Title

Minimal information for studies of extracellular vesicles 2022 (MISEV2022): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2018 guidelines.

Authors and affiliations

To be written on completion of survey.

10

Abstract

To be written on completion of survey.

Keywords

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

Funding details

Disclosure statement

1 What MISEV IS and IS NOT

Since MISEV2018 appeared, there has been much discussion of what the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines mean to the extracellular vesicle (EV) field and where they should or should not be applied. Informed by that discussion, here is a summary of what MISEV IS and IS NOT.

MISEV IS:

1. *a set of recommendations to increase rigor and reproducibility during study design and reporting.*
2. *a tool for reviewers and editors to assess the strengths and weaknesses of proposals, funding applications, conference presentations, and manuscripts.*
3. *a source of strength and validation when thoughtfully applied to an individual project.*
4. *a wealth of examples of various techniques and measurements that are listed and briefly reviewed.*
5. *a support for innovative research to cross new boundaries and enable the field to move forward.*
6. *a document applicable to all sorts of EV research, not just academic "basic science" or studies of mammals.*
7. *an indication of current, broad consensus in the EV field, as well as areas of uncertainty and growth.*

MISEV IS NOT:

1. *a one-size-fits-all blueprint or a substitute for common sense.*
   Each researcher knows their system and should seek to apply MISEV appropriately, rather than trying to conform to a perception of MISEV expectations that does not make sense for a given experimental system.

2. *a checklist of "dos and don'ts" or "musts and must-nots".*
   There is no technique that is absolutely required or prohibited by MISEV. Similarly, MISEV does not mandate the 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.

   Reviewers, please do not reject a paper by claiming that MISEV mandates the use of Western blots or measuring CD63 or showing an NTA plot. This is simply incorrect.

3. *a "free pass" to publication via a box checking exercise.*
   Rather than being prescriptive, MISEV encourages the use of a combination of approaches appropriate to the system in question. For example, in the characterization of EVs, the most relevant markers should be employed. Similarly, in a study of many samples or multiple conditions, MISEV is not satisfied by showing a single vesicle in an electron micrograph, or a single NTA plot or lane of a Western blot.

4. *a barrier to innovation.*
   When introducing a new EV technique or application, some aspects of the approach may not fit precisely into the existing MISEV framework, or more likely, into a reviewer's interpretation of it. MISEV should not stifle innovation, but rather inform the presentation and validation of new approaches.

5. *a cudgel to prevent publication or funding of a particular project.*
   Just as MISEV should not stifle innovation, it should also not be used to prevent research from being shared with the community.

   Reviewers, please do use and emphasize the enabling aspects of MISEV. For example, an "*exosome*" paper that does not prove biogenesis can be re-cast as an EV paper, or an "*EV*" paper without full

characterization as an extracellular particle (EP) paper. If EVs cannot be linked to specific effects using suitable controls, it might suffice to acknowledge the caveats.

6. *an irrelevance to clinical research including production of therapeutic EVs.*
   MISEV has been suggested to be of no significance to clinical studies and applications, however, there is broad agreement that EV therapeutics should demonstrate:
   a. standardized production processes.
   b. EV hallmarks, and absence of other entities, for example, EPs, viruses and DNA.
   c. responsibility of EVs for the therapeutic effect.
   d. quality control attributes (QCAs) mirroring the therapeutic mechanism as much as possible, especially the outcome of potency assays.

   There is a lack of MISEV support for uncharacterized cell releasates being described as "*exosome*" therapies. The recommendations listed above are, however, perfectly consistent with the spirit of MISEV, so perhaps it is simply an issue of communication.

7. *a specialist set of rules only applicable to mammalian sources.*
   Past MISEV documents have focused on mammalian EVs, however, the basic principles are applicable to EVs from all sources, for example, wherever possible, good nomenclature, source definition, separation and concentration details, characterization, controlled functional studies, and comprehensive reporting.

Distilled Down

For those working with EVs, the spirit of MISEV is embodied in just a handful of questions.

1. We want to speak the same language, so what terms are you using?
2. Where are your EVs from, how did you separate them and why?
3. What can you tell us about your EVs and what else is in the mix?
4. To what extent can you attribute your biomarker or function to EVs versus other material?
5. How have you shared data and reported methods to allow replication and reproduction?

2 Introduction

Minimal Information for the Study of Extracellular Vesicles (MISEV) 2022 seeks to build on the criteria and guidelines set out in MISEV2014 [1, 2] and MISEV2018 [3] for the rapidly developing field of extracellular vesicle research. MISEV2014 was the first EV position paper designed to give robustness to EV analysis. MISEV2018 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 characterization.

In MISEV2022, we have sought to combine a succinct summary of MISEV2018 with refinements and new areas of development in EV isolation and assay specific recommendations. We have also focused more on how the secretion, uptake and functions of EVs are analyzed *in vitro*, since some of the approaches employed complement biochemical studies of EVs. And finally, we have also discussed *in vivo* analysis in a new **Section 12,** since *in vitro* and *in vivo* studies are increasingly informing each other, and therefore adding to the understanding of EV biogenesis and function.

3 ISEV Initiatives & Endorsement

Founded in 2012 in Sweden, the International Society for Extracellular Vesicles (ISEV) (https://www.isev.org) is the leading professional society for researchers and scientists involved in the study of extracellularly secreted vesicles.

