensus: 70.4% (703) of MISEV2023 survey respondents agreed "completely," and 28.5% (284) agreed "mostly" with Section 3: Collection and pre-processing: pre-analytical variables through to storage. 0.1% (1) "mostly" disagreed, and 1.0% (10) stated that they had no opinion and/or expertise. No respondents disagreed "completely."

4 | EV SEPARATION AND CONCENTRATION

EVs are typically characterised and used after one or more separation or concentration procedures. Trends in these approaches have been previously assessed by ISEV (Gardiner et al., 2016; Royo et al., 2020). Separation/concentration can be performed according to the EV biophysical characteristics of size, density, charge and surface composition (specific surface molecules). Other terms that are sometimes used for these procedures include 'enrichment', 'purification' and 'isolation'. The material captured after separation/concentration is an 'EV-containing preparation' or 'EV preparation' that may require storage prior to analysis or use. Any separation method should be chosen based on the known properties of the specific EV sources and the desired EV yield and specificity. When separating complex biofluids, quantification of yield and specificity for total EVs will likely be estimates, since particle number quantification is not always EV-specific and/or typically relies on surrogates of EV abundance such as spike-in populations or measurement of detectable subpopulations. Figure 2 shows the position of some commonly used methods for EV preparation on a yield (recovery) versus specificity grid. This section provides information and suggestions on some of these methods. More detailed information can be found in the literature (Hendrix et al., 2023).

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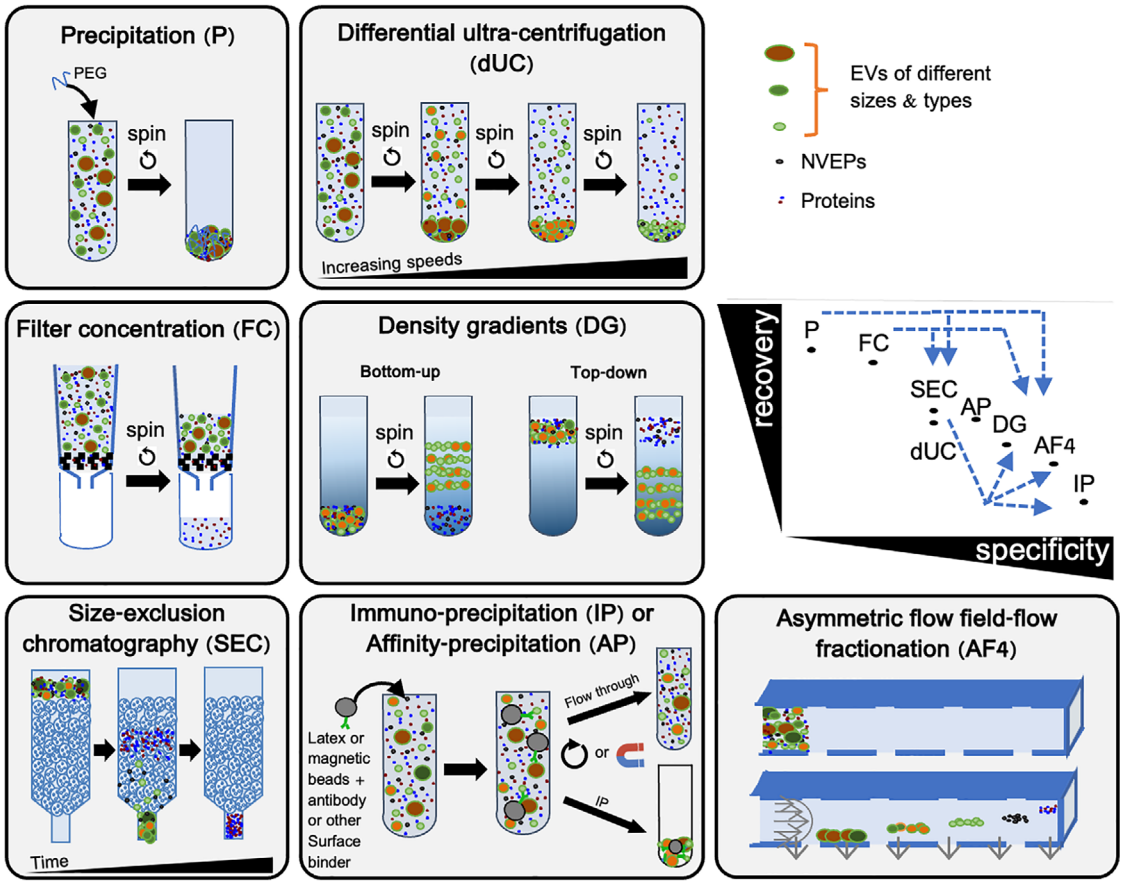

FIGURE 2 Position of some EV separation and concentration methods on a recovery (yield) versus specificity grid. Dashed blue arrows indicate combinations of methods resulting in increased specificity. Specificity can be of different types: Size exclusion chromatography (SEC) separates EVs by size from many (but not all) NVEPs, but all EV types are recovered together, while differential ultracentrifugation (dUC) separates EV subtypes based on their size/weight, but also co-isolates NVEPs at high speeds. Note that many 'exosome purification' kits use precipitation (P), thus do not isolate pure exosomes or even EVs but a mixture of EPs, while some use affinity precipitation (AP), which may be more specific to EVs but not exosomes. Those who develop new methods should consider positioning their EV outcomes on such a graph.

EVs can sometimes be studied or used directly and immediately in the source matrix. In biomarker studies, for example, there may be no need to separate or concentrate EVs from a biological matrix if sufficient specificity and sensitivity are reached with the unfractionated sample. In some cases, EVs can also be analysed specifically and directly in a biological fluid (Duijvesz et al., 2015; Woud et al., 2022). However, to show exclusive EV association of a proposed biomarker or function, separation may be required in the first instance, and further guidance on this is provided here.

4.1 | EV concentration

Concentration in EV studies is the act of increasing the particle number:sample volume ratio. Concentration may be needed in various settings. Large volumes of source materials like CCM, urine, milk may require concentration before EVs can be separated from other EPs. For example, chromatography columns may have a maximum loading volume, while some separation methods may be more efficient if material is first concentrated (e.g., some immunoisolation procedures). Concentration methods may, but do not necessarily, also achieve some degree of separation of particle types.

Concentration can be done by several approaches. Polymer-based methods of precipitation reduce the availability of biomolecules to solvent, 'crowding out' water molecules. This allows suspended/dissolved materials including EPs to be pelleted by low-speed centrifugation. Some commercial kits that are described as 'exosome isolation' kits in fact rely on such polymer precipitation and do not strictly 'isolate' EVs, much less subtypes of EVs. Precipitation methods may not achieve any appreciable separation of EPs (Gámez-Valero et al., 2016; Karttunen et al., 2019; Lobb et al., 2015; Paolini et al., 2016).

In filtration, a suspension passes through a filter by, for example, gravity, centrifugation or vacuum: water and molecules smaller than the molecular weight cut-off of the filter pass through, while EPs larger than the cut-off are recovered in the concentrated

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fluid compartment of the filter. A variety of filter cut-offs are available, including 3, 10, 100 and 1000 kDa, allowing filtration to achieve some degree of size separation, not just concentration. A cut-off of 100 kDa retains EVs while removing many proteins, while a cut-off of 1000 kDa may allow passage of some smaller EVs. However, another consideration is recovery, since different filters/tubes may allow different levels of EV 'sticking' and thus recovery (Vergauwen et al., 2017). Please note that filtration may also be performed to retain microbes ('sterilization') or large EVs/EPs in the pre-filter compartment; although care should be taken to avoid extrusion. Tangential flow filtration (TFF, also called cross-flow filtration) is a filter-based concentration method in which liquid and molecules smaller than the pores pass through the filter perpendicularly to the flow applied to the EV-containing fluid. This allows continuous flow and repeated passages of the fluid unless and until the filter is clogged, and thus allows processing of large volumes of fluid. As for other filtration methods, size-based separation can be achieved based on the molecular weight cut-off of the filter. TFF has been successfully and reproducibly used for large-scale EV production, for example, for therapeutic applications (Busatto et al., 2018; Lamparski et al., 2002). Finally, concentration can also be obtained by (ultra)centrifugation, for which parameters are described in the next section.

Summary: Concentration

- Can be done by polymer-based precipitation, filtration including tangential flow filtration, and (ultra)centrifugation.
- Leads to EV-containing preparations containing variable amounts of NVEPs and proteins, depending on the exact method and variables such as filter cut-off (size or molecular weight).

Reporting recommendations: for concentration, report the following:

- nature of the material used for concentration;
- initial and final volumes of biofluid;
- time of processing (incubation with polymer, centrifugation through filters or directly);
- flow rate (for TFF);
- size or molecular weight cut-off (for filtration/concentration);
- temperature during concentration.

4.2 | Differential (ultra)centrifugation

The principle of differential ultracentrifugation (dUC) is to apply increasing relative centrifugal forces (RCF = g-force) to the EV-containing fluid, from which intact donor cells or tissues have first been eliminated by one or more low speed centrifugations. The aim is to pellet sequentially EPs of decreasing sedimentation coefficients. Since the sedimentation velocity of a sphere is proportional to its diameter squared and to the density contrast between the particle and the medium (Stokes' law equation), the largest and/or densest EPs tend to be pelleted in the first (medium speed/short time) steps, while the smallest and/or least dense are recovered predominantly after higher speed/longer centrifugation. However, in practice, perfect EV separations are not achieved by this method, and pellets from different centrifugation speed have overlapping properties and variable biochemical and physical parameters.

Whatever the centrifugation steps used, as detailed in MISEV2018, report speed in rpm and rotor type (to allow calculation of adjusted k-factor), time of centrifugation (to allow calculation of the sedimentation coefficient of the pelleted particles), and temperature. Instrument acceleration and deceleration settings should also be reported. In typical dUC workflows reported in the literature, a maximal force of around 10,000 to 20,000 × *g* is applied for between 10 and 90 min to enrich putatively larger/denser EVs, while a maximal force of around 100,000 to 200,000 × *g* is applied for 45 to 150 min to pellet putatively smaller/lighter EVs. These figures can be used to calculate the sedimentation coefficient (S) of the particles recovered by these different protocols: S = adjusted K factor of the rotor/Time of centrifugation. Theoretically, particles with S coefficients in the range of 15–150 are recovered by the 'larger EV' centrifugation conditions, and those in the range of 2 to 5 by the 'smaller EV' conditions. Particles with smaller S can be recovered by extending the speed and time of centrifugation, at the cost of increasing NVEP/free protein co-isolation. Depending on the centrifugation parameters, the resulting pellets may be enriched for large/dense or for small/light EVs, but complete separation of these populations is not achieved. Yield of smaller EVs may also be low, especially when suspended in protein-rich fluids such as blood products and complex culture medium components, and this problem may not be resolved by simply increasing centrifugation time or speed (Driedonks et al., 2019; Zhang, Borg, et al., 2020). Examples of dUC protocols (with or without density gradient, see next section) and downstream comparison of EVs include (Jeppesen et al., 2019; Kowal et al., 2016; Lischnig et al., 2022; Martin-Jaular et al., 2021).

The majority of published studies have focused on smaller EVs and thus discard and/or do not analyse the pellet(s) obtained with lower-speed centrifugation. To allow comparison between studies and to avoid pelleting larger particles and potentially introducing artefacts, however, it is recommended to perform these first centrifugations. The strong g-force of high-speed UC has also been shown to induce aggregation of EVs (Linares et al., 2015), but this may not be observed for all sources of EVs. When

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analysing a new source of EVs, retain the intermediate centrifugation pellets and analyse them side-by-side at least once with the final, highest-speed pellet to determine whether the molecules or activity of interest are specifically enriched in small EVs or are also present in other subtypes.

Summary: dUC

- Enriches for EV subtypes that are separated according to their sedimentation coefficient, proportional to their diameter and density.
- Co-isolates NVEPs that have the same sedimentation coefficient as EVs, especially after high-speed and lengthy ultracentrifugation.
- May induce aggregation of EVs.

Reporting recommendations: for differential (ultra)centrifugation, report the following:

- speed, rotor type, and time of centrifugation, to allow calculation of the adjusted k-factor (to apply to other rotors) and the sedimentation coefficient of the pelleted EPs;
- tube type and sample volume in the tube;
- temperature during centrifugation;
- acceleration and deceleration (brake) settings.

4.3 | Density gradient/cushion

Density gradients or cushions can be used to separate certain NVEPs and proteins from EVs based on the characteristic densities of different classes of EPs (Raposo et al., 1996). Gradients are prepared of layers consisting of different ratios of a selected dense medium (like sucrose, iodixanol or iohexol) and aqueous buffers, with density decreasing from bottom to top of the gradient, whereas cushions consist of a homogeneous layer of dense material below an aqueous column. EV-containing materials can be loaded beneath a gradient ('bottom-up') or onto the top of a gradient or cushion ('top-down') and then ultracentrifuged. In the bottom-up approach, the EV-containing preparation is mixed with high-density medium, loaded at the bottom of a centrifuge tube, and overlayed with layers of decreasing density; the preparation may also be underlaid under a prepared gradient. As ultracentrifugation proceeds, particles that are less dense than the surrounding medium float upwards. With sufficient time, particles will ultimately reach a density fraction corresponding to their buoyant density. Since smaller EVs travel at a relatively slower rate than larger EVs, especially in viscous media, the bottom-up approach in velocity sucrose density gradient UC can also be used to separate EVs according to size (Aalberts et al., 2012). In top-down settings, the EV-containing preparation in a low-density medium is loaded onto the top of a gradient or cushion: for gradients, particles travel into the gradient at a rate corresponding to their density and size until their equilibrium buoyant density is reached; for cushions, particles that reach the cushion remain at the interface if less dense than the cushion material but continue into and through the cushion if they are denser. The cushion approach is thus easier to implement but separates EPs by a threshold of density. Importantly, for gradients, lengthy ultracentrifugation may be needed for optimal separation [e.g., longer than 48 h (Aalberts et al., 2012; Palma et al., 2012)], but shorter spins may suffice for some applications [e.g., 1–2 h (Kowal et al., 2016), 16 h (Aalberts et al., 2012; Liao et al., 2019)].

Following separation by gradient, fractions must be collected carefully to avoid disrupting the gradient. It is good practice to confirm density of final fractions, for example, by weighing given volumes or measuring refractive index. Before performing most downstream assays, the density medium must be removed. This can be done, for example, by diluting the fractions with buffer and ultracentrifuging, or by using SEC. Recovery after density gradient and fraction washing is relatively low.

Summary: density gradients and cushions

- Can be implemented in different settings (top-down, bottom-up) depending on the aim, that is, to separate EVs from proteins, or from NVEPs, or to separate EV subtypes.
- Leads to low recovery of high-purity material (based on density).

Reporting recommendations. For density gradients and cushions, report the following:

- density material, buffer composition, and exact method of gradient/cushion preparation;
- volume and concentration of material loaded, as well as method of loading onto or at the bottom of the column;
- exhaustive description of centrifugation parameters (same as for dUC);
- details of collection procedure, final densities of fractions (where relevant), and washing.