ISEV's mission is to advance extracellular vesicle research globally. This is being facilitated by providing essential training and research opportunities for those involved in EV research. ISEV engages a diverse group of researchers across the world through its scientific Annual Meeting, peer-reviewed journals, online learning programs, workshops, and industry partnerships.

The ISEV community has disseminated current best-field practices and considerations through the publication of ISEV Position Papers. These have been consensus documents of the ISEV community, in the form of MISEV[1, 3], inter-society position papers[4], as well as focused groups of research-specific experts [5-10], **Table 5**. ISEV also endorses the use of external standards, such as MIQE for real-time PCR (RT-PCR) analyses [11], and reporting and atlas tools such as EV-TRACK[12, 13], and Vesiclepedia [14].

In 2019, a formal structure was outline for ISEV Rigor and Standardization Task Forces [15]. There are currently 12 Task Forces (https://www.isev.org/page/RigorStandardization) spanning a range of topics that include different biofluid standardization considerations, reference materials, and regulatory affairs.

4 Nomenclature

MISEV2018 endorsed the term extracellular vesicle ("*EV*") as the catch-all term "for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate, i.e., do not contain a functional nucleus." The term extracellular particle ("*EP*") was suggested if EV identity could not be confirmed by the criteria outlined in MISEV2018. MISEV2018 also discouraged uses of terms such as "*exosome*" and "*microvesicle, ectosome, microparticle*" denoting EVs from the endosomal system and plasma membrane, respectively, unless subcellular origin could be proven. Since markers of subcellular compartments often overlap or are not the same in different cells and conditions, and since different classes of EVs have overlapping sizes, it is difficult to establish subcellular origin. Even so, the term "*exosome*" has continued to be misused broadly to refer to all small EVs, total EVs, and general cell releasate/EPs in studies that do not establish EV biogenesis or purity [16]. MISEV2018 encouraged instead the use of "operational terms" (**Table 1**) if a portion of the population was being isolated for studies, for example, small EV ("sEV"), with clear explanations of how different procedures restrict size in each study. There currently exists no universal diameter to define EV subsets. However, as the sensitivity of detection has increased it has been observed that many EV sources have a modal point below 100 nm [17-19].

Since MISEV2018, a variety of EV subtypes have been proposed, which have specific properties, and/or subcellular or cellular origin. These include large EVs involved in protective clearance mechanisms, e.g. exophers from brain [20], heart cells [21] and in *Caenorhabditis elegans*, muscle [22], which are implicated in removing dysfunctional mitochondria and protein aggregates. There are also EVs generated from specific structures, such as cilia-derived vesicles [23, 24] and filopodia-derived vesicles [25], large vesicle-containing vesicles called migrasomes [26] as well as cholesterol-rich particles released during cellular locomotion [27], as well as *en bloc* released MVB-like small EV clusters [28]. Other EVs are being annotated according to their biogenesis such as Rab11a-labelled exosomes from recycling endosomes [29]. It is noteworthy that platelets of large EV size are produced from megakaryocytes by budding from cytoplasmic extensions [30], but it remains unclear whether this process is related to the mechanisms described above.

A number of new EPs have also been defined. The tethers formed by neutrophils have been shown to detach and form elongated neutrophil-derived structures (ENDS) within blood vessels, which are described as microparticles. ENDS have a membrane initially, but this seems to break down with time [31]. Other cellular extensions called flow-induced protrusions (FLIPRs) have also been reported to be produced by platelets [32]. Exomeres, which are small EPs with a diameter of ~50 nm or less, were mentioned in MISEV2018 [33] and have since been isolated by others [14] and characterized further [34, 35]. These and more recently discovered nanoparticles, termed supermeres (supernatant of exomeres) [36], which are smaller in size than exomeres, are thought to be non-vesicular extracellular particles (NVEPs), but still contain proteins, lipids (at least for exomeres) and nucleic acids that can be transferred to target cells. Both can be recovered as functional nanoparticles by extended high-speed ultracentrifugation. The mechanism by which cytotoxic T cells kill infected and cancerous cells has been shown to involve extracellular multiprotein complexes, encased within a glycoprotein shell, and termed supramolecular attack particles (SMAPs)

[37]. They act as autonomous killing entities. Modified forms of extracellular neutral lipid droplets, termed microcarriers, have also been shown to have signaling roles in reproduction [38].

MISEV2018 specified that EVs are "naturally released from the cell," prompting questions about nomenclature of special classes of vesicles. Engineering cells might lead to production of quantitatively or qualitatively different vesicles. Cells might also be stimulated physically or chemically, or even extruded, to make vesicles. After production, EVs can be modified chemically or by fusion with lipoplexes. And what about the ultimate case of engineering, where vesicles are built synthetically?