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4.4 | Size exclusion chromatography

SEC separates nanoparticles including EVs based on size (Boing et al., 2014; Karimi et al., 2018). In SEC, a sample is placed onto the top of a column loaded with a matrix that contains passages with defined pore size. Driven by gravity or by pressure from a pump, larger particles pass through the matrix quickly, without entering the pores, and can be collected as early fractions, while smaller particles (smaller than the matrix pore size) are retained longer and elute predominantly in later fractions. Certain SEC matrices allow separation of EV-sized particles (EVs, viruses, larger lipoprotein particles) from small NVEPs and free proteins.

Variables that affect the degree of separation by SEC include the matrix composition and pore size, column packing method, the ratio of column length to diameter (or volume), flow rate (gravity vs. defined pressure), and applied sample concentration and volume. Size exclusion columns can be home-made or purchased. Commercial columns are often packed under strictly controlled conditions and may allow more reproducible results than home-made columns. Abundance and purity of EVs and other NVEPs in collected fractions must be established through careful characterisation, as for all other methods. SEC dilutes the sample, increasing volume compared with the input material, so concentration of a sample before or after SEC may thus be needed. SEC size separation can be combined with affinity methods by modifying the matrix. The related method of bind-elute chromatography combines size-based separation with selection by charge or molecular affinity and permits a single elution (with retention of unwanted materials) that may be amenable to high-throughput separations, for example, in multi-well plates. In some cases, SEC matrices can be reused after thorough cleaning.

Summary: SEC

- SEC is an easily accessible technique for size-based separation of particles.
- Columns can be packed with a variety of matrices and at different scales, depending on desired capacity and resolution of separation.

Reporting recommendations. For SEC, report the following:

- type of matrix and pore size; height and diameter (or volume) of matrix-containing column;
- method of column packing (or source of commercially-available columns);
- source, volume and particle concentration of pre-SEC sample, including any prior separation/concentration steps;
- buffer composition;
- specify gravity flow or pressure. If pressure, indicate pump system and pressure parameters;
- void volume and numbers and volume of fractions collected;
- any post-SEC concentration methods;
- if columns are re-used, method of column regeneration and number of times the column has been used.

4.5 | Fluid flow-based separation

Fluid flow-based techniques separate EVs and other particles based on one or more particle properties, but without relying on a 'matrix' or stationary phase. The two main categories of flow-based techniques currently used in EV studies are field-flow fractionation (FFF, Giddings et al., 1976) and free-flow electrophoresis (FFE, Preußer et al., 2022). These techniques can be applied to highly heterogeneous input materials, and the absence of a solid phase allows high particle recovery. They are also among the gentlest of separation methods and may thus be used to study molecules that are loosely associated with EVs.

The most prevalent FFF approach in EV studies is asymmetric flow FFF (AF4, Sitar et al., 2015), in which particles in a sample are transported by fluid flow with a parabolic pattern through a long, thin channel, while a field perpendicular to the direction of transport tends to concentrate particles against the bottom of the channel. Smaller particles, diffusing more rapidly, are more likely to enter the higher-velocity flow regions in the middle of the channel, and particles are thus separated by hydrodynamic size. Some degree of purification may also be achieved by a channel bottom consisting of a molecular weight cut-off filter. Although AF4 can precisely separate particle populations with small differences in size (Hood et al., 2014; Zhang et al., 2018), it is not specific to EVs in its standard configuration. AF4 is also not a preparative technique. In contrast, FFE combines flow with electrophoresis, adding separation by, for example, isoelectric point (Preußer et al., 2022). Introducing separation buffers with different pH or other characteristics across the separation channel allows high-resolution separation of different EV and other EP populations. FFE can be done at various scales.

Summary:

- Fluid flow-based separations such as AF4 can achieve size-based separations with high resolution.
- Lacking a solid phase and with limited applied forces, flow is gentler than most EV separation techniques.

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- Size-based separation can be combined with separation by other principles by applying different types of fields.
- Preparative scales can be reached with flow techniques such as free-flow electrophoresis.

Reporting recommendations. For flow-based separations, report the following:

- all instrumentation, including pumps and collection devices;
- composition of all buffers and their filtration. Especially in FFE: how properties of the buffers were confirmed;
- all field characteristics such as flow rates and pressures, pH gradients, electric field;
- dimensions and composition of separation chambers, including any molecular weight cutoff plates;
- all relevant details of fraction collection.

4.6 | Charge and molecular recognition-based separations

The common principle of all affinity methods is to capture EVs based on their surface charge or molecular composition. Ion-exchange chromatography takes advantage of a very simple affinity of particles/surfaces of opposite charge: EVs and/or NVEPs have affinity for a matrix based on negative (anion-exchange) or positive (cation-exchange) surface charge (Saari et al., 2023). In contrast, the term 'affinity separation' as commonly used in molecular biology refers to methods that harness the specific recognition of one macromolecular complex for another. In this context, affinity probes include heparin and various lectins (which bind glycans, Balaj et al., 2015); specific full-length proteins with affinity for a particular lipid or protein (e.g., Tim4 for phosphatidylserine, (Nakai et al., 2016); peptides that bind specific EV surface proteins (Bai et al., 2014; Gao et al., 2018; Gobbo et al., 2016; Joy et al., 2018; Liu et al., 2019; Pham et al., 2021; Suwatthanarak et al., 2021) or the membrane more generally (Gori et al., 2020; Ishida et al., 2020; Yang et al., 2022), including curvature-sensing peptides that select for EVs in certain size ranges (Saludes et al., 2012); aptamers [short single-stranded DNA or RNA molecules that are developed to bind specific targets (Zhang, Yue et al., 2019)]. Antibodies that are raised to recognise specific EV surface molecules are the most commonly used affinity reagents, and their most-used targets are the tetraspanins (Kowal et al., 2016; Mathieu et al., 2021).

In molecular recognition-based affinity approaches, the EV-containing fluid (which may have first been concentrated according to Section 4.1) is introduced to affinity probes before or after the latter are bound to a matrix, such as a membrane or beads. Beads, in turn, can be placed in a column or tube to facilitate binding and washing. Molecular target-displaying materials are bound by affinity probes to the matrix ('pull-down'), while unbound material flows away ('flow-through'). Non-specifically bound materials may be removed by one or more washes. If EVs are the intended target, detergent should not be present in the dilution and washing buffers unless at very small concentrations (0.001% or less) to minimise non-specific binding to the capturing matrix or between EVs. To evaluate the efficiency and specificity of recovery of the targeted EVs, it is recommended, at least during protocol development, to compare side-by-side the flow-through and the pull-down by biochemical analyses, measuring the affinity motif and a few EV markers (see Section 5).

Bound EVs can be dissociated from the matrix and recovered by a variety of techniques, ranging from changing the properties of the buffer, to adding an excess of target molecules (e.g., sugars or lipids), to eliminating factors required for efficient binding (e.g., using EDTA to chelate calcium). However, some affinity reagents may bind tightly to their EV target and require removal by, for example proteases. In some cases, the EV-binding molecule and/or the matrix may be recovered together with the EVs. This may not be an issue if downstream analyses are not affected by these materials (e.g., nucleic acid analysis of EVs contaminated with a protein-based affinity probe), but it may be in other cases, (e.g., in functional uses, since the EV surface is modified by an EV-binding molecule). Antibodies are particularly difficult to separate from EVs. Low pH treatment classically used to separate antibodies and antigens will likely affect the structure of EVs, and protease treatments may also digest proteins on the surface of the EVs.

In any molecular affinity approach, it is important to understand the degree to which the target molecule is associated with EVs versus NVEPs, or with one EV subtype versus others, and to assess the specificity of the capture reagent. For example, the use of CD9 or CD63 affinity capture for urinary EVs (uEVs) excludes uEVs from cells of the proximal nephron (Blijdorp et al., 2021; Limbutara et al., 2020). In another example, the literature on L1CAM affinity (a putative neuronal EV marker) has developed substantially since MISEV2018. Although L1CAM has been investigated as a membrane-associated antigen to separate putative neuronal EVs from peripheral blood samples, it has more recently been described as being in a cleaved, mostly soluble form in certain EV sources (Norman et al., 2021). It is also found on EVs from a variety of sources, not just neurons, and a widely-used anti-L1CAM antibody might also recognise other targets (Gomes & Witwer, 2022; Norman et al., 2021).

Summary: charge and molecular recognition affinity-based methods

- Separate components of EV preparations according to surface charge or exposure of a specific molecular determinant.
- Will co-isolate all EV subtypes or NVEPs which expose a given charge or molecular determinant: specificity and recovery depend on the specificity versus universality of exposure of the chosen molecular determinant.

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- Antibody-based affinity separation leads to co-isolation of the determinant-exposing EPs with the antibody and/or isolation beads.
- Efficiency and selectivity must be quantified when establishing the protocol by quantifying material recovered in pull-down versus flow-through.

Reporting recommendations: for affinity-based separation, report the following:

- molecule used as affinity probe (nature, source);
- matrix (beads, gel, column);
- incubation times;
- buffer and number of washes;
- elution process (such as elution buffer composition, time).

4.7 | General considerations and caution on kit-based approaches

Due to overlapping biophysical characteristics of EPs (Geeurickx et al., 2019; Karimi et al., 2018) and the abundance of many NVEPs in various EV sources, complementary separation techniques are increasingly applied sequentially (Benedikter et al., 2017; Stam et al., 2021; Zhang, Borg, et al., 2020) (arrows in Figure 2), which allows increased specificity. Examples of methods used to separate EVs from protein aggregates and other NVEPs include size exclusion chromatography, density gradient ultracentrifugation (Jeppesen et al., 2019), asymmetric flow field-flow fractionation (AF4) (Zhang et al., 2018) and ultra-high-speed ultracentrifugation (Zhang, Higginbotham, et al., 2019; Zhang et al., 2021). However, some of these studies suggest that several proteins previously proposed to be sEV markers are equally, if not more, abundant in NVEPs, thus calling for re-evaluation of the achieved EV selectivity.

Conversely, a growing realization since MISEV2018 is that some molecules that co-isolate with EVs, including proteins, nucleic acids, sugars, and lipids, could be viewed not as 'contaminants', but rather as a part of a dynamic EV 'corona' (Buzas, 2022; Palviainen et al., 2020; Tóth et al., 2021). Molecules and even biological nanoparticles such as lipoproteins (Busatto et al., 2022; Sódar et al., 2016) may adsorb to the EV surface where they may serve as biomarkers or contribute to EV function (Musicò et al., 2023; Radeghieri et al., 2022). The EV corona may be removed in part or in full by separation processes including dUC and SEC (Singh et al., 2020; Wolf et al., 2022). Ongoing studies of the EV corona may change how we view contaminants and the perceived need for highly pure EVs; this point is also relevant for the next MISEV section, on EV characterization.

Only methods using readily (i.e., commercially) available devices and instruments are described in this section. However, new developments of separation methods, including those involving equipment built in individual laboratories, are occurring constantly and are strongly encouraged by ISEV. When establishing a new separation/concentration workflow, a good practice is to assess the extent of EV separation/concentration with methods discussed in Sections 5 and 6 and with careful and complete book-keeping. Comparing the results with those of another already established method is also recommended. For example, EV marker proteins can be tracked and related to total isolated protein to determine fold enrichment over total protein reported and account for EV marker losses (Geeurickx et al., 2019; Zhang, Borg, et al., 2020). Results will indicate recovery and degree of enrichment and will also show whether the separated EV population is representative of the original population or has been selectively obtained.

Finally, some cautionary notes on commercial kits. Numerous kits are advertised as obtaining specific types of EVs (usually 'exosomes') or EVs from specific types of sources. These kits may or may not achieve EV separation or concentration based on a variety of principles, including polymer precipitation, membrane affinity, antibody capture and filtration. These kits may be helpful under certain circumstances, but EV researchers should be aware of several major caveats. Kits that do not disclose details of the principles of EV separation/concentration may produce results that are difficult to interpret, not least because they may introduce unknown contaminants (e.g., polyethylene glycol for some polymer precipitation kits). Extra work may be needed to compare these methods with results from other techniques and to place the results on the recovery/specificity grid (Figure 2) for better interpretation. Precipitation-based kits in particular will concentrate all EPs in a mixture, even many free proteins, resulting in a highly impure preparation, especially from complex, NVEP-rich sources such as blood plasma and serum. Use of such kits is strongly discouraged unless for volume reduction alone (Gámez-Valero et al., 2016; Karttunen et al., 2019; Lobb et al., 2015; Paolini et al., 2016). In contrast, affinity-based methods may isolate only subtypes of EVs, and the specificity of the affinity reagents may be difficult to assess if the exact reagents are not disclosed. Generally, kits that disclose contents and principles should be preferred over kits that make unsubstantiated claims (e.g., 'exosome' isolation) and do not provide details.

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Recommendations

- If separation/concentration is not done, indicate why. Otherwise, justify why each separation/concentration method was selected in terms of yield and specificity.
- Provide sufficient methodologic detail to allow replication of each separation and concentration step.
- Report any measurements that are used to assess the separation/concentration process(es). Where applicable and feasible, and especially when establishing a new workflow, check EVs before and after each step. For example, track EV marker protein levels relative to total protein to estimate fold enrichment and yield for each step.
- For affinity-based EV separation approaches, establish molecular specificity of reagents and EV/EV subtype-specificity of all targeted markers.

> Consensus: 74.4% (743) of MISEV2023 survey respondents agreed "completely," and 24.8% (248) agreed "mostly" with Section 4: EV separation and concentration. 0.1% (1) "mostly" disagreed, and 0.6% (6) stated that they had no opinion and/or expertise. No respondents disagreed "completely."

5 | EV CHARACTERIZATION

EV characterization is needed for estimation of EV quantity, to establish the presence of EVs, and to assess the contributions of non-EV components to an EV preparation. Characterization is challenged by small particle size, heterogeneity of EV size and molecular heterogeneity, a lack of universal EV identification methods, and the non-EV-specificity of many measurement techniques. As a result, no single measurement or method is able to satisfy all EV characterization requirements, and use of orthogonal methods (those that do not have the same measurement limitations) is recommended.

If making claims about an EV preparation, the extent to which a sample will need to be characterised to justify the claims may depend upon the source of the material (see Section 4). This may mean additional characterization steps are needed with different samples and may also mean that additional reporting information is required to allow the influence of other preanalytical variables on EVs to be assessed.

Overall EV composition (contribution to total mass of proteins, lipids, nucleic acids and other biomolecules) varies by EV source. While measurement of these individual molecular classes can be used to estimate EV abundance, these values do not necessarily perfectly correlate with EV concentration, nor is there universality across source materials; thus, they should not be used as a sole measure of EV concentration.

Just as no single molecular class measurement can quantify all EVs, there are also no universal molecular markers of EVs or EV subtypes. Markers must be chosen based on source- and type-specific evidence. Currently, no generic marker is known to identify all EVs irrespective of source. Although several proteins have been proposed as putative markers of EV biogenesis pathways (e.g., Annexin A1 (Jeppesen et al., 2019), SLC3A2, and BSG (Mathieu et al., 2019) for purported ectosomes, and Lamp1 (Mathieu et al., 2021)) for purported exosomes, the universality of these markers is not yet clear or accepted. Note that affinity-based protocols involving the tetraspanins CD9, CD63 and CD81 are not specific for exosomes as an EV subtype; using antibodies to each of these tetraspanins enriches EV populations that do not completely overlap in molecular composition (Kowal et al., 2016; Mathieu et al., 2021). Additionally, not all EVs display these tetraspanins, therefore tetraspanin enrichment does not capture all EVs.

Orthogonal methods measurements of the same parameter are unlikely to have the same biases; for example, the derivation of diameter from optical versus non-optical methods. Characterization of EV samples using orthogonal methods is critical to provide evidence that co-isolates are not responsible for biomarker or functional findings. Due to many EV characterization methods being either not EV specific or unable to detect all EVs, transparent reporting of methods and results is needed for reproducibility of EV data. A framework for reporting EV data has been previously developed and updated in the form of EV-TRACK (EV-TRACK Consortium et al., 2017; Roux et al., 2020). Standardization of EV characterization has been supported by ISEV workshops, the ISEV Rigor and Standardization Task Forces, and ISEV position papers (Clayton et al., 2018; Nieuwland et al., 2020; Welsh, Van Der Pol, Arkesteijn, et al., 2020).

In the following sections different approaches to EV characterization are discussed, with each section providing recommendations if that characterization approach is taken. Overall recommendations for characterization, regardless of the method, are summarised below.

Recommendations

- Each EV preparation should be defined by quantitative measures of the source of EVs (e.g., number of secreting cells, volume of biofluid, mass of tissue).
- Approximations of the abundance of EVs should be made (particle number, protein, and/or lipid content).

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- EV preparations should be tested for the presence of components associated with EV subtypes or EVs generically, depending on desired specificity one wishes to achieve.
- Establish the degree to which non-vesicular, co-isolated components are present.
- Provide an indication of the instrument/method limit of detection (LOD) when EVs are characterised with quantitative metrics.

5.1 | Quantification of particle number concentration

EV number can be used along with volume measurement to define the number concentration (in particles/mL), a metric that is widely reported and used for assay input standardization, assay output measurements, and in vivo dosing. However, it is often unreliable, since many techniques lack specificity for EVs and sensitivity for all EVs.

The ISEV Rigor and Standardization EV Reference Material Task Force recently outlined the considerations in measurement techniques, along with the challenges faced by the field in moving towards traceable measurements, for the development and reporting of well-characterised EV reference materials (Welsh, van der Pol, Bettin, et al., 2020). A key highlight of this work is the need to report assay LOD, allowing others to validate findings irrespective of the sensitivity limit. Note that reported EV concentration in blood plasma spans six orders of magnitude depending on the measurement method (Johnsen et al., 2019). Greater confidence in EV concentration measurements may be achieved by using orthogonal methods, each with defined LODs, for example, detecting light scattering intensity, fluorescence intensity and physical size, since orthogonal methods do not share the same measurement limitations (Arab et al., 2021; Silva et al., 2021). For example, for resistive pulse sensing (RPS) techniques that are calibrated with size-standards, a LOD can be reported in diameter. The lower LOD for RPS will most likely be due to sensitivity limitations, while the upper LOD will be influenced by the pore size. For optical techniques such as flow cytometry, the LOD may be reported in diameter, derived from light-scattering optical models, or molecules of equivalent soluble fluorophore (MESF), derived from fluorescence intensities. These approaches result in concordant data across instruments and sensitivities (Welsh, Van Der Pol, Arkesteijn, et al., 2020; Welsh, Jones, et al., 2020; van der Pol, Sturk, et al., 2018). Currently, there is no method to derive a traceable LOD for nanoparticle tracking analysis (NTA), DLS, or imaging flow cytometry, due to the number of variables involved in particle detectability. Techniques that output concentration measurements without any phenotypic characterization, such as the use of membrane dyes, can lead to overestimation of EV concentration due to dye self-aggregation and an inability to differentiate between EVs and other co-isolates (Takov et al., 2017). A membrane dye lacking these problems could lead to underestimation unless it universally stained all EVs, irrespective of composition and derivation, and such a dye has not yet been reported. Further instrument and assay-specific recommendations can be found in Section 6.

For techniques that cannot differentiate EVs from other potential co-isolates or suspension contaminates, it is recommended that concentration be reported as 'particle or EP concentration' and not 'EV concentration', regardless of upstream separation steps.

Recommendations

- Report the LOD of each assay, or state that it is not quantifiable or known.
- Where possible, report data from dilution series to demonstrate that concentration derivations were in the linear region of system measurement.
- Where possible, use orthogonal methods to determine particle number.
- Unless methods are highly specific for EVs, the output of these measures should be described as pertaining to 'particles' or 'EPs'.

5.2 | Quantification of particle size

Measurements of EV size (in nm radius or diameter) rely on assumptions, such as of sphericity or mobility, and output can be influenced by upstream variables (Tian et al., 2020). Common high-throughput methods assume that EVs are spherical. These include flow cytometry, NTA, RPS, multi-angle light scattering and dynamic light scattering (DLS). While 'size' and 'diameter' are often used interchangeably between measurement methodologies, the way in which they are derived can also result in consistent differences in measurement techniques. For example, techniques relying on the mobility of particles, such as NTA or DLS, measure hydrodynamic diameter, resulting in an overestimation of size compared with an imaging method such as cryo-EM (Chernyshev et al., 2015; Skliar et al., 2018). Few if any methods are able to measure EV size accurately throughout the entire possible EV diameter range, from tens of nanometres to microns. For example, while high-resolution imaging by cryo-EM is one of the most accurate methods (Yuana et al., 2013), it is relatively low-throughput, and many larger EVs that tend to be orders of

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magnitude less abundant may not be quantified. The ability to quantify low contrast EVs below 100 nm may also be a limiting factor.

As more researchers use dedicated single-particle techniques with increased sensitivity, it is becoming increasingly clear that many EV preparations display an asymmetric right-skewed distribution, for example, a log-normal distribution, with the majority of EVs <100 nm in diameter (Bachurski et al., 2019; Dong et al., 2020; Lennon et al., 2019; Tian et al., 2018, 2020; van der Pol, Coumans, Grootemaat, et al., 2014). Most single-particle analysis techniques cannot resolve the full population of EVs, so the detected EV diameter distribution should be shared, not just a summary metric such as mean, mode, or median size, which can be easily skewed depending on the LOD and the asymmetric size distribution (Welsh, van der Pol, Bettin, et al., 2020). Be aware that the modal size statistic, as measured, for example, by NTA for low refractive index particles, may better approximate the instrument LOD than the true modal diameter of the EV population (Bachurski et al., 2019). Techniques using software with proprietary algorithms to determine particle diameter may also result in variation between software versions or software platforms (van der Pol, Coumans, Grootemaat, et al., 2014); software and version should therefore be reported. Techniques deriving size from refractive index assumptions may result in variation due to differing compositions and cargo. Derivation of size from fluorescent probes, such as membrane intercalating dyes, may result in variation due to different dye intercalation based on different membrane lipid compositions. For techniques that cannot differentiate EVs from co-isolates/contaminants, it is recommended that diameter be reported as 'particle' or 'EP' diameter and not 'EV diameter', regardless of upstream separation steps.

Recommendations

- Where possible, make orthogonal measurements to increase confidence in size distribution.
- EV diameter distribution of a population should be shared, not just mean, mode, or median.
- Consider the LOD of the method chosen and how this may influence the data.
- Report instrument settings, software platforms and versions and possible influence of measurement reagents, especially intercalating dye.

5.3 | Quantification of total protein

Total protein (in μg, or μg/mL for concentration) in an EV preparation can be approximated by colorimetric assays, fluorometric assays, global protein stain on SDS-PAGE, or absorbance readings, each with differing sensitivities and accuracies (Vergauwen et al., 2017). As a bulk analysis technique, total protein quantification often overestimates EV concentration due to co-isolated protein, especially for less specific methods of EV separation or complex biofluids. Conversely, highly purified, low-yield EV preparations may challenge assay sensitivity. Since measured protein concentration may vary depending upon whether intact or disrupted EVs are measured, details of physical disruption and the nature and concentration of any detergent should be indicated.

Protein concentration as a surrogate of EV concentration should be used with caution and is generally not recommended, as enrichment of some proteins per EV may occur with different cellular phenotypes or stimulations. Since protein:particle ratios also depend on the LODs lower concentration limit of detection or lower size limit of detection of each assay/instrument, it is recommended to provide absolute protein and particle concentrations separately if ratios are reported.

Recommendations

- Report output by 'particles' or 'EPs' unless evidence of upstream processing is highly specific for EVs.
- Report the lower concentration limit of detection of each assay to facilitate interpretation.
- For ratios, report the original constituent measurements, not just the ratio.
- Protein concentration should be within the linear range of the reference curve, which should also be reported.
- Report whether intact or disrupted preparations are used.

5.4 | Quantification of total lipids

Total lipid quantification of EV samples can be achieved by colorimetric assays (Visnovitz et al., 2019), fluorescence of membrane intercalating dyes, total reflection Fourier-transform infrared spectroscopy (FTIR) or chromatography (Mihaly et al., 2017). However, intercalating dye methods and FTIR may be insufficiently sensitive for small amounts of EVs, and some methods require highly specialised equipment. It remains unknown whether these techniques detect all EVs independent of lipid composition. Total lipid measurements may overestimate EVs due to co-isolated NVEPs such as lipoproteins.

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Recommendations

- Consider the LOD of your assay.
- Consider how co-isolated NVEPs may influence your measurement.

5.5 | Quantification of total RNA

RNA is a frequently studied EV-associated molecule (see Section 6.5), so basic characterization of EV preparations may include total RNA quantification as a quality control component or for normalization in profiling and functional studies. Quantification of total EV RNA can be done by capillary electrophoresis and other methods. However, using total RNA as a surrogate for EV concentration or purity is difficult to recommend due to vastly more abundant extra-EV RNA in many EV sources. Some methods of RNA quantification do not distinguish between RNA and DNA. Isolation kits have also been demonstrated to influence downstream results (Eldh et al., 2012). An early ISEV RNA position paper recommended the use of sensitive techniques such as Agilent Bioanalyzer pico chip or Quant-iT RiboGreen RNA Assay for EV RNA quantification over less sensitive methods such as NanoDrop (Hill et al., 2013). However, several nucleic acids dyes, such as RiboGreen, are not specific for RNA over DNA. Additionally, small RNAs require specialised Bioanalyzer kits. Other sensitive methods include the Qubit microRNA Assay kit, which has sensitivity for small RNAs. Pre-treatment with RNase-free DNase may be useful for accurate RNA quantification since many techniques are sensitive to DNA contamination. However, DNase treatment may not completely remove all DNA contamination (Verwilt et al., 2020).

Recommendations

- Consider the ability of your assay to discriminate between RNA and DNA, and the limits of detection of your chosen method.
- Report any enzymatic pre-treatments of the sample, for example, with DNase.

5.6 | Characterization of EV morphology

EV morphology is currently best assessed for smaller EVs using high-resolution imaging techniques such as: scanning electron microscopy (SEM) (Cavallaro, Hååg, et al., 2021) , transmission electron microscopy (TEM) (Théry et al., 2006), cryo-EM (Arraud et al., 2014; Stoner et al., 2016; Wu et al., 2015); and scanning-probe microscopy (SPM), including atomic force microscopy (AFM) (Sharma et al., 2011). EVs that are much larger than the light diffraction limit ($\gtrsim$200 nm diameter) might be assessed by conventional light microscopy. These techniques are not necessarily interchangeable or capable of attaining comparable image quality. For example, desiccated conditions may cause EVs to form an artefactual cup shape, not seen under hydrated conditions. Imaging techniques may allow assessment of EV purity, at least at the particle level, if they can visualise co-isolated NVEPs equally well. Imaging techniques are often limited by low throughput and the potential for bias based on field-of-view selection (Rikkert et al., 2019).

Recommendations

- Irrespective of imaging technique, report all experimental details. These include the instrument, software version, acquisition and analysis settings, sample preparation processes, how the imaged areas were selected, and controls and calibration information where relevant. Further details can be found in Sections 6.4.1 and 6.4.4.

5.7 | Characterization of EVs by protein composition

Because of the heterogeneity of EVs, MISEV2023, like MISEV2018, cannot recommend molecular markers of specific EV subtypes. MISEV2023 recommends the five-component framework introduced in MISEV2018 for reporting claims about the protein content of EVs (Table 3). Categories 1 and 2 assess the presence of EVs features. Category 3 assesses purity from common contaminants. Categories 4 and 5 provide additional information on possible intracellular origins of EVs (4) or co-isolates (5). Ideally, enrichment or depletion of markers in EV preparations versus unfractionated source material should be shown. To avoid perceived restrictions on which EV proteins should be analysed, MISEV2023 gives only a few nominative examples (Table 3). Other putative marker proteins can be assigned to one of the categories using databases such as Uniprot (https://www.uniprot.org/), where the section 'Subcellular location' indicates subcellular compartments or extracellular location, and 'features' indicates

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TABLE 3 Protein content-based EV characterisation.