In fact, several studies present synthetic EV-like vesicles as reference standards for molecular characterization and functional assessment of EVs [39-42]. Synthetic EVs are based on vesicular liposomes, contain biomolecules in their lumen and present EV proteins or fragments on the surface. They mimic biophysical properties of natural EVs (e.g., laterally mobile membrane proteins, luminal structure, lipid shell), are commercially available or are manufacturable on laboratory and industrial scales [43]. A key difference compared to cell-derived EV reference materials is their defined character, featuring narrow size distributions, low batch-to-batch variations, pre-defined composition and biorthogonal tagging modalities (e.g., biotin-tags, fluorescent tracers) [40, 42]. Synthetic EVs are applied as separate controls or spiked into EV preparations as internal reference material for EV purification and characterization [43]. Furthermore, they can be equipped with functional EV components to study their signaling, targeting or therapeutic mechanism under defined conditions. Comprehensive reporting on the use of synthetic EV-like vesicles should include size distribution and zeta potential analysis, lipid compositions, incorporated fluorophores or chemical tags, particle concentration and nucleic or amino acid sequences included. Of note, synthetic EVs do not fully recapitulate the physiological properties and functions of 'natural' EVs, but provide complementary information to other approaches when used for functional analysis and standardization; analysis using synthetic EVs should be carried out following the technical considerations detailed elsewhere [44].

If the products of these processes are true vesicles, and extracellular, should we call them EVs? These cases show that the MISEV2018 definition applies to "naturally produced" or "native" EVs, but that other types of EVs can be made in the laboratory. It is thus recommended that authors clearly specify any special circumstances associated with EV production.

In summary, since MISEV2018, more discrete functions have been assigned to EVs from specific cell types and a greater range of cell surface structures implicated in EV release. In addition, an increasing range of non-vesicular structures with biological activity have been defined, which might also be present in EV preparations. In MISEV2022, in addition to the term EP, we propose the use of the operational term "*non-vesicular extracellular particles*" (NVEPs) for those particles shown not to contain a lipid bilayer, e.g. exomeres, supermeres, SMAPs, lipoproteins [45] and protein aggregates [46].

To conclude, exceptions to the MISEV2018 definition of EVs at the beginning of this section might include fully synthetic particles. Any engineering, cell stimulation or destruction, or synthetic biological processes should be clearly specified to distinguish such from native EVs. In addition, there are no universal markers to establish biogenesis across cell types and conditions—certainly not in complex biological samples—so biogenesis-related terms such as "exosome" and "ectosome" should be used only in well-controlled cell biology settings, not in biomarker studies and probably not for therapeutic applications. In these situations, EVs or sEVs are likely to be the relevant operational term and neither equates to exosomes.

5 Task Force Initiatives

ISEV's commitment to increasing and maintaining rigor and reproducibility in EV research is exemplified by its establishment of a Rigor and Standardization Sub-Committee in 2019, under the umbrella of which 9 Task Forces have been formed. Recognizing that different sources of EVs will have specific (as well as general) pre-analytical considerations, among these are task forces dedicated to cell culture conditioned medium, blood, urine, cerebral spinal fluid, saliva, synovial fluid, milk and bacterial EVs. We recommend using the following link (https://www.isev.org/rigor-standardization) for information on the Sub-Committee and the task forces, their

membership, what they are working on, and their outputs. Of note, for various reasons including how long a given task force has been in existence and the activity in that specific research area, some task forces are at a more advanced stage than others. However, the intention is that such task forces will continue to exist for as long as appropriate to help ensure rigor and standardization and additional task forces will be created as relevant. Indeed, members of the EV community are invited to propose new task forces if they wish. Below we consider each of the EV sources for which ISEV had established a Task Force by end of 2021. Also outlined is the work of ISEV Task Force on Regulatory Affairs and clinical use of EV-based Therapeutics and the guidance they offer.

5.1 Regulatory affairs and clinical use of EV-based therapeutics

The task force on Regulatory Affairs and Clinical Use of EV-based Therapeutics works with stakeholders from regulatory authorities, academia, clinical research, and other research institutions to contribute to the development of applicable regulatory guidance. Its overall vision is to jointly accelerate achieving the ultimate goal of safe and efficient evaluation of EVs in clinical studies towards proven EV-based therapeutics. This task force's products to date have included a Public Safety Notification on Exosome Products accessible on the FDA's (https://www.fda.gov/vaccines-blood-biologics/safety-availability-biologics/public-safety-notification-exosome-products) and ISEV's website, a Patient Information and Safety Notice on EVs /exosomes and unproven therapies (https://www.isev.org/patient-information-and-safety-notice--extracellular-vesicles-exosomes-and-unproven-therapies) , and a combined statement between ISEV and the International Society for Cellular and Gene Therapies (ISCT) on EVs from mesenchymal stromal cells and other cells in relation to their potential therapeutic relevance to suppressing coronavirus disease-19 [47].

"massivEVs" ISEV's hybrid workshop on massive product on EVs was held at the end of October 2021. Its topics presented and discussed by academics, clinicians, and industry attendees- addresses EV production as therapeutics, nutraceutics, cosmetics and nanotechnologies; upstream and downstream processing; validation, standardization, and regulatory issues. Several information products including guidelines are planned, arising from this workshop.

In conclusion, MISEV2022's recommendation is to report on EV research accurately and honestly, while avoiding over-claiming, sensationalizing and, in any way, misleading other researchers or the public at large.

6 Collection and pre-processing: pre-analytical variables

It has been well established by members of the EV research community that most, if not all, EV-containing fluids encounter some of the same challenges and so many of the same considerations are relevant. Some challenges common to all include checks to maximize quality of starting material, how to separate EVs from soluble proteins and non-EV particles, how to determine recovery and purity of EVs, and how to define and measure contaminants. Establishing complete answers to these questions is still on-going. We recognized that best practices for collection, handling, and storage are not all established yet and that this can only be achieved by the EV community performing well designed experiments and accurate reporting.