Category				
1- Transmembrane (or GPI-anchored) proteins associated with plasma membrane and/or endosomes All EVs Non-exhaustive examples, categorized a, b, c: by decreasing strength of membrane association.	**2- Cytosolic proteins in EVs** All EVs	**3- Major components of non-EV co-isolated structures (NVEPs)** All EVs as purity control	**4- Transmembrane, lipid-bound and soluble proteins associated with intracellular compartments other than PM/endosomes** Subtypes of EVs and/or pathologic/atypical state, and/or novel separation method	**5- Secreted proteins recovered with EVs** Corona or functional component of EVs
1a: multi-pass transmembrane proteins. Tetraspanins (CD9, CD63, CD81, CD82); other multi-pass membrane proteins (CD47; heterotrimeric G proteins GNA*, TSAP6)	**2a: with lipid or membrane protein-binding ability**. ESCRT-I/II/III (TSG101, CHMP*) and accessory proteins: ALIX (PDCD6IP), VPS4A/B; ARRDC1; Flotillins (FLOT1/2); caveolins (CAV*); syntenin (SDCBP)	**3a: lipoproteins**. Produced mostly by liver, abundant in plasma, serum. Apolipoproteins	**4a: nucleus**. Histones (HIST1H**); Lamin A/C (LMNA/C)	**5a: blood-derived corona proteins**. Partially overlapping with 3a/3b: apolipoproteins, complement, fibrinogen
1b: single-pass transmembrane proteins. Major Histocompatibility Class I or II, Integrins (ITGA*/ITGB*), transferrin receptor (TFR2); LAMP1/2; heparan sulphate proteoglycans including syndecans (SDC*); EMMPRIN (BSG); ADAM10	**2b: promiscuous incorporation into EVs (and possibly NVEPs)**. Heat shock proteins HSC70 (HSPA8), and HSP84 (HSP90AB1) note that both are abundant also in NVEPs; cytoskeleton: actin (ACT*), tubulin (TUB*); enzymes (GAPDH)	**3b: protein and protein/nucleic acid aggregates**. Immunoglobulins (blood); Tamm-Horsfall protein (Uromodulin/UMOD; urine); albumin. YWAH* (14-3-3*) and AGO* (can be present in EVs but generally more abundant in NVEPs).	**4b: mitochondria**. VDAC, cytochrome C (CYC1); TOMM20	**5b: cytokines and growth factors**. e.g., TGFB1/2; IFNG, VEGFA, FGF1/2, PDGF*, EGF, interleukins (IL*)
1c: GPI- or lipid-anchored proteins. Glypicans (GPC1), 5′nucleotidase CD73 (NT5E), complement-binding protein CD59		**3c: exomere or supermere-enriched components**. HSP90AA/B, TGFBI, HSPA13, LDHA/B	**4c: secretory pathway**. Endoplasmic reticulum, Golgi apparatus: calnexin (CANX); Grp94 (HSP90B1); BIP (HSPA5), GM130 (GOLGA2)	**5c: adhesion and extracellular matrix proteins**. Fibronectin (FN1), Collagens (COL**), MFGE8; galectin3-binding protein (LGALS3BP), CD5L; fetuin-A (AHSG)
			4d: others. Autophagosomes, cytoskeleton… LC3 (MAP1LC3A), Actinin1/4 (ACTN1/4)	

At least one protein of categories 1, 2 and 3 should be analysed as EV hallmarks and to assess the presence of NVEPs in an EV preparation. Analysis of proteins of category 4 is optional, as they may be present in some subtypes of EVs, or under certain conditions, with no general rule. Proteins of category 5 may bind to EVs after their release and may be part of the recently described EV 'corona'. ***Please note that this table provides a limited number of examples only*** for proteins commonly found in mammalian cell-derived EVs. Other proteins that fall into the given categories may be equally valid, particularly for analysis of EVs from prokaryotic (bacteria) or non-mammalian eukaryotic sources (including parasites and plants). For most proteins of interest, their subcellular location in intracellular compartments (for categories 1 and 4), or their transmembrane or lipid-anchored nature (for categories 1 and 2), is provided in the Uniprot database (www.uniprot.org). *XX* = human gene names. *XX** or *XX*** used for families of multiple proteins, for example, for integrins: *ITGA** indicates any integrin alpha chain.

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topological and transmembrane domains. Although these categories apply to EVs regardless of analysis method, some of these markers may not be technically usable in some single-EV analysis techniques, which may require other controls. A variety of methods exist to determine the presence of protein markers. The sensitivity, specificity, and reliability of these methods can vary. Current assay- and instrument-specific reporting considerations are outlined in Section 6.

Recommendation

- Utilise the five-component framework (Table 3) for reporting claims about EV protein content.

5.8 | Non-protein markers of EVs

Non-protein markers, such as phosphatidylserine, glycans or specific nucleic acids, are seldom EV-specific but in some cases may add support for the presence of a lipid bilayer or cytosolic components. Co-localization with protein markers may also provide stronger evidence for the presence of EVs, for example, a membrane-intercalating dye and a tetraspanin-positive event, especially for single-particle measurements. Non-protein markers may be detected directly with techniques such as lipid mass spectrometry or Raman spectroscopy (Section 6.7), or indirectly using fluorescent probes such as membrane labels or intraluminal dyes. Recommendations for the reporting of EV labelling with non-protein markers is outlined in Section 6.6. The non-EV-specificity of most non-protein component markers urges caution. Membrane dyes may complex with any lipids, including those of NVEPs; dyes that are activated by intraluminal enzymes such as esterases may not be present in all EV preparations or subtypes; nucleic acid dyes have been used for EVs, but recommendations on controls and specificity are still needed (Liu et al., 2022).

Recommendations:

- If non-protein markers are used, consider using protein colocalization.

5.9 | Localization of EV-associated components

EV-associated components such as proteins, nucleic acids and glycans, may be luminal, in the membrane, or external to the EV. Knowledge of topology may be important for understanding the biology. For example, must an EV fuse with a recipient cell to deliver a luminal cargo, or can the EV simply present a surface-associated molecule to a receptor? The location of putative active components should therefore be determined by performing mild digestions, permeabilizations or affinity reagent accessibility by adopting or adapting previously published methods (Bonsergent & Lavieu, 2019; Cvjetkovic et al., 2016; Lai et al., 2015; Mateescu et al., 2017; McKenzie et al., 2016; Osteikoetxea et al., 2015; Sharma et al., 2010; Sung & Weaver, 2017).

Recommendations:

- Consider how topology can be determined in method design.

> Consensus: 72.3% (722) of MISEV2023 survey respondents agreed "completely," and 27.0% (269) agreed "mostly" with Section 5: EV characterization. 0.3% (3) "mostly" disagreed, and 0.4% (4) stated that they had no opinion and/or expertise. No respondents disagreed "completely."

6 | TECHNIQUE-SPECIFIC REPORTING CONSIDERATIONS FOR EV CHARACTERIZATION

As utilization and expertise has expanded across a broad range of EV detection assays and instrumentation, the identification of pertinent reporting criteria has also grown to ensure reliable and reproducible interpretation of data. A collated list of minimal assay and instrument-specific reporting considerations are detailed here. These are generally applicable irrespective of experiment design. The techniques listed in the following section are not exhaustive, and many detection technologies are under development or being actively researched. The techniques listed are, however, all commercially available, with existing literature from multiple researchers. These recommendations are not exhaustive, and further criteria are likely required due to subjective experimental parameters.

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6.1 | Flow cytometry-based methods

6.1.1 | Bead-based flow cytometry

Bead-based flow cytometry has been used widely to interrogate EV surface proteins. Large beads capture particles regardless of their surface composition (e.g., surfactant-free aldehyde/sulphate beads) (Théry et al., 2006), or antibody-conjugated beads capture particles exposing the corresponding antigen. Commercially available EV multiplex kits allow interrogation of 30 or more surface antigens (Koliha et al., 2016; Wiklander et al., 2018). After capture, bead-associated particles are labelled with a fluorescently conjugated affinity reagent (or mixture of several) for detection. Differences in staining intensity are semi-quantitative only, since signal derives from multiple particles captured by individual beads. A difference in signal intensity might thus mean different particle concentration, epitope density, diameter distribution or relative abundances of EV subsets.

When reporting bead-based approaches, controls should include isotypes as detection antibodies, or isotype-conjugated capture beads, and capture beads with detection antibody alone (for antibody-coated capture beads). Multiple EV input concentrations may be used to demonstrate titration of signal and rule out non-specific binding (Wiklander et al., 2018; Welsh et al., 2022). Stained beads as a percentage is not a valid statistic; reporting normalised bead median fluorescence intensities is recommended (Welsh et al., 2022). Reporting data and median fluorescent intensity statistics in molecules of equivalent soluble fluorophore (MESF) (as with single EV flow cytometry) from singlet gated beads is recommended to allow standardization of data across instrument platforms and settings. If preparing beads in-house, reagents and conjugation chemistry should be reported, while for commercial capture bead reagents, catalogue and lot numbers should be reported. Other reporting parameters include: total bead number, the sample-bead incubation time, post-bead incubation wash methodology, detection reagent staining time and post-staining wash methodology.

Recommendations:

- Controls should include isotypes as detection antibodies, or isotype-conjugated capture beads, and capture beads with detection antibody alone (for antibody-coated capture beads).
- Multiple input EV concentrations should be used to demonstrate titration of signal.
- If making beads, reagents and conjugation chemistry should be reported. For commercial capture beads reagent catalogue and lot numbers should be reported.
- Report normalised bead median fluorescence intensities.
- Report data and median fluorescent intensity statistics in molecules of equivalent soluble fluorophore (MESF) (as with single EV flow cytometry) from singlet gated beads.
- Report full and detailed methodology.

6.1.2 | Single-EV flow cytometry

Flow cytometry is an optical technique that has demonstrated detection of vesicles down to ~40 nm in specialised cases (Zhu et al., 2014) and ~100 nm using many modern conventional cytometers by light scatter and fluorescence (Morales-Kastresana et al., 2019; Sandau et al., 2020; Stoner et al., 2016; Welsh, Killingsworth, et al., 2021). Through calibration of data, flow cytometry has been demonstrated to be capable of characterising particle diameter (Stoner et al., 2016; Tian et al., 2020; van der Pol, de Rond, et al., 2018; Welsh, Horak, et al., 2020), epitope abundance (Gorgens et al., 2019; Welsh, Jones, et al., 2020), epitope density (Welsh, Jones, et al., 2020), effective refractive index (Pleet et al., 2023; van der Pol, de Rond, et al., 2018) and number concentration within standardised size ranges (van der Pol, Sturk, et al., 2018). In 2023, a tri-society working group (EV Flow Cytometry Working Group) initiative involving the International Society for Extracellular Vesicles, International Society for Advancement of Cytometry, International Society for Thrombosis & Haemostasis, published a single-EV flow cytometry compendium to comprehensively address the considerations for developing a single-EV flow cytometry assay (Welsh et al., 2023).

Calibration of fluorescent and light scatter parameters is critical for interpretation and replication of single-EV flow cytometry results. If particle concentrations are reported using single-EV flow cytometry, define the upper and lower LOD to allow replication and interpretation of data using orthogonal techniques. Currently, imaging cytometers use a dynamic triggering method that makes determination of the lower LOD difficult to define and therefore standardise.

In 2020, a comprehensive experiment and reporting framework was developed (MIFlowCyt-EV) and published as a position paper by the EV Flow Cytometry Working Group (van der Pol et al., 2022; Welsh, Tang, et al., 2021; Welsh, Van Der Pol, Arkesteijn, et al. 2020). The MIFlowCyt-EV reporting framework is split into categories of: preanalytical variables and experimental design, sample preparation, assay controls, instrument calibration & data acquisition, EV characterization, FC data reporting and FC data sharing. This reporting framework and learning resources for implementing the MIFlowCyt-EV framework can be found on the

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EV Flow Cytometry Working Group website (www.evflowcytometry.org). Complete the MIFlowCyt-EV spreadsheet and attach it as supplementary material for any manuscript with single-EV flow cytometry. The MIFlowCyt-EV framework is applicable to all flow cytometers, including conventional, spectral, imaging and single-photon-detecting cytometers.

Recommendations:

- Refer to the ISEV-ISAC-ISTH MIFlowCyt-EV framework Position Paper and utilise the reporting framework as supplementary material for any manuscript utilising single-EV flow cytometry.
- Ensure correct calibration of volume and fluorescent and light scatter parameters.
- Define upper and lower limits of detection to allow others to validate your work.

6.2 | Genetic protein tagging

EV proteins can be genetically labelled by introducing a genetic construct from which a tag, such as GFP, is ultimately co-translated with a protein or protein domain of interest (Corso et al., 2019; Heusermann et al., 2016; Joshi et al., 2020; Mittelbrunn et al., 2011). The tagged protein may be chosen based on its status as a general EV or EV subtype marker (Section 5.7), and numerous markers have been labelled (Corso et al., 2019; Dooley et al., 2021). Tagged proteins have been used to interrogate EV/subtype release and uptake pathways (Mathieu et al., 2019; Mathieu et al., 2021) and to enable overall biodistribution and pharmacokinetics studies. Importantly, the tag itself or alterations in expression of the tagged protein may affect EV biogenesis (Fan et al., 2020), loading, release, or function, so unlabelled EVs are recommended as a control to assess these possibilities. The fusion protein may also affect subcellular localization or cellular functions. Localization of the chimeric versus wildtype protein should be confirmed. Certain tags (e.g., GFP) may be subject to quenching in acidic compartments (Corrigan et al., 2014). Construct maps should be provided and, where possible, plasmids deposited in Addgene (www.addgene.org) or other repositories.

Recommendations:

- Carefully consider the selection of the tagged protein and its suitability as an EV or EV subtype marker.
- Assess the subcellular localization and function of the chimeric versus wildtype protein in the cell and the EV by comparing engineered and wildtype cells and labelled/unlabelled EVs.
- Report construct maps and deposit plasmids with a repository.

6.3 | Mass spectrometry proteomics

Mass spectrometry (MS) measures mass-to-charge ratio of molecules and, in EV studies, is commonly used to detect and characterises EV-associated proteins in both discovery and targeted applications (Aebersold & Mann, 2003; Hoshino et al., 2020; Pocsfalvi, Stanly, Vilasi, et al., 2016; Sodar et al., 2017). Targeted analyses are typically performed on a triple quadrupole (QQQ) liquid chromatography (LC)-MS platform, while untargeted proteomics is commonly performed using Time-of-Flight (ToF) or Orbitrap MS platforms (Liebler & Zimmerman, 2013). Targeted and untargeted proteomic approaches have nuances in terms of applications, advantages and limitations in sample processing, data acquisition, and analysis (Granvogl et al., 2007; Klont et al., 2018). Untargeted proteomic studies are used to identify all detectable ions within the sample, whether from EV-related proteins or contaminant matrix proteins. This approach provides a comprehensive understanding of the sample protein composition and is ideal for applications such as biomarker discovery (Nakayasu et al., 2021). For characterization of MISEV EV purity (Category 1, 2) and matrix contamination (Category 3) markers (Section 5.7), targeted peptide analysis may be more suitable, demonstrating the presence or absence of each analyte above a pre-specified detection threshold. It can also quantify absolute protein abundance. Multiplexing, for example, as in LC-MS workflows, can provide high sensitivity for limited sample volumes, such as for samples from clinical trials (Newman et al., 2022). Targeted proteomics may be more suitable to quantify changes in protein abundances, such as in a disease or therapeutic intervention (Pocsfalvi, Stanly, Fiumeet, al., 2016; Rodrigues et al., 2021). Inclusion of stable isotope labelled (SIL) peptide standards enables absolute quantification of the corresponding endogenous analyte when used in combination with 'light' peptide calibrators prepared in a matched matrix (Liebler & Zimmerman, 2013).

Instrument settings, including collision energy, gas flow and temperature, and capillary voltage, are platform and analyte-specific and, as such, should be optimised and then kept constant for the duration of a project. MS instruments are sensitive to contamination by ion-pairing reagents, buffer salts, and detergents, reducing sensitivity and assay performance. As such, EV peptide samples for targeted LC-MS analysis should be prepared in low-salt buffer/MS-compatible solvent matrix and an appropriate concentration of SIL peptide standard. Positive controls containing proteins of interest and negative controls, such as EVs from alternative species or cell lysates not expressing a protein of interest, should be included in targeted analyses (Abbatiello et al.,

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2013; Bereman, 2015; Nakayasu et al., 2021). Report the sequences of target peptides and the strategy for peptide selection. The linearity of response and limits of detection and quantification should be defined using synthetic light and heavy-labelled peptides spiked into an appropriate matrix. Report normalization, for example, by total protein, volume of starting material or particle count from which proteins were digested and injected for MS analysis. When reporting results from either targeted or untargeted proteomic studies, follow the Minimal Information About a Proteomic Experiment (MIAPE) guidelines for harmonization of methodology and rigor/reproducibility (Gandham et al., 2020; Kreimer et al., 2015; Taylor et al., 2007). All sample preparation techniques should be reported with reproducible experimental descriptions for each step. All data software and versions used should be reported to understand how data were processed. Filters, scores and confidence levels for both identifications and quantitation should also be reported, as well as the method used for quantitation if relevant (Martinez-Bartolome et al., 2013). Data and metadata should be uploaded to a data repository to ensure that data generation and reporting remain rigorous and potentially reproducible for EV experiments.

Recommendations:

- Optimise instrument settings and keep them constant for the duration of a project.
- In targeted LC-MS protein analyses, include both positive controls (containing proteins of interest) and negative controls, such as EVs from alternative species or cell lysates not expressing a protein of interest.
- Spike SIL peptide standards into the EV matrix to assess matrix effects and to demonstrate the concordance of retention times and quantifier-to-qualifier ion transition ratios between standards and endogenous analytes.
- For targeted MRM analyses, monitor at least one quantifier and one (preferably two) qualifier ion transitions.
- Define linearity of response and limits of detection and quantification using synthetic light and heavy-labelled peptides spiked into an appropriate matrix.
- Report sequences of target peptides and the strategy for peptide selection, as well as the isotopic purity and source of synthetic peptides.
- Sample preparation techniques, including the normalisation approach used, should be reported with detailed experimental descriptions for each step in the workflow.
- Follow the reporting recommendations of the Minimal Information About a Proteomic Experiment (MIAPE).
- Upload data and metadata to a data repository.

6.4 | Microscopy-based methods

6.4.1 | Atomic force microscopy

Atomic Force Microscopy (AFM) provides label- and stain-free imaging of individual EVs and co-isolated nanoparticles (Bordanaba-Florit et al., 2021; Obeid et al., 2019; Sharma et al., 2018). AFM imaging requires analytes to be deposited on a solid surface (substrate). Measurements can then be performed after either drying the sample or keeping it submerged in liquid, such as saline or cell culture media. AFM morphometry can be used to obtain EV size distribution and ultrastructural details and to check for the presence and relative amounts of contaminants (Cavallaro, Pevere, et al., 2021; Paolini et al., 2016, 2020; Parisse et al., 2017). In addition, AFM is one of the very few techniques capable of measuring single-vesicle nanomechanical properties (Gautron et al., 2021; Piontek et al., 2021), which were found to correlate with EV identity and function (Bortot et al., 2021; LeClaire et al., 2021; Romanò et al., 2022; Sorkin et al., 2018; Vorselen et al., 2018; Whitehead et al., 2015; Ye et al., 2021). The unique mechanical fingerprint of EVs can also be used to discriminate them from NVEPs of similar size and shape (Ridolfi et al., 2020).

Minimal reporting requirements for the AFM imaging of EV samples comprise detailed information on the preliminary sample deposition procedure, substrate type and pre-treatment, immobilization method, sample concentration and deposition times, plus details on any rinsing and/or drying steps. AFM imaging mode, acquisition conditions, and probe information including expected tip curvature radius and spring constant should also be provided. If quantitative morphometry is performed, the heuristics employed to select the measured objects, as well as the procedure to extract morphological descriptors from them, should be described. Reporting the height of the detected particles, for example, greater than or less than the thickness of two lipid bilayers (~8 nm) may help distinguish between deformed EVs and non-EVs/collapsed EVs. In addition, EV mechanical studies should describe the assumed contact mechanic model (Calò et al., 2014; Ridolfi et al., 2021; Vorselen et al., 2017), and, ideally, provide enough data for the reader to be able to test alternative models.

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Recommendations

- Report preliminary sample deposition procedure, substrate type and pre-treatment, immobilization method, sample concentration and deposition times, plus details on any rinsing and/or drying steps.
- Provide AFM imaging mode, acquisition conditions and probe information, including expected tip curvature radius and spring constant.
- If quantitative morphometry is performed, describe the heuristics used to select the measured objects, as well as the procedure to extract morphological descriptors.
- EV mechanical studies should describe the assumed contact mechanic model (Calò et al., 2014; Ridolfi et al., 2021; Vorselen et al., 2017), and, ideally, provide enough data for the reader to be able to test alternative models.

6.4.2 | Diffraction-limited fluorescence microscopy

Applications of fluorescence microscopy techniques can range from live cell imaging to single-molecule localization. These approaches, including Total Internal Reflection Microscopy (TIRF-M), confocal microscopy and more recently, light-sheet microscopy, have been used to evaluate cell-EV interactions such as EV release and uptake (Christianson et al., 2013; Elgamal et al., 2020; Feng et al., 2010; Heusermann et al., 2016; Joshi et al., 2020; Lai et al., 2015; Mittelbrunn et al., 2011), as well as the composition of single EVs (Corso et al., 2019; Han, Kang, et al., 2021). As a general consideration, since TIRF microscopy is limited to imaging the surface at the glass interface and has high signal-to-noise ratio that facilitates single molecule detection, it may be the most suitable system for analysing EV content (Han, Kang, et al., 2021). Confocal and light-sheet microscopes, especially the most recent models, are capable of single-molecule detection for calibration (Willy et al., 2021) and dynamic studies, but are more suitable for live cell imaging experiments (Elgamal et al., 2020; Mittelbrunn et al., 2011). These methods and potential drawbacks have been extensively reviewed (Chuo et al., 2018; Colombo et al., 2021; Gallego-Perez et al., 2016; Panagopoulou et al., 2020).

In microscopy experiments, report the type of microscope, magnification, laser power and exposure time because fluorescently labelled samples have a limited number of labelled molecules. Each labelled sample can provide only a finite number of photons before photobleaching, so each experiment must be optimised to maximise the amount of information obtained from a limited 'photon budget' (Li et al., 2015). Consequently, the sample is exposed for a short time using minimal excitation to perform live-cell experiments (Coffman & Wu, 2014; Heddleston et al., 2021) or at higher excitation power and longer camera exposure for single-molecule detection (Elgamal et al., 2020). While calibration of the system is mandatory for quantitative microscopy experiments (Montero Llopis et al., 2021; Willy et al., 2021), we recommend where possible to extend it to any microscopy approach to obtain unbiased evaluation of sensitivity of the instrument. Calibration to a single fluorescent dye or labelled protein molecules is a well-established approach that permits one to infer the total number of proteins or RNAs present on or in EVs (de Voogt et al., 2021; Higginbotham et al., 2011) and ensure that even molecules retained in few copies in EV can be detected. The software used to detect EVs should be reported including the specific parameters used to threshold the object intensities. Code developed for these purposes should be deposited and made accessible to the community. Available algorithms (Aguet et al., 2013; Elgamal et al., 2020; Jaqaman et al., 2008) take advantage of the small size of EVs, which are in general diffraction-limited objects. These assume the same shape as the point spread function (PSF) of the imaging system and can be approximated to a Gaussian function in confocal, TIRF and light-sheet microscopy.

Recommendations

- Report the type of microscope, magnification, laser power and exposure time.
- Calibration of the system is mandatory for quantitative microscopy experiments, but is also recommended for any microscopy approach to obtain unbiased evaluation of the sensitivity of the instrument.
- The software used to detect EVs should be reported, including the specific parameters used to recognise the objects and, if applicable, threshold the object intensities. Any code written for these procedures should be made publicly available.

6.4.3 | Dynamic light scattering

DLS, also known as photon correlation spectroscopy (PCS) and quasi-elastic light scattering (QELS), is a technique capable of determining the hydrodynamic diameter of sufficiently monodisperse particles in dilute aqueous dispersions (Berne & Pecora, 1976; Hackley & Clogston, 2011; "Particle size analysis — Dynamic light scattering (DLS)" 2017; Stetefeld et al., 2016). DLS can be performed as a cuvette analysis or as an inline analysis when connected to a fluidic pump, such as high-performance liquid chromatography (HPLC). The hydrodynamic diameter is defined as the diameter of a solid sphere that would exhibit the same

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diffusion coefficient as the measured particle of interest. DLS measures the autocorrelation function of the intensity of laser light scattered by multiple particles in solution. The autocorrelation function carries information about the diffusion coefficient of the particles, which is related to the hydrodynamic diameter via the Stokes-Einstein theory of Brownian motion.

Various algorithms can be used to derive the diffusion coefficient from the measured autocorrelation function. The most common method, the cumulant analysis, assumes a monodisperse size distribution, which EV samples do not have. Other approaches, such as the CONTIN algorithm, attempt to handle the drawbacks of the cumulant analysis (Provencher, 1982), but for polydisperse size distributions of EV samples (van der Pol, Coumans, Grootemaat et al., 2014), derivation of the diffusion coefficient distribution from the autocorrelation function becomes an ill-posed mathematical problem. This implies that DLS should not be used to determine quantitative properties, such as the average hydrodynamic diameter, of EV samples, unless DLS is applied to a monodisperse size fraction of EVs, such as an EV sample fractionated by flow field-flow fractionation using an inline analysis. On the other hand, DLS can be used to qualitatively confirm the presence of submicrometer particles and possible aggregates that may be present in EV samples (Palmieri et al., 2014). In either case, please follow the recommendations on nomenclature and reporting of DLS measurements from the international standard ISO 22412:2017 ("Particle size analysis — Dynamic light scattering (DLS)" 2017).

Recommendations

- DLS should not be used to determine quantitative properties, such as the average hydrodynamic diameter, of EV samples, unless applied to a monodisperse size fraction of EVs.
- DLS can be used to qualitatively confirm the presence of submicrometer particles and possible aggregates that may be present in EV samples.
- Follow the recommendations on nomenclature and reporting of DLS measurements from the international standard ISO 22412:2017 ("Particle size analysis — Dynamic light scattering (DLS)", 2017).

6.4.4 | Electron microscopy

Electron microscopy (EM) variants are among the few techniques capable of detecting EVs irrespective of size. The throughput of EM, however, means that larger EVs are statistically underestimated as compared with smaller EVs (van der Pol et al., 2022). While EV characterization by SEM (Cavallaro, Hååg, et al., 2021; Wu et al., 2015), TEM (van der Pol, Coumans, Grootemaat, et al., 2014), and cryo-EM (de Vrij et al., 2013; Hoog & Lotvall, 2015; Linares et al., 2015) are all high-resolution methods, they are not necessarily interchangeable or capable of providing images of comparable quality. For example, cryo-EM clearly shows the lipid bilayer, better maintains EV morphology than the dehydrating conditions used to fix samples for TEM, and may be more quantitative, as all particles in a given volume can be imaged, not just those that adhere to a surface (the grid). TEM should be performed with a protocol adapted to EVs, which includes contrasting and embedding in a mixture of uranyl compounds and methylcellulose to maintain the lipid bilayer morphology (Théry et al., 2006). SEM shows the surface aspect of EVs of any size, but images obtained at the highest magnification required to visualise the smallest EVs may be more difficult to analyse.

There have been limited standardization studies across EM methods to determine minimal reporting requirements. For TEM, three major parameters should be reported: fixation, adsorption, and negative staining methods (Rikkert et al., 2019). Fixation includes: the fixative used, its concentration, and incubation time. Adsorption includes the grid material, mesh size, film type, coating, incubation time, and wash details. Negative staining details should include substance, concentration, and incubation time. Both low- and high-magnification images should be shared, along with selection criteria.

Recommendations

- TEM should be performed with a protocol adapted to EVs, which includes contrasting and embedding in a mixture of uranyl compounds and methylcellulose to maintain the bilayer morphology.
- Three major criteria should be reported for any electron microscopy technique used: fixation, adsorption, and negative staining methods.
- High- and low-magnification images should be supplied for both high-resolution EV images and an assessment of the broader quality of the sample.

6.4.5 | Nanoparticle tracking analysis

NTA, also known as single particle tracking, is a widely utilised optical technique in the EV field to estimate particle size and concentration. The use of NTA to determine effective refractive index and epitope existence has also been demonstrated (Gardiner

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et al., 2014; van der Pol, Coumans, Sturk, et al., 2014). NTA derives hydrodynamic diameter by measuring a particle's diffusion coefficient, usually implementing an algorithm that reduces variation in diameter distribution. It should be noted that the FTLA algorithm used on some platforms was developed to better represent monodisperse mixtures, of which EVs are not, and can result in artefactual multi-modal distribution (van der Pol, Coumans, Grootemaat, et al., 2014; Walker, 2012). Currently, there is no method of determining or reporting a set LOD for NTA. Several standardization studies have been conducted comparing results between users and instruments (Bachurski et al., 2019; Hole et al., 2013; Vestad et al., 2017). The use of NTA to measure the diameter distributions and concentration of complex biofluids should be interpreted with caution due to counting of co-isolates such as lipoproteins and large protein complexes, and EVs larger than a few hundred nanometers in diameter are difficult to quantify. Detection of particles with NTA can be done using light scattering, relying on refractive index and diameter, or fluorescence. Fluorescence NTA depends on removal of unbound label, photobleaching resistance of the dye, and the presence of detectable levels of dye per particle.

For NTA reporting, include instrument model, camera type, camera settings, laser wavelength, laser power, software version, analysis settings, and particles per frame. As outlined in Section 5.2, NTA diameter distributions are preferred over a single diameter statistic, since NTA statistics are easily skewed by the LOD. If known, the algorithm used to produce diameter distributions should be reported due to potential for differing results depending on the algorithm used (Kestens et al., 2017; Walker, 2012). A buffer-only control is recommended in the case of light scatter or fluorescence detection modes. For fluorescent NTA, report the number of total particles in light scatter mode along with the number of labelled particles in fluorescence mode, along with label removal method and a buffer/reagent control to assess labelling artefacts. Report sample injection fluidics and settings if used.

Recommendations

- Instrument model, camera type, camera settings, laser wavelength, laser power, software version, analysis settings and particles per frame should be reported.
- Report NTA diameter distributions rather than a single diameter statistic.
- If known, report the algorithm used to produce diameter distributions.
- A buffer-only control is recommended for both light scatter and fluorescence detection modes.
- When using fluorescent NTA, it is recommended to report the number of total particles in light scatter mode, the number of labelled particles in fluorescence mode, along with label removal method, Use a buffer-only/reagent control to assess labelling artefacts.
- Report injection fluidics and settings if used.

6.4.6 | Single-particle interferometric reflectance imaging sensing

Combined interferometric imaging/fluorescence imaging (Bachurski et al., 2019; Crescitelli et al., 2021; Daaboul et al., 2016; Dogrammatzis et al., 2021) involves particle capture by affinity agents (e.g., antibodies, peptides, aptamers) onto a multiplexed array of micron-sized spots. In interference reflectance imaging sensor (IRIS) mode, interference patterns from scattered light are used to derive the size and number of captured particles (Young et al., 2018). Converting interference to nominal size depends on refractive index (RI), which can vary across EV populations (de Rond, Coumans, et al., 2018). Current SP-IRIS platforms assume a constant RI (~1.45), which may result in variation across orthogonal measurements and may undersize EVs with lower RI. It is thus recommended that software version and estimated refractive index parameter be reported.