In the meantime, our fundamental recommendation here, as for studies on EVs from all sources, is to perform well designed studies and give due consideration and report influences that the methodology used to collect, store, and pre-process the EV source may have on the EVs that are subsequently collected. Adding this context will increase the value of the new knowledge generated. The importance of this is exemplified by studies of cerebral spinal fluid (CSF). Specifically, human donor demographics reported to affect CSF biomarkers include age [48-52]; patients' medication [53, 54] and social [55, 56] history; gender [52]; genotype [52]; and ethnicity [57]. CSF protein concentrations change throughout development, with high CSF protein levels in neonates declining to low levels in children, and then increasing again from adolescence through adulthood [48, 49, 57]. In addition, for biomarkers that exhibit changes with circadian rhythm, the time of day for CSF collection will greatly affect their expression levels [58]. Such parameters are also likely to affect CSF EVs. Furthermore, pre-analytical variables identified as important in studies of other biofluid EVs are also very likely to be relevant to CSF EVs. These include the type of sampling and storage tube, time and holding temperature between collection and storage, centrifugation prior to freezing, number of freeze-

thaw cycles, and use of additives [59]. So, in the case of studies of EVs in body fluids –regardless of the origin of the biofluid- all such information, as well as body-mass index, disease (if any known), medication, etc., should be collated and reported. In fact, readers may find Table 2 in the position paper by ISEV's Urine Task Force [10] a useful example for reporting, which would be tailored as appropriate.

Overall, considerations around EV-containing biofluids, indicated and somewhat generalized in MISEV2018, remain valid. However, each biofluid can also have its own complexities and so the biofluids for which an ISEV Task Force is now operational are outlined below with any recommendations developed to date.

6.1 Cell culture (eukaryotic and prokaryotic) conditioned media

Evidence indicates that all types of cells cultured *in vitro* release EVs that can be separated from their conditioned media (CM). This includes both eukaryotic cells from pluri- and uni-cellular organisms and prokaryotic cells from both gram-positive and -negative bacteria and from *Mycobacteria*. Many recommendations listed here also apply to bacteria conditioned media, but more specific details on bacteria EVs is provided in **Section 6.3**.

Cultured cells' EVs (CM-EVs) that have potential to be used as therapeutic products in regenerative medicine have become of substantial interest. The importance of various culturing parameters has previously been noted [7, 60-62], building on MISEV2018 guidelines that highlighted key parameters to be reported regarding collecting CM [3]. However, many papers on CM-EVs are still unclear and/or incomplete with regards to reporting of cell culturing parameters (CCP). Thus, a focus of the CM Task Force has been on establishing considerations for minimal criteria required for reporting CCPs.

CCPs are, by definition, all parameters in the cell cultures including the producing cells (e.g., their name, viability, passage number, etc.), supplements used in their media (e.g., nutrients, micronutrients, and chemicals), culture conditions, harvesting approaches, and any contaminating infections. This holds true for any cell culture system, whether eukaryotic or prokaryotic. There are numerous examples showing that CCPs affect EVs yield and/or potency, directly or indirectly, and supplements added to culture media may be taken up by cells and re-packaged into released EVs [63-75]. Serum or platelet lysate are often added to culture media for mammalian cells, but they can prove to be particularly challenging supplements as they are rich in EVs and many other undefined entities including DNA fragments and micronutrients [74, 76]. However, consideration must also be given to challenges associated with efforts to use EV-depleted serum/plate lysate or serum-free media, with regards to their influence on cell physiology and EV production/release [74]. It is also important to be mindful that the effectiveness of EVs removal from serum or platelet lysate is somewhat controversial [77]. If serum-free media is chosen, it must be considered that supplements used to compensate for the lack of serum can carry miRNAs that co-purify with EVs [78]. Due to the complex nature of serum, checking the efficiency of EVs removal is also problematic [10]. Thus, we recommend that the detailed process of EV depletion and any change in serum/platelet lysate supplementation of cells should be comprehensively reported. Cell culture repositories (e.g., the **American Type Culture Collection**, European Collection of Authenticated Cell Cultures) from which the cells are obtained recommend specific growth medium and cultured conditions for the cells. Regardless of whether those recommendations are adhered to or are modified, the growth medium and culture conditions used should be reported, as well as the method (e.g., short tandem repeat) used for confirming the cellular identity. CM contaminations may affect almost all the characteristics of producing cells [79] and can be re-packaged in the derived EVs [75]. For example, contaminating microorganisms such as *Mycoplasma* may also release EVs into the CM [80]. Collectively, it is strongly recommended to provide as much information as possible about the CCPs used for culturing the cells *in vitro*.

Although some effects of CCPs on the EVs yield or potency have been investigated, the full extent of the influences of CCPs on EVs is not yet fully understood. CCPs may influence biological processes such as osmotic pressure, senescence, and apoptosis [81-83] which, in turn, may also influence cellular EV production. Comparisons of EVs yields and/or potency between different biological situations is also tricky/risky and quantifiable features assessed by reproducible and standardized assays for checking the confluency, viability, EV

depletion efficiency, etc. are required, but not yet agreed upon. Furthermore, if relevant to the scientific question being addressed, the CM Task Force recommends the use of high-throughput approaches for analysis of changes in EVs' proteins, nucleic acids, lipids, and metabolites in response to different CCPs and culturing systems. However, it is accepted that such global analysis is not possible for all studies.