In fluorescence mode, captured particles labelled with fluorescent probes are detected in one or more color channels. Some aspects of this mode require careful consideration of calibrations and control experiments to obtain rigorous results. For particles smaller than the diffraction limit, for example, <~250 nm in diameter for visible light, validate the detected events to confirm that single particles were detected, for example, with a dilution series to ensure that fluorescence intensity per particle does not scale with solution concentration. To confirm that fluorescence is associated specifically with EVs, vesicle-disrupting surfactant treatments can be used; however, consider that surfactants can also disrupt lipoprotein particles (Botha et al., 2022). For fluorophore detection, reporting recommendations are indicated below.

Recommendations

- Report details of affinity reagent(s) printed onto the chip.
- Report software version and estimated refractive index parameter.
- For particles smaller than the diffraction limit, detection of single events should be validated.
- To confirm that fluorescence is associated specifically with EVs, surfactants can be used to disrupt vesicles (although they may also disrupt certain NVEPs).

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- For fluorophore detection, report affinity reagent (e.g., antibody clone), conjugated fluorophore type, incubation concentration, light-source wavelength, bandpass filter cut-offs, analysis software version and fluorescence cut-offs along with the method of choosing these cut-offs. Negative controls such as nonspecific IgG capture spots or chips incubated with EV-depleted materials are recommended for choosing these cut-offs.

6.4.7 | Super-resolution microscopy

To resolve fluorescence emitters events that are closer together than the diffraction limit of light, fluorescent super-resolution microscopy methods modulate the light to ensure that neighbouring molecules do not emit simultaneously. A resolution 10-fold below the diffraction limit can be achieved using two main approaches: (1) stimulated emission depletion (STED) (Hell & Wichmann, 1994; Klar & Hell, 1999), which spatially regulates activation of an ensemble of fluorophores using a synchronised two-laser system with a phase plate; and (2) single molecule localization microscopy (SMLM) techniques, such as (d)STORM (Rust et al., 2006; Wombacher et al., 2010) and (f)PALM (Betzig et al., 2006; Hess et al., 2006), which temporally regulate stochastic activation of single fluorophores. The nanometer scale resolution of STED and SMLM is well suited for detecting and characterising individual EVs and their components, including EV membranes (Nizamudeen et al., 2018; Sharma et al., 2020; Zong et al., 2018), proteins (Avalos-Padilla et al., 2021; Chen et al., 2016; Lennon et al. 2019; Maire et al. 2021; Mondal et al., 2019; Sanada et al., 2017; Wang et al., 2018; Zong et al., 2018), DNA fragments (Maire et al. 2021) and miRNAs (Chen et al., 2018; Oleksiuk et al., 2015). Using quantitative analysis, these methods have been further used to define EV size (Mondal et al., 2019; Zong et al., 2018; Nizamudeen et al. 2018; Lennon et al. 2019; Sharma et al., 2020) and to quantify protein content (Lennon et al., 2019) and number of localizations of miRNA (Oleksiuk et al., 2015) and DNA fragments in EVs (Maire et al., 2021). Additionally, STED and SMLM have been used to image cellular uptake (Chen et al., 2016; Chen et al., 2018; Polanco et al., 2018; Pfeiler et al., 2019; Toda et al., 2020) and release of EVs (Ambrose et al., 2020) or EV clusters (Valcz et al., 2019).

Super-resolution microscopy methods comprise tailored approaches for sample preparation, sample imaging, and data analysis. To prepare samples for SMLM and STED imaging, EV membranes or cargo molecules are labelled with reagents that contain appropriate photo-controllable fluorophores. Four typical strategies for labelling EVs are affinity labelling, genetic labelling, covalent labelling, and uptake of lipophilic molecules/lipid analogues. Reported details of labelling should include type of labelling, appropriate reagent controls and/or references, reagent concentration, incubation times/buffers, and method for removal of excess fluorescent reagents). If applicable (e.g., for isolated EVs), reporting should include coverslip modifications/coatings, the protocol for incubation of EVs on coverslips, fixation protocol, and controls for affinity separation (e.g., isotype or non-fouling surface). Reported imaging parameters should include the major microscope components: laser lines, camera, filters, objectives and other relevant optical path components. Descriptions of protocols should include detailed imaging parameters such as laser powers, relevant microscope configuration and imaging conditions (including buffer for SMLM). Reports on multicolour imaging should detail the alignment between channels and any applied correction for chromatic aberration (Churchman & Spudich, 2012; Hebisch et al., 2017).

In STED, the resulting images consist of intensity maps, and analysis typically relies on approaches established in confocal microscopy (Gould et al., 2012); relevant processing/analysis parameters should be reported. SMLM images are reconstructed from the determined coordinates (i.e., localizations) of single molecules, and EV analysis typically employs segmentation and/or clustering algorithms (Khater et al., 2020). To quantify detected molecular densities and molecular organization with SMLM, it is important to define the photophysical properties of fluorescent reporters (e.g., average number of localizations per molecule, maximal dark time) (Khater et al., 2020). Thus, SMLM reporting should include details on image processing parameters, photophysical characterization of relevant fluorescent reporters, and data analysis parameters/algorithms. Newly developed analysis methods should be evaluated (e.g., using simulations or another validated approach), and custom written codes should be made publicly available.

Recommendations

- Reporting details of the EV labelling protocol.
- Where applicable, report coverslip modifications/coatings, the protocol for incubation of EVs on coverslips, fixation protocol and controls for affinity separation.
- Report the major microscope components and imaging protocol parameters such as laser power, relevant microscope configuration and imaging conditions.
- Reports on multicolor imaging should detail the alignment between channels and any applied correction for chromatic aberration.
- SMLM reporting should also include details of image processing parameters, photophysical characterization of relevant fluorescent reporters and data analysis parameters/algorithms.
- Newly developed analysis methods should be evaluated, and custom written codes should be made publicly available.

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6.5 | Nucleic acid characterization

Nucleic acids (NAs) are among the most commonly assayed EV constituents because of perceived biomarker potential and functional roles. RNA has been studied much more frequently than DNA, although there are more recent reports on EV DNA in intercellular communication (Clancy et al., 2022; Sansone et al., 2017) and as disease biomarkers (Cambier et al., 2021; García-Silva et al., 2019; Möhrmann et al., 2018; Qu et al., 2019; Vagner et al., 2018) including in microbial infections (Bitto et al., 2017; Kameli et al., 2021). Some early EV studies reported DNA inside the EV lumen (Cai et al., 2013; Kahlert et al., 2014; Lee et al., 2014; Thakur et al., 2014), whereas some recent studies have suggested mostly EV surface-association of DNA (Bitto et al., 2017; Lázaro-Ibáñez et al., 2019; Liu et al., 2022; Maire et al., 2021; Saari et al., 2020). These seemingly contradictory findings might be due to the lack of standardised methods for protecting EV surface DNA from digestion during EV separation and characterization (Lázaro-Ibáñez et al., 2019). Whether the major type of EV DNA is ssDNA or dsDNA is also still debated (Balaj et al., 2011; Lázaro-Ibáñez et al., 2019; Liu et al., 2022; Thakur et al., 2014).

Challenges for RNA studies including input quantities, normalization and sensitivity are also relevant for EV DNA research. Most characterization of EV RNAs involves one or more of: detection, identification, quantification, localization (inside or outside the EV) and enrichment (packaging). Low-input RNA sequencing (RNA-Seq) and quantitative PCR (qPCR) are commonly used to identify specific sequences in EV preparations. ISEV has previously provided guidance on aspects of EV RNA studies ranging from sample collection to bioinformatic analysis (Hill et al., 2013; Soekmadji et al., 2018; Witwer et al., 2013), as has the US NIH Extracellular RNA Communication Consortium (ERCC) (Ainsztein et al., 2015; Das et al., 2019).

Regardless of RNA characterization method, biases may be introduced by RNA purification and pre-assay preparations. Some RNA purification methods isolate mostly longer RNAs (>200 nt), while others are biased by design to concentrate short RNAs. For RNA-Seq, library preparation methods may select for RNAs or inserts within a particular size range. Reverse transcription protocols may also select for specific RNAs such as polyA-tailed transcripts. Tiled probe-based imaging of longer RNAs becomes less sensitive for shorter/degraded transcripts. Adapter ligation-based small RNA library preparation methods optimised for miRNAs will also enrich other RNAs containing 5′-phosphates and 3′-OH, while RNAs bearing different end-chemistries will be underrepresented. Highly structured RNAs such as full-length tRNAs are not efficiently reverse-transcribed unless using thermostable enzymes. Accurate interpretation and reporting of results thus depend on understanding and reporting techniques with enough detail to assess biases.

Due to its ability to detect and measure small amounts of nucleic acids, reverse transcription real-time quantitative PCR (qPCR) is widely used in the EV field. We recommend that qPCR experiments follow the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) where possible, and the ISEV EV RNA checklist in the 2017 ISEV position paper (Mateescu et al., 2017). When sharing qPCR results, raw cycle of quantitation (Cq) values should be reported in addition to normalised or processed data for readers to assess abundance of the target RNA and reliability of the assay. Although Cq values depend on many variables and may not by themselves be informative, they tend to correlate with abundance, especially in liquid samples, and where the input sample volume can be reported. Possibly, not all MIQE principles broadly apply to extracellular samples. For example, when samples contain only minute amounts of carrier-specific RNA, having identical input RNA levels in every sample might not always be possible. Some prefer to normalise by sample input volume, given the liquid nature of extracellular samples. Normalization strategy can greatly impact interpretation of the results and should be reported. Digital PCR, including droplet digital PCR (ddPCR), provides absolute quantification and has been shown to improve reproducibility and accuracy of EV RNA detection compared with conventional qPCR (Wang et al., 2019). Absolute quantification may also circumvent issues with normalization.

Recommendations

- For qPCR-based analysis, report sequences of reverse transcription adapters or primers as well as primers and probes (where relevant) for amplification steps; experimental design with biological and technical replicates; exact cycling conditions; and data inclusion and exclusion criteria.
- For RNA-Seq, report all details of nucleic acid fragmentation, reverse transcription, adapters and adapter attachment (ligation or ligation-free), amplification and multiplexing, as well as clean-up or size selection.
- For sequencing data analysis, report pre-processing, read mapping, overlapping annotations and database quality, quantification and normalization/differential expression analysis.

6.6 | Protein- and non-protein labelling of EVs

Most EV labelling reagents include fluorescence moieties, but other modes of detection are available and should share similar controls. Due the small size and thus limited cargo capacity of EVs, the detection of protein and non-protein markers is diffi-

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cult and can easily be confounded by unbound reagents from the labelling process or co-isolates from the separation method. The degree to which unbound label requires removal increases with the sensitivity of the techniques. For techniques that can detect <10 molecules of a reagent, for example, super-resolution microscopy, SP-IRIS and single EV flow cytometry, the presence of unbound dye may easily lead to false positive events.

Lipid dyes are routinely used to bind to/insert into the EV membrane (de Rond, van der Pol, et al., 2018; Feng et al., 2010; Lundy et al., 2015; Sandau et al., 2020; Stoner et al., 2016). Lipid-specificity does not guarantee EV-specificity, since NVEPs such as lipoproteins may be co-isolated and stained, and some reagents may also label proteins. Supplementation with an EV protein marker is therefore recommended. Lipid labels may also self-aggregate (de Rond, van der Pol, et al., 2018; Pužar Dominkuš et al., 2018) and vary in affinity for EVs with different membrane composition.

Protein-reactive dyes that label the EV surface (Lim et al., 2021; Roberts-Dalton et al., 2017; Tian et al., 2010) may also label free protein and protein-containing NVEPs. If the EV separation method does not completely remove non-EV components, this possibility should be recognised and/or assessed. As above, a lipid marker might be used to complement protein labelling. When protein artefacts are a possibility, a low-concentration detergent can be used to assess the lability of the EV membrane and reduction of associated signal (Gyorgy et al., 2011).

For antibodies, manufacturer-matched isotype controls, used at the same concentration as the specific antibody, are one way to support specificity. Negative EV controls, for example, from cells that do not express the antibody epitope, are also useful controls.

In assays where purification is required after staining, procedural controls should be used to demonstrate before/after consistency of the EV population, that the purification procedure did not introduce artefacts, and that the dye was removed. For example:

1. Analyse a buffer with reagent control before and after label depletion method (e.g., SEC) to assess free/aggregated label removal.
2. Analyse unstained EVs before and after the label depletion method to demonstrate that it does not change or selectively enrich the EV population.
3. Analyse the reagent-stained sample after label removal and compare with unstained results (above) to assess possible dye-induced changes. Staining may noticeably increase the diameter or density of small EVs in particular.

Recommendations

- Use a buffer/label-only control to identify false-positive artefacts arising from unbound label. However, label-only artefacts are not the only potential labelling artefacts.
- For antibodies, manufacturer-matched isotype controls may be used at the same concentration as the specific antibody to evaluate binding specificity. Negative EV controls (lacking the antibody epitope) may also be used.
- Be aware that EV protein labelling methods may also label free proteins and protein-containing NVEPs, and that lipid dyes may label lipid-containing NVEPs. Ensure that the EV separation method is appropriate for the downstream analysis. If the EV separation method does not completely remove non-EV components, this possibility should be recognised and/or assessed.
- To identify the contribution of non-EV labelling artefacts, consider using protein and lipid labelling concurrently.
- In assays where purification is required after staining, procedural controls should be used to demonstrate before/after consistency of the EV population, that the purification procedure did not introduce artefacts, and that excess dye was removed.

6.7 | Raman spectroscopy

Raman spectroscopy (RS) is a label-free analytical optical technique capable of qualitatively and quantitatively resolving the chemical composition of a small volume of a sample based on inelastically scattered photons originating from the sample upon irradiation with a narrow-linewidth laser (Smith & Dent, 2005). A Raman spectrum is essentially a chemical fingerprint of the interrogated small volume of the sample within the focus of the laser beam. RS enables chemical specific, non-destructive probing, minimal to no sample pre-processing, and it is relatively inert to aqueous content of the measured sample (Smith & Dent, 2005). A strategy to overcome the weak signals of RS is the use of surface-enhanced Raman scattering (SERS), which is a nano plasmonic-assisted amplification derivative of RS (Langer et al., 2020; Jones et al., 2019). This method uses metal nanostructures to boost Raman scattering by many orders of magnitude. Both spontaneous and surface-enhanced Raman methods have demonstrated utility for basic research and translational EV analyses (Carlomagno et al., 2021; Enciso-Martinez et al., 2020; Gualerzi et al., 2017; Gualerzi et al., 2019; Kwizera et al., 2018; Lee et al., 2018; Ma et al., 2018; Park et al., 2017; Rojalin et al., 2020; Smith et al., 2015).