6.2 Biological fluids

It is important to highlight that although some biofluids are not discussed below because ISEV does not have a Task Force dedicated to that topic, they are important source of EVs too. Examples include EVs termed prostasomes from seminal fluid [84], epididymosomes [85], which are reported to participate in regulating sperm motility activation, capacitation, and acrosome reaction. Likewise, in the female reproductive tract, follicular fluid, oviduct/tube, and uterine cavity EVs are considered as vehicles to carry information during oocyte maturation, fertilization, and embryo-maternal crosstalk [86]. Another example is EVs from exhaled breath condensation. However, studies of this source have been few in numbers and detail, and the origin of the EVs (whether mammalian or bacterial cells) has yet to be further investigated [87, 88].

6.2.1 Blood

At present, blood is the most studied biofluid for biomarker research. Most of these studies are of human blood. Blood is a complex fluid containing not only EVs but also high concentrations of non-EV particles, including platelets, lipoproteins, and chylomicrons. These non-EV particles overlap in size (diameter) and/or density with EVs, which hamper straightforward isolation and analysis of EVs. Based on a survey questionnaire and discussions, the Blood Task Force published a roadmap for collecting, handing and storage of blood-derived plasma and serum for blood EV research; see Clayton et al [89] for details on this survey and discussion. This Task Force is now defining quality controls, again based on a survey questionnaire and discussions, useful to monitor the quality of plasma and serum samples that are used for EV research. For example, there are multiple and often slightly different (local) centrifugation protocols to prepare plasma, but their efficacy to remove platelets is unknown. By measuring and reporting the platelet concentration in the prepared plasma samples, one gets insight not only in the efficacy of the applied centrifugation protocol to remove platelets but also in the presence of a main confounder. The goal is to summarize recommendations about quality controls as minimal reporting requirements for blood-based studies on EVs. Obviously, these reporting requirements do not replace detailed description of pre-analytical parameters, which are clearly essential as exemplified by Berckmans et al. [90] and Dong et al. [91]. Other future projects that are relevant to the EV community may involve anti-coagulants, blood stabilization tubes, and development of reference plasma samples. For now, in additional to the recommendations common to all biofluids listed above and including donor demographics and all samples collection, handling and storage, this Task Force's agreed upon guidelines are those described in Clayton et al [89].

6.2.2 Urine

To date, urine is the second most analyzed biofluid after blood. Urinary EVs (uEVs) are very attractive as potential multiplex biomarkers as they are easily accessible non-invasively, in large quantities and in serial sampling. As such, they are seen as promising information source on the health status of the kidney, urogenital tract and possibly other organs and systems [10]. Indeed, as molecular mirrors of their cell of origin they are investigated as disease and site-specific markers in many kidney and urologic diseases [92]. Several uEV candidate biomarkers have been identified for kidney disease processes [93-95]. However, validation is still needed to advance EVs for clinical application, for examples as liquid biopsy in glomerulonephritis and transplantation or early disease markers in diabetic nephropathy [93]. For prostate cancer, the Federal Drug Administration in the US has approved a uEV RNA signature as a non-invasive screening method [96].

Besides their role in diagnosis and prognosis, uEVs are studied increasingly as mediators of disease pathogenesis in various urological and nephrological diseases and during kidney regeneration. For examples, intra-nephron communication through (u)EVs is investigated to understand how glomerular and tubular damage

leads to acute and chronic kidney disease [97]. As a regenerative tool, uEVs have also been found to improve renal recovery of glycerol induced acute kidney injury [98].

uEV's diverse origin and dynamic composition, however, present an enormous analytical challenge which are object of intense investigation [99]. During the last decades many uEV separation and analysis techniques have been developed [10]. This enabled high-throughput uEV characterization for single and bulk uEV analysis, but also led to increased complexity of data. The Urine Task Force was formed to provide guidance on urinary EV research and recently published a Position Paper that presents the current state of the art and discusses critical knowledge gaps [10]. The position paper also provides recommendations regarding biospecimen handling, processing, normalization issues and reporting requirements to improve reproducibility and interoperability, which we recommend as minimal guidelines. It is unlikely that a universal pre-analytical procedure for all uEVs studies will be established, but by advancing rigor and standardization of uEV analysis clinical application of uEVs will be accelerated [10].