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Inter- and intra-device variability in Raman spectra can arise for several reasons, including laser variations and non-uniform response of each of the optical elements, including the detector, to different light energies (known as spectral response). Raman systems should therefore be carefully calibrated (Raj et al., 2020). Modern commercial Raman systems have automatic calibration routines, but older and lab-built systems do not, thus adding to the issue of reproducibility. Several aspects of the measurement should be reported, including laser wavelength and power, calibration routines, make/model of major optical components, numerical aperture and magnification of the objective (if applicable), probe type and specifications (typically for non-microscope setups and measurements), and physical size of the laser spot. Spectra acquisition parameters should also be mentioned, for example, total number of spectra collected on each sample or sampled spot, signal collection time per one spectrum (also called as integration or acquisition time), and for scanning, the dimensions of the scanned area/volume (e.g., 100 × 100 area, step size of 400 nm, total scanned area 40 μm × 40 μm). Lastly, it is recommended to report all pertinent parameters of sample preparation. As EV samples are typically suspended in aqueous solutions with different concentrations of dissolved compounds, and thus osmotic pressures, there is a need to consider and report the EV formulation and whether the EVs were measured in suspension or dry. For example, EVs can be measured in suspension using SERS nanoprobes or dried onto a quartz glass slide for RS spectra acquisition (Cameron et al., 2018). It is unclear if there is an advantage to wet versus dry measurements (Butler et al., 2016), so both approaches are considered feasible provided that the EV sample preparation steps are detailed.

Along with instrument and sample considerations, data analysis and statistical procedures can impact the endpoints and conclusions of RS studies. All data analysis software and versions should be reported. If custom-made program suites and algorithms are employed, it is recommended that the code be deposited in an online data repository for transparency and re-usability. After acquisition (and before downstream analyses), spectra that are meant to be compared with each other should be postprocessed using identical data manipulation parameters. For example, if baseline correction and/or background subtraction is implemented, all related parameters should be kept constant for all spectra. All downstream spectral analyses and further statistical testing (e.g., multivariate analysis, machine learning, statistical hypothesis testing) should be reported in full and with data openly available.

Recommendations

- Report all instrument and measurement parameters.
- Report sample preparation/application parameters including buffer composition and wet/dry measurement.
- Report data analysis software and versions. Deposit any code for custom-made program suites and algorithms in an online data repository for transparency and re-usability.
- Report downstream spectral analyses and further statistical testing.

6.8 | Resistive pulse sensing

Resistive pulse sensing (RPS) is a non-optical technique utilising the Coulter principle to determine the concentration and diameter of particles (Hogg & Coulter, 1967), along with zeta potential on some platforms. Current implementations of RPS include pre-calibrated fixed pores in a microfluidic cartridge format and uncalibrated stretchable pores, both with detection limits down to ~50 nm in diameter and the capability to measure particles up to several microns. The use of RPS to measure the diameter distributions and concentration of EVs in complex biofluids should be interpreted with caution, since co-isolates, such as lipoproteins and large protein complexes, are also counted and cannot be differentiated from EVs. RPS measurements do, however, have very high concordance with TEM data (van der Pol, Coumans, Grootemaat, et al., 2014).

When reporting RPS data it is recommended that instrument model, pore size, calibration bead diameter and source, and software version be reported. For stretchable pores, the applied voltage, applied stretch, and procedure to optimise settings should be shared (Coumans et al., 2014). For microfluidic RPS, appropriate dilution buffer to lower the surface tension of water should be considered and reported (Cimorelli et al., 2021). As outlined in Section 5.2, it is preferable to report RPS diameter distributions rather than a single diameter statistic for EV data, due to RPS statistics being easily skewed by the LOD. The inclusion of buffer-only controls to identify background, along with detergent-lysed samples run at the same concentration to determine label events is also recommended (Osteikoetxea et al., 2015). Due to RPS techniques being easily clogged by larger particles, preanalytical steps such as centrifugation or filtration may be used to remove larger particles. Since these approaches may alter the EV population being analysed and affect comparison with orthogonal methods, any preanalytical procedures should be clearly stated.

Recommendations

- Report any preanalytical procedures applied prior to RPS.
- For microfluidic RPS, appropriate dilution buffer should be considered and reported.
- Include buffer-only controls and detergent-lysed samples run at the same concentration as the untreated sample.

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- Report all instrument and software details.
- Report RPS diameter distributions rather than a single diameter statistic.

6.9 | Western blotting

Western blotting is a commonly used method to detect proteins in EV-containing preparations. Proteins are first separated by gel electrophoresis, then transferred to a membrane and probed with affinity reagents, usually antibodies. Input is often normalised by some aspect of the EV preparation (total protein, particle count) or some aspect of the EV source (biofluid volume, cultured cell number): the former allows comparison of amounts of EV cargo between similar groups of EVs, while the latter might also assess overall differences in EV production/uptake balance in the source system. For cell culture EVs, cell lysates, either in specified protein amount or in cell-equivalent amounts, should be loaded onto the same gel to assess enrichment/depletion in EVs versus producing cells. This comparison, however, can be easily performed only for analysis of EVs from cell culture-conditioned medium, since for other sources of EVs (e.g., biological samples), the source cells cannot be easily identified or recovered.

Where possible, known antigen-positive and -negative control samples should be included beside the experimental samples. Controls for assessing the purity of the sample preparation should also be included if claiming the protein is present on or in EVs; see Section 5.7. Antibody information (specificity, clone, source, labelling concentration, incubation time), sample denaturing conditions, presence, and nature of reducing agent, transfer methodology, membrane type, buffers, and imaging equipment and parameters should all be reported. For transparency, it is recommended that uncropped images of Western blots (including controls and a molecular weight ladder) be provided at a minimum as supplementary information.

Recommendations

- Provide details of protein enrichment and quantification.
- Where possible, include antigen-positive and -negative controls
- If claiming EV-association of a protein, include measures of the purity of the EV preparation.
- Report all details of input normalization, gel electrophoresis, transfer methodology, probing and imaging/analysis. These include but are not limited to antibody information, sample denaturing and reducing conditions, transfer methodology, membrane type, buffers, and imaging equipment and parameters.
- Provide uncropped images of all Western blots (e.g., as supplementary information if published in a journal).

> Consensus: 70.6% (705) of MISEV2023 survey respondents agreed "completely," and 27.5% (274) agreed "mostly" with Section 6: Technique-specific reporting considerations for EV characterization. 0.4% (4) "mostly" disagreed, and 1.5% (15) stated that they had no opinion and/or expertise. No respondents disagreed "completely."

7 | EV RELEASE AND UPTAKE

7.1 | Approaches to modulate EV release

EV release can be visualised by a range of methods, including those employing fluorescent tags and dyes (Sections 6.2, 6.6), which permit real-time imaging [reviewed in (Verweij et al., 2021)]. MISEV2018 discussed inhibition of EV release with a range of genetic manipulations and drugs, for example, *RAB27A/B* knockdown (Ostrowski et al., 2010), neutral sphingomyelinase inhibition (Trajkovic et al., 2008) and ARRDC1 inhibition (Mackenzie et al., 2016; Wang & Lu, 2017). More recent genetic and pharmacological manipulations are reviewed elsewhere (Catalano & O'Driscoll, 2020; Dixson et al., 2023; Zhang, Lu, et al., 2020). Some cellular manipulations can also stimulate EV release (Taher et al., 2019). While these treatments are often claimed to be specific for EVs of particular biogenesis pathways, they may affect EV formation and membrane trafficking more generally. It is thus difficult to exclude an impact on other EVs and/or non-EV cellular processes (Izumi, 2021; Mathieu et al., 2019; Puca et al., 2013; Xiang et al., 2021). MISEV2018 highlighted the importance of identifying biogenesis machinery that is confined to particular EV subtypes, and this remains a priority, with very few specific additional regulators identified. Using complementary methods to attenuate and/or enhance the production of specific EV subtypes can add strength to data suggesting their association with specific functions. The resulting EVs and control preparations should be analysed using the physical and molecular methods described in Sections 5 and 6, with particular attention to normalization methods (e.g., based on the number/protein mass of secreting cells, or EV number, etc.), identification of unchanged as well as altered markers, where possible, for specificity, and the use of multiple cell types to test whether the mechanism is generic or cell type-specific.

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Recommendations

- For genetic and pharmacological manipulations used to inhibit or stimulate EV secretion, report potential effects on other secretory or cell biological processes. For example, confirm that there is no change in cell viability, proliferation and secretion of non-EV-associated factors.
- Where possible, assess whether inhibiting a specific EV production pathway leads to a change in other EV release mechanisms by assessing EV-specific cargoes or activities.
- Identification of unchanged markers and the use of appropriate normalization methods are important for rigorous comparative analysis of EV preparations.

7.2 | EV interaction with cells

EVs can interact with target cells at different levels: binding, internalization, and fusion/content delivery. EVs contact the surface of cells, which might be referred to as 'EV binding'. In contrast, 'EV uptake' encompasses several outcomes. It can mean fusion of the EV with the cell membrane and release of contents into the cytoplasm. It can also mean internalization into the endocytic and/or other intracellular compartments of the cell, with or without EV-cellular membrane fusion. EV-mediated effects on the recipient cell might thus be occasioned by EV binding to receptors at the cell surface or internally and/or by release of contents into the cell at the surface or internally. The relative importance of these different interactions remains unclear, even though most reports of EV function have assumed content delivery. However, EV uptake may occur only at a low rate (Bonsergent et al., 2021; Somiya & Kuroda, 2021a, 2021b) in some target cells, necessitating a high ratio of EVs to target cells to visualise this process (Jurgielewicz et al., 2020; Ragni et al., 2019).

How can these different modes of action be interrogated? Some fluorescence microscopy methods can identify subcellular fluorescent events associated with cells, while flow cytometry mostly detects EV 'capture' without discriminating between binding and uptake. For all methods, the long-lived nature of EV labelling substances may not accurately reflect the presence of EVs in target cells, lipophilic dyes might change EV properties (Section 6.6), and detection of downstream receptor-mediated cell signalling induced by EVs does not discriminate between different modes of action. While covalently bound dyes cannot be exchanged between EVs and cell membranes without fusion, lipophilic dyes can be exchanged without actual EV transfer, resulting in false positive signals (Simonsen, 2019). New approaches for assaying cargo delivery (including endosomal escape) have been developed since MISEV2018, for example, anti-GFP fluobodies (Joshi et al., 2020), proteolytic cargo cleavage (Perrin et al., 2021), split-luciferase reporters (Somiya & Kuroda, 2021a), CRISPR-Cas9 reporters (de Jong et al., 2020), Cre reporters (Borghesan et al., 2019), trans-activator delivery (Somiya & Kuroda, 2021b) and knockout of a cargo gene in recipient cells (Taha et al., 2020). By labelling specific EV subtypes, blocking their biogenesis and assaying cargo delivery, it may be possible to determine how EV-target cell interaction mechanisms vary between different EV subtypes and EV donor-acceptor combinations. Going forward, inhibition of specific EV ligand-receptor interactions may establish discrete phenotypic effects: for example, by genetic approaches or addition of blocking antibodies or inhibitory compounds. Blockade of specific intracellular trafficking pathways will suggest which are critical for EV function.

Recommendations

- Assess the suitability of the labelling/reporting system in terms of the impact on normal cellular processes, the stability of the EV-cell association, and longevity within an intracellular environment.
- Report EV:recipient cell ratios and the physiological relevance of the delivered dose.
- Report incubation conditions, exposure time, cell densities, and configuration, for example, 2D/3D.
- Evaluate binding, uptake and content transfer to identify critical mechanistic elements driving the cellular response(s).

> Consensus: 69.6% (695) of MISEV2023 survey respondents agreed "completely," and 24.3% (243) agreed "mostly" with Section 7: EV release and uptake. 0.2% (2) "mostly" disagreed, and 5.8% (58) stated that they had no opinion and/or expertise. No respondents disagreed "completely."

8 | FUNCTIONAL STUDIES

MISEV2018 recommendations on functional studies of EVs continue to hold for MISEV2023. Because of the great diversity of functional studies in vivo and in vitro, we provide only general recommendations. First, physiologically informed dose-response and time-course studies are encouraged. Second, carefully selected EV negative controls are needed to assess the contribution of 'background' EV activity (such as EVs present in culture medium components) and/or non-specific activity of EVs other than those of interest. For cell culture-derived EVs, this might mean unconditioned medium that has been processed in the same way

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as conditioned medium (i.e., to separate any EVs that may be present in culture medium components). For EVs from a specific cell type, EVs from another cell type might serve as an appropriate control. For engineered EVs, consider EVs from unmanipulated cells or cells engineered with an irrelevant component (cell engineering) or EVs that have not been modified (post-production engineering). For patient disease studies, use EVs sourced from healthy, matched or untreated donors. Third, controls consisting of non-EV-containing, EV-depleted or enzymatically treated EV separation fractions can help to identify if a function is specific to EVs or associated with co-isolating materials. Possibly complicating this analysis, evidence has emerged since MISEV2018 for a functional role of certain loosely tethered coronal elements, as discussed in Sections 3.4 and 4.7, and EV co-isolates may indeed contribute along with EVs, additively or synergistically, to effects. Finally, the influence of EV separation/concentration, storage and formulation factors on EV activity should be studied, with the goal of maximising activity. Importantly, it is not expected that all conceivable controls will be studied simultaneously in any given system. Instead, potency assays (Gimona et al., 2021; Nguyen et al., 2020) can be used (or developed) to identify the most informative controls for pre-clinical and clinical studies.

Recommendations

- Perform dose-response and time-course studies to assess specificity, kinetics and saturability.
- Report and justify the method(s) used to normalise input.
- Evaluate negative EV controls where possible to rule out effects of 'background' EVs (e.g., from culture medium) and to evaluate the specific effects of EVs from a certain source or of specific EV elements.
- Evaluate appropriate non-EV (e.g., NVEP, soluble protein) negative controls to understand the EV-association of specific activities.
- Assess the effects of pre-analysis factors, especially storage and formulation, on EV activity.

> Consensus: 71.1% (710) of MISEV2023 survey respondents agreed "completely," and 25.1% (250) agreed "mostly" with Section 8: Functional studies. 0.3% (3) "mostly" disagreed, and 0.1% (1) "completely" disagreed. 3.4% (34) stated that they had no opinion and/or expertise.

9 | EV ANALYSIS IN VIVO

In vivo EV studies can provide mechanistic insights into EV release, biodistribution, pharmacokinetics and function (Verweij et al., 2021) and may be performed in a wide variety of species, including but not limited to model organisms that recapitulate aspects of human health and disease. In genetically tractable organisms, progress may be facilitated by EV tags and cellular reporter systems (Section 6.2). The relative ease of genetic manipulation of invertebrate and vertebrate model organisms allows hypothesis testing and specific EV labelling approaches (Beckett et al., 2013; Budnik et al., 2016; Fan et al., 2020; Gross et al., 2012; Verweij et al., 2019), including for EV subtype-specific mechanisms (Beer et al., 2018; Fan et al., 2020). Table 4 presents non-exhaustive examples of in vivo models for EV studies, each of which has specific strengths and limitations. For example, enlarged endosomal compartments in secondary cells of the fruit fly *Drosophila melanogaster* allow visualization of intraluminal vesicle biogenesis (Corrigan et al., 2014; Fan et al., 2020), while larval motor neurons express multiple EV cargoes with known physiological roles, such that EV regulatory mechanisms can be tested through functional assays (Koles et al., 2012; Korkut et al., 2013; Walsh et al., 2021). The transparent nematode *Caenorhabditis elegans* has also provided insights into the cellular, developmental, and behavioral roles of EVs in addition to EV biogenesis (Beer & Wehman, 2017; Wang et al., 2014; Wehman et al., 2011). EV separation and concentration are challenging for small invertebrates but have been reported from nematode worms (Nikonorova et al., 2022; Russell et al., 2020) and fruit flies, (Thomas et al., 2018; Tsai et al., 2019). By virtue of its transparency, the zebrafish embryo can be used for real-time biodistribution and uptake studies (Hyenne et al., 2019; Verweij et al., 2019). In contrast, larger mammalian models may be needed to recapitulate some aspects of human physiology and disease processes. A key strength of in vivo models is the opportunity to assess the release of physiological levels of EVs and their interaction with target cells.