6.2.3 Cerebrospinal Fluid

Cerebrospinal fluid (CSF) serves as a conduit for biomarkers of multiple neurological disorders [100-102]. As the molecular cargo of EVs can reflect the state of the CNS [103], the past few years have seen a marked increase in publications that investigate the utility of CSF EVs as disease biomarkers for a range of indications affecting the nervous system [104-107], [108], [109, 110], [111], [112, 113], [114, 115]. Studies performed using total CSF (i.e., not on separated EVs) suggest that differences in pre-analytical factors can affect the measurement of biomarkers by 20-30% [116-118]. To increase rigor in CSF EV studies, these pre-analytic factors must be carefully considered and fully reported; some of which are specific for CSF. For clarity and context, CSF moves from the brain down the spinal cord to the lumbar sub-arachnoid space. This unilateral flow establishes a rostro-caudal gradient [119], with lower concentrations of brain proteins (e.g. S-100β, total Tau, and phosphorylated Tau) in the lumbar region than the cisternal region, depending on the type of neurological disorder [120, 121]. Thus, it is highly recommended for biomarker studies that both the collection site (e.g., lumbar puncture compared to ventriculoperitoneal shunt) and the volume of CSF drawn [59, 122] are standardized. Importantly, the effect of this gradient on CNS-derived EVs, and a comparison of lumbar to central CSF, have yet to be performed. Another factor that greatly affects CSF biomarker levels is blood contamination, given that protein concentrations in serum and plasma are 200-400 times greater than in CSF [123]. Markers of blood contamination in CSF include hemoglobin, catalase, peroxiredoxin, carbonic anhydrase I, apolipoprotein B-100, IgM, apolipoprotein B-100, fibrinogen, and haptoglobin [119, 123], and the current recommendation is to exclude CSF samples that contain >500 erythrocytes/μL from biomarker studies [122].

The effects of pre-analytical variables on CSF biomarkers have been predominantly, if not exclusively, established with total CSF and not specifically for CSF EVs. We, therefore, recommend that controlled studies be performed to address the effect of these variable on CSF EVs. Ultimately, controlling for and accurately reporting pre-analytical factors will be an essential step in designing and performing reproducible and informative CSF EV biomarker studies for neurologic diseases.

6.2.4 Saliva

Saliva is an easy accessed and non-invasive body fluid from which to collect EVs to be evaluated both for their function and their potential role as biomarkers [124]. Healthy adults produce 500-1500 mL saliva per day, but this varies with pathological and physiological conditions [125]. Furthermore, saliva consists of water (94-99%), cells, cellular components, proteins such as enzymes and antibodies, electrolytes, food debris, and eukaryotic EVs. However, it also included bacteria and bacterial EVs [124-128]. [129]. Saliva is produced by three major pairs of salivary glands –parotid, submandibular and sublingual– as well as 300-750 minor salivary glands located within the mouth [127, 130]. The different glands secrete different types of saliva and the final composition of the saliva, and so the EVs within, depends on the relative activity and contribution of the different glands.

Although saliva is collection is non-invasive, there are several different parameters that affect the composition of the collected saliva that should be considered and reported on. These include generic donor demographics (mentioned above) that are common to EV studies in all biofluids, and others which are more specific to EV studies in saliva e.g. whether whole saliva or saliva from one type of gland only is collected, the method of choice for saliva collection [130-132], and the stimulus, if any, to which the individual has been subjected [133]. From studies of whole saliva it has already been established that age [134], gender [135], smoking [136], stress [137], exercise [138], oral hygiene, medical conditions and medications, and mental health status [127, 139] affect viscosity, pH, concentrations of different proteins, and saliva flow rate. However, it is not known how these parameters affects the concentration and composition of saliva EVs. We, therefore, recommend that studies be performed to evaluate how pre-analytical factors affects the EV concentration and composition in saliva.

6.2.5 Synovial fluid

EVs have also attracted attention in the research of joint diseases, because of their potential as biomarkers and therapeutic agents [140]. Synovial fluid, as opposed to peripheral blood, as starting material to analyze EVs is attractive as it close to the relevant immunopathological processes of the disease. Comparative studies of EVs of different cellular origin in rheumatoid arthritis have shown differences between blood and synovial fluid [141].

Since the publication of MISEV2018 guidelines, there have been several publications reporting the analysis of EVs from synovial fluid. Most of them used samples from humans with rheumatoid arthritis, osteoarthritis, and healthy controls. Horse synovial fluid EVs analysis has also been reported [142]. There is substantial heterogeneity in the literature regarding the procedures of collection and pre-processing. Most publications report on synovial fluid samples that have been frozen for later analysis of EVs after thawing. However, centrifugation before freezing, to remove cells and debris, has not been consistent between studies [143, 144]. Some groups use hyaluronidase to decrease viscosity of the synovial fluid, others do not [145].

The very high protein content of synovial fluid is an obstacle for analysis of the proteome of EVs, as centrifugation does not result in removal of proteins such as albumin, fibronectin, apolipoprotein A-I. Size exclusion chromatography has been reported to result in higher purity of EV fractions when compared to ultracentrifugation-based enrichment methods [146].

A recent study demonstrated sex-specific differences in protein content of synovial fluid derived EVs from patients with osteoarthritis [147]. Differences in EV numbers and protein content have been described between different diseases e.g., rheumatoid arthritis versus osteoarthritis and healthy controls [148]. In addition, how active the disease is may influence EV numbers and protein content [149]. The Synovial Fluid Task Force agrees that studying EVs from synovial fluid may contribute to a better understanding of the pathogenesis of joint diseases and has identified limitations in some studies reported to date. This is a relatively new field of EV research, and the Task Forces is committed to working towards ensuring validity and reproducibility rigorous methodological standards are established. However, it has not yet established specific recommendations other than maximum reporting of all methods used.

6.2.6 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 [150]. EVs separated from milk of up to 14 different species have thus far been reported, with human and bovine milk mostly studied. As for other complex biological fluids, proper collection and pre-processing of milk prior to storage or EV collection, where this is intended, are important in order to increase the purity of milk EVs and reduce the chances of co-isolating other milk components that share EV characteristics such as density and size [151]. Generally, removal of MFGs, cells and cellular debris by centrifugation prior to storage or EV separation are applied, whereby preventing the disintegration of MFGs and cells is encouraged by short-term storage after collection while keeping milk at body temperature if possible [152].

As milk of ruminant species in particular is highly abundant in casein micelles (which are more abundant and overlap in size with EVs), various protocols have been established to efficiently remove or disrupt these prior to EV collection. Casein micelles can be precipitated by lowering the pH of the milk with acidic acid or hydrochloric acid to pH 4.6, after which aggregated caseins can be pelleted [153-155]. They can be coagulated by enzymatic treatment [156] or dissociated by sequestering calcium via the addition of EDTA [156] or sodium citrate [157]. Following these pre-processing steps, cleared milk supernatant can be stored (See **Section 6.2.6**) until EV separation. Currently, there is no preferred method for pre-clearing milk from casein micelles and this remains to be determined.

Additionally, raw milk, consumers milk (which is industrially processed by heating and homogenization), or powdered milk (including infant formula) might require different approaches in terms of pre-processing, as the colloidal properties of milk are different between these milk types and storage times until processing might differ considerably [158]. Furthermore, the effects of long-term storage or storage temperatures of pre-processed milk on recovery and efficient isolation of milk EVs is yet to be determined. For now, the only recommendation of the Milk EV Task Force is to provide as much detailed information as possible on collection and pre-processing, etc., which include donor information and technical procedures.

6.3 Bacteria

The Bacterial EV Task Force is currently conducting an in-depth survey focused on bacterial EVs. The diversity of bacterial species lends itself to broad generalizations with respect to the production and characterization of EVs and classification of EVs typically aligns with the basic structure of the bacterial cell membrane to encompass gram-negative, gram-positive and mycobacteria. Depth of knowledge on pre-analytical preparation, separation, and storage of bacterial EV is still limited with respects to most bacterial species [159, 160].

Most of the considerations for eukaryotic EVs and gram-negative bacterial EVs (historically referred to as outer membrane vesicles, OMVs) translate loosely to gram-positive and mycobacterial EVs. This includes the implicated effect of media composition, oxygenation/aeration, as well as culture format (such as standing, shaking, roller bottle, bioreactor). Importantly, and often not specified, is information pertaining to growth phase at harvest. Maximal EVs release has been observed during log-phase growth [161]. However, transition into late-log phase can increase bacterial lysis and skew downstream analytics. Thus, we recommend that a measure of cell lysis, such as defined cytoplasmic protein or lactate dehydrogenase, be performed in the harvested supernatant. Bacterial EV samples derived from *in vivo* sources (infected cell culture, body fluid) should be treated initially as other eukaryotic samples, as the content are primarily host derived [162, 163]. Considerations include limiting prolonged storage prior to enrichment, especially if left unfiltered, as well as additives introduced during sample collection.

While same methodologies used to enrich eukaryotic EVs can be used to enrich bacterial EVs, improper selection can introduce significant bias in downstream analyses. The added complexity with bacterial EVs is that bacterial EVs' heterogeneity includes biophysical properties (such as size and density, which is cause by the content) and also heterogeneity between species. Guidelines for the correct density to collect the particle-rich fractions are unavailable, as this has to be worked out for each bacterium and all growth conditions. The variability in EV sub-populations is vast between bacteria, much more so than seen for mammals that are genetically and molecularly more similar [164]. However, the use of laminar flow hoods during culture filtrate harvest, to avoid EVs from other organisms, is encouraged.

[Of note: While some of the information here may be relevant to all single celled organisms, the focus of this Task Force to date has been on bacteria].

6.4 Tissue

Although the work of the Tissue Task Force has been on animal tissues and so animal tissue is the focus here, plant EVs are also emerging as an interesting and important area of research.

To fully understand the role of EVs in regulating organ or tissue function, EVs need to be collected from that specific tissue for the purpose of characterizing them, and for different types of functional experiments. Several groups have started on the journey of isolating EVs from tissues, and primarily tumor tissues from humans or experimental animals have been used [165-172]. Indeed, for those aiming to identify EV biomarkers of different diseases, careful characterization of tissue EVs could potentially be a highly enriched source. It is noteworthy that EVs have also been collected from both frozen as well as fresh tissues, for example in the case of brain [168, 173-177].

Two principles for EV isolations have been attempted for extracting EVs from tissues i.e., a relatively slow approach that involves culturing tissue slices over days *ex vivo*, whereafter the EVs are collected from the tissue culture supernatant [165, 171, 178]. The alternative is a quicker approach to collect fresh EVs directly out of tissues [166, 167, 169, 170, 172, 173, 175, 179, 180]. Both approaches have benefits and shortcomings that should be considered by any researcher attempting to collected EVs from tissues. Regardless of which type of methods is used, it is important to be aware that any dissection of the tissue can disrupt cells, allowing for intracellular molecules and intracellular vesicles to leak into the collected. One crucial passage showing high risk to destroy cell membrane is the tissue dissection step. In some papers, authors used the homogenizer [168, 176, 177, 179]. Alternative techniques are vortexing [181] or slicing [172, 174, 175, 182]. It is important to be aware that demonstrating that the collected vesicles were extracellular (i.e., EVs) before tissue processing, rather than intracellular vesicles released by cells broken up by the tissue disruption step, is still difficult. However, as intracellular vesicles should have the opposite membrane orientation as EVs (i.e., expose the cytosolic, rather than the extracellular side of transmembrane proteins), analyzing membrane orientation of the recovered EVs may be an appropriate approach when easy single EV-analysis processes become available. As with any EV source it is, thus, important to carefully characterize the resulting EVs, to determine whether any signs of non-EV contaminants contribute to the content in the isolates.