Some in vivo studies examine endogenous EVs, usually using fluorescent (Estrada et al., 2022; Hegyesi et al., 2022; Neckles et al., 2019; Nørgård et al., 2022) or bioluminescent tags (Gupta et al., 2020; Luo et al., 2020; Rufino-Ramos et al., 2022). Pre-clinical studies with syngeneic models and human cancer cell line xenograft models have allowed tumour and other EVs to be specifically labelled and traced (Driedonks et al., 2022; Hyenne et al., 2019; Liu et al., 2016; Pucci et al., 2016; Wiklander et al., 2015). Functions have been assigned to these EVs, such as roles in metastasis, by pharmacologically or genetically manipulating putative EV biogenesis regulators (Costa-Silva et al., 2015; Peinado et al., 2012; Wen et al., 2016); however, see caveats on blocking biogenesis that are discussed in Section 7.1 and on the relationship between uptake and function discussed in Section 7.2. Attempts to assess cytoplasmic delivery of EV cargo have involved, for example, EV-loaded mRNA for the DNA recombinase Cre and its detection in target reporter cells (Zomer et al., 2015). Parabiosis, whereby the circulations of two animals are joined, permits labelled EVs from one mouse to be visualised in the other (Liu, Kou, et al., 2018; Zhang et al., 2022).

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TABLE 4 Studying EV biology in vivo.

In vivo models	EV-releasing cells or other EV source	Other specific strengths	Genetic tractability	Genetic similarity to humans
Budding yeast *Saccharomyces cerevisiae*	Unicellular yeast (Oliveira et al., 2010; Zhao et al., 2019)	Whole organism analysis in vivo	++++	+
Green algae *Chlamydomonas reinhardtii*	Flagellated unicellular algae (Wood et al., 2013)	Cilia biology	++++	+
Flowering plant *Arabadopsis thaliana*	Leaf cells (Baldrich et al., 2019; He et al., 2021)	Plant immunity	++++	+
Nematode *Caenorhabditis elegans*	Embryonic cells (Beer et al., 2018; Wehman et al., 2011)	EV release mechanisms; whole organism analysis in vivo	++++	++
	Larval epithelial cells (Liégeois et al., 2006; Hyenne et al., 2015)	EV release mechanisms; whole organism analysis in vivo		
	Ciliated sensory neurons (Clupper et al., 2022; Nikonorova et al., 2022; Razzauti & Laurent, 2021; Wang et al., 2015)	Cilia biology; whole organism analysis in vivo; reproductive functions		
Fly *Drosophila melanogaster*	Larval wing imaginal disc (Beckett et al., 2013; Gradilla et al., 2014; Gross et al., 2012; Matusek et al., 2014)	Wnt/Hedgehog morphogen signalling	++++	++
	Larval motor neuron axon terminals (Koles et al., 2012; Korkut et al., 2013; Walsh et al., 2021)	Synaptic function		
	Larval haemocytes (Tassetto et al., 2017)	Adaptive immune system		
	Adult male secondary cells (Corrigan et al., 2014; Fan et al., 2020; Marie et al., 2023)	Large MVBs: exosome subtype biogenesis; reproductive functions		
	Adult muscle cells (Jewett et al., 2021)	Neurodegeneration		
Zebrafish *Dario rerio*	Embryonic yolk syncytial layer (Verweij et al., 2019)	Transparent embryos: EV imaging in bloodstream; target cell biodistribution; metabolic functions	+++	+++
	Adult osteoblasts (Kobayashi-Sun et al., 2020)	Fracture healing		
	Larval and adult cardiomyocytes (Scott et al., 2021)	Cardiovascular disease		
	Tumor cell lines (Hyenne et al., 2019)	Melanoma		
Chicken *Gallus gallus domesticus*	Chorioallantoic membrane (CAM) cells (Sung et al., 2015)	High-resolution live imaging of cell migration	+	+++
Mouse *Mus musculus*	Endothelial cells (McCann et al., 2020)	Cell type-specific EVs in plasma	++	++++
	Red blood cells; heart (Valkov et al., 2021)	Ischaemic heart		
	Mouse tumour cells	Pre-clinical metastasis (syngeneic grafts) (Ge et al., 2021; Ghoroghi et al., 2021)		
	Human tumour xenografts (Costa-Silva et al., 2015; Hoshino et al., 2015; Peinado et al., 2012; Zomer et al., 2015; Zomer et al., 2016)	Metastasis		

A non-exhaustive list of cellular models from different organisms, with particular emphasis on those that are widely used in genetic studies. Nomenclature: genetic tractability and genetic similarity to humans are rated from: weak ('+') to strong ('++++'). Please note that citations are examples only.

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Other in vivo studies introduce exogenous EVs into an organism. These EV may be unlabelled when a disease or physiologic outcome is targeted and imaging is not done. For studies with imaging, EVs are often fluorescently or bioluminescently labelled (Alexander et al., 2015; García-Silva et al., 2021; Kang et al., 2021; Long et al., 2017; Royo et al., 2019). Exogenous EVs have also been labelled, for example, with species-specific RNAs (Ciullo et al., 2022) and by substances compatible with magnetic resonance imaging (MRI), X-ray computed tomography (CT) imaging, magnetic particle imaging (MPI), single-photon emission computed tomography (SPECT) or positron emission tomography (PET) (Arifin et al., 2022; Skotland et al., 2022). There are several caveats to the exogenous approach. Specific labels may affect biodistribution patterns and detectability thresholds (Lázaro-Ibáñez et al., 2021), necessitating standardization (Herrmann et al., 2021). Exogenous EVs may also differ from endogenous EVs in route and timing of administration (bolus/continuous), dose, non-EV components of the administered preparation, and of course composition, and physiologic relevance should be carefully pondered (see also Section 8) (Ridder et al., 2014).

For detection and tracking endogenous and exogenous EVs, several additional technical considerations apply. In vivo EV tracking and ex vivo detection will be limited by technique-specific sensitivity and spatial resolution, for example, a fluorescent signal may represent a single EV, clustered EVs or non-EV labelled substances. Caveats associated with genetic labels such as the common CD63-GFP approaches are discussed in Section 6.2 and elsewhere (Verweij et al., 2021). They include the potential disruption of protein, EV or cellular biology through fusion protein (over)expression; possible quenching in acidic compartments; labelling of only specific EV subtypes; labelling of different EV subtypes by a specific marker in different cell types and species; and possible separation of the tag from its host protein. A knock-in strategy, by which a fluorescently tagged fusion construct (e.g., CD63-GFP) replaces the respective EV gene in its endogenous locus, or the use of multiple EV markers, provide possible solutions to some of these problems.

Recommendations *(Note: These recommendations are broad, as this section of MISEV2023 is meant to raise awareness of the diversity of in vivo studies and not to make prescriptive guidelines. Innovative new approaches should thrive in diverse organisms to move the field forward.)*

- Report all details of labelling and detection/imaging technologies to allow replication studies.
- For exogenous EV administration, report all parameters of administration, including anatomical site, timing (bolus/continuous) and dose.
- Consider and control for the possible effects of EV labelling on EV biodistribution, pharmacokinetics and function.
- Consider that pharmacologic or genetic manipulations meant to block EV production in vivo may have off-target consequences.
- Consider the possibility of different behaviour of endogenous and exogenous EVs.

> Consensus: 65.5% (654) of MISEV2023 survey respondents agreed "completely," and 21.6% (216) agreed "mostly" with Section 9: EV analysis in vivo. 0.1% (1) "mostly" disagreed, and 12.7% (127) stated that they had no opinion and/or expertise. No respondents disagreed "completely."

10 | CONCLUSIONS

Consensus building was achieved for MISEV2023 through a lengthy process. Suggestions for the new MISEV were gathered from the ISEV community and MISEV2018 authorship through a 2020 survey that received >750 responses (Witwer et al., 2021). A five-member MISEV2023 organising committee was then formed during the strategic planning session of the ISEV Board of Directors in November 2020, consisting of Deborah Goberdhan, Lorraine O'Driscoll, Clotilde Théry, Joshua Welsh and Kenneth Witwer. An initial MISEV2023 draft went through rounds of review and revision by members of the ISEV board and other individuals, including task force members, who were invited by the organising team because of their subject expertise relevant to specific sections. An exhaustive MISEV2023 survey was circulated to ~5700 EV researchers, and 1025 responses were received. Refinements were made to the manuscript by the organising committee and invited co-authors based on these responses. The manuscript was then submitted to the Journal of Extracellular Vesicles. The journal selected more than 30 individual experts to review the manuscript, and reviews were shared with the organising committee along with editorial suggestions. The manuscript was then revised by the organising committee and subject experts, and the ISEV Board of Directors was consulted on matters of timing and logistics. At the request of the ISEV Board, the revised manuscript was sent via survey to all who were involved in developing the guidelines and who had indicated willingness to accept co-authorship. The results of this authorship survey were used to gauge consensus on each section and to determine the final author lists before resubmission to the journal. The consensus statements at the end of Sections 1 through 9 reflect the complete answers of 998 unique MISEV2023 authorship confirmation survey respondents. There were 1039 responses in total, including several duplicates, one triplicate, three declines, and several incomplete responses. Note that several confirmed authors did not complete the survey for reasons that were deemed valid, including technical issues.

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MISEV2023 has compiled recommendations for EV research, from basic to advanced, state-of-the-art technologies and methodologies. As such, it can serve both as a handbook for those new to EV research and also as an inspiration for more advanced science in the field. In generating this document, an overarching goal has been to reach a high degree of agreement from a large group of scientists within the EV community. As with any consensus document, not every co-author necessarily agrees with every section or every recommendation. We also recognise that new methods will appear, while some advanced techniques may become easier to use: the field is dynamic, not static. Nevertheless, we propose that MISEV2023 describes the current best practice in the field and represents the current consensus position of the extracellular vesicle community.

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CONFLICT OF INTEREST STATEMENT

Pierre Arsène is CEO of Mursla Ltd. and Chair of Exosla Ltd; Antonella Bongiovanni has filed the patent (PCT/EP2020/086622) related to microalgal-derived extracellular vesicles and is co-founder and CEO of the spin-off company EVEBiofactory srl; Paul C Boutros sits on the scientific advisory boards of Sage Bionetworks, Intersect Diagnostics Inc and BioSymetrics Inc; Xandra O Breakefield is Scientific Advisor for Evox and MGB-Cannon; Edit I Buzas is a member of the Scientific Advisory Boards of Sphere Gene Therapeutics Inc (Boston, MA, USA) and ReNeuron (UK); David RF Carter is an Evox Therapeutics Ltd, employee and stock option holder; Anna Cifuentes-Rius was employed by Exopharm Ltd when the survey was conducted ACR is a shareholder of Exopharm Ltd; Rossella Crescitelli has developed multiple EV-associated patents for putative clinical utilisation and they own equity in Exocure Sweden AB; Andrew Devitt is Chief Technical Officer, co-founder, and director of EVolution Therapeutics; Erez Eitan works and has equity in NeuroDex, a company that develops EV-based diagnostics; Samir EL Andaloussi is co-founder of Evox Therapeutics; Ludwig Ermann Lundberg is an employee of BioGaia; Susanne Gabrielsson has a patent on B cell derived EVs in immune therapy and is part of the Scientific Advisory Board of Anjarium Biosciences; Ernesto Gargiulo is a medical writer at Novo Nordisk A/S; Bernd Giebel is a member of the Scientific Advisory Boards of Mursla Ltd, ReNeuron, and PLBioscience and is the founding director of Exosla Ltd; André Görgens is a consultant for and has equity interest in Evox Therapeutics (Oxford, UK) and is an inventor on several patent applications and patents related to EV isolation, modification, and analytics; Ahmed GE Ibrahim owns stock in Capricor Therapeutics; Marzena Kurzawa-Akanbi Kurzawa-Akanbi is an academic founder and Chief Scientific Officer at ESP Diagnostics Limited; Quentin Lubart is an employee of Abbelight (Cachan, France), which constructs and sells super-resolution microscopes to characterize EVs; Fabrice Lucien receives consulting fees from Mursla Bio and Early is Good; Elisa Lázaro-Ibáñez is employed by AstraZeneca R&D; Jan Lötvall is co-founder of two companies aiming to develop EV-based therapeutics, Exocure Sweden AB and Nexo Therapeutics AB, has been or is a scientific consultant for NanoSight, Clara Biotech and ExoCoBio, and was Editor-in-Chief of the Journal of Extracellular Vesicles during the development and publication of MISEV2023; Eduardo Marbán has founder's equity in Capricor Therapeutics Inc; Maurizio Muraca is a consultant for EXO Biologics (Liège, Belgium); Irina Nazarenko is a scientific adviser of CapCO Bio GmbH; D Michiel Pegtel has research funding from Takeda, Amgen, Abbvie, and Gilead, is an advisor of Y2Y BV, and has equity in Y2Y BV; Janusz Rak is inventor on a patent on oncogene-carrying EVs that is licensed to NXPharmaGene; Gregory E Rice is Chief Scientific Officer, Inoviq Ltd; Andrew Rowland is a recipient of investigator-initiated research funding outside of the scope of this publication from AstraZeneca, Boehringer Ingelheim, and Pfizer and is a recipient of speakers fees from Boehringer Ingelheim and Genentech; Susmita Sahoo performs research funded by Evox Therapeutics; Randy Schekman is a member of the Scientific Advisory Boards

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of companies involved in the analysis and diagnostic/therapeutic application of various forms of synthetic or native extracellular vesicles in diagnostics: Sail (formerly Senda) Biomedicines, Invaio Sciences, Mercy BioAnalytics, and Esperovax; Raymond M Schiffelers is CSO of Excytex bv; Johan Skog is an employee of Bio-Techne and an inventor on patents for exosome isolation and analysis; Vera A Tang is a consultant for Beckman Coulter on small particle flow cytometry; Clotilde Théry is an inventor on a submitted patent on therapeutic use of EVs; Edwin van der Pol is cofounder and shareholder of Exometry, Amsterdam, The Netherlands; Joshua A Welsh is an inventor on patents and patent applications related to EV analysis; Oscar PB Wiklander has stock options with Evox Therapeutics; Kenneth W Witwer is or has been an advisory board member of ShiftBio, Exopharm, NeuroDex, NovaDip, and ReNeuron; holds NeuroDex options; privately consults as Kenneth Witwer Consulting; and conducts research under a sponsored research agreement with Ionis Pharmaceuticals.

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