When pieces of tissues are maintained in cell culture medium ex vivo for one or several days, there is a risk that cells will undergo apoptosis and so releases apoptotic bodies into the medium. This could result in a mixture of vesicles in the supernatant produced by those cells that continue to proliferate in the culture medium and/or those that are undergoing cell death. It is also possible that the EV-producing cell phenotype may change under these conditions. This should all be considered when content or function of the EVs is studied.

An alternative approach is to attempt to retrieve fresh EVs relatively quick from tissues, by allowing tissue pieces to release EVs ex vivo after careful slicing and enzymatic treatment to disrupt interstitial structures, followed by brief incubation (1 hour) in culture medium [171]. Compared to the first approach, this method may capture different EVs populations with different characteristics and functions. It is possible that EVs collected directly from tissues, without allowing the cells from the tissues to expand in vitro, better represent EVs present in the tissue, although no direct comparisons have been performed.

Considering the approaches taken to date, collection of EVs from tissues seems feasible, but has multiple hurdles to overcome and that need to be considered. Experiments directly comparing the characteristics of EVs released after tissue culture over a day(s) versus those collected more rapidly from tissues could be very helpful. As highlighted at the start of this Section, as for all EV sources, how the tissue is procured, stored, and processed could have substantial influences on the released EVs. Consideration should be given for example to potential cell death during initial enzymatic digestion that is aimed at generating a single cell suspension and how this may influence the type of EVs that are subsequently collected. These could be considered as technical influences, rather than true biological influences, if trying to develop the basis of an understanding from ex vivo studies as to the involvement of EVs in physiological processes or how they contribute to -or are altered in- disease. EVs have also been isolated and analyzed from plants tissues EVs. Methods to isolate and purify plant EVs from the leaves of the genetically amenable mustard plant, *Arabidopsis thaliana*, have been developed using intercellular apoplastic washes [183, 184]; these methods involve combinations of differential ultracentrifugation, sucrose density gradients and immuno-isolation. There are, however, divergent views on the details of these approaches, for instance the optimal

differential centrifugation speed, and consequently, there is some debate concerning the functional conclusions that can be drawn (discussed in [185]). *Arabidopsis* EV-associated small RNAs (sRNAs) have been implicated in host immunity and suppression of fungal virulence (reviewed in [186]), although it has recently been reported these sRNAs may primarily be located on the outside of EVs [187]. Developmental roles for plant EVs have also been reported, such as the deposition of protective suberin lamellae between the plasma membrane and plant cell wall [188]. Beyond their biological functions, there is significant interest in the potential of EVs obtained from juice of edible plant tissues for biomedical applications. As with other biotechnological developments involving EVs (**Section 12.1**), the primary focus in this area is on improving quality control and productivity [189].

6.5 Storage

How samples are stored prior to EVs separation -if EVs are to be collected- and how the resulting EVs are stored could substantially influence EVs (collectively or sub-populations) in many ways. This could include their yields, their contents, their functionality, whether they exist as single EVs or as EV aggregates. For example, from milk studies it has been reported that the quantities of EVs decrease to approximately 50% when human milk is stored at 4 ºC for 4 weeks [190]. However, if milk is stored at 4 ºC many components of the overall milk matrix will have changed in this time -given that milk will sour. This, in itself, could contribute to reduced quantities of EVs and/or decreased ability to separate out the EVs. The presence in milk of bacterial EVs and EVs from spores of fungi must also be considered. Thus, context must always be kept in mind.

EVs may also stick to certain plastics and so be lost depending on the make-up of the tubes/storage vessel walls [191]. Where details on EV storage are reported, which is not always done -but which we would strongly recommend- typically storage is at -80 ºC. These samples are sometimes snap-frozen in liquid nitrogen first, sometime not, and in different types of buffers e.g., containing cryoprotectants or not [192, 193]. A recent study investigating the impact of storage on EVs from human plasma and those released cultured murine microglia cells found that whether or not preservatives or cryoprotectants are included or not, -80 ºC storage reduces EVs concentration, and sample purity in a time-dependent manner while increasing particle size/EVs fusion and size variability and modifies EVs zeta potential [194]. If freezing, we recommend avoiding repeated freeze-thawing. EV lyophilization is also under investigation [195], but there is currently inadequate data on which to make recommendations.

Some stability studies of EVs, from different sources, have been reported. For example, a study of saliva EVs reported them to be stable at 4°C for up to 20 months, retaining membrane integrity and protein content. In this same study it was reported that saliva could also be stored at 4°C for a month prior to EV extraction, although because of the presence of bacteria it is unclear how this can be achieved without any determinantal effects on the subsequent analyses. Freeze-thawing of the EVs was also reported to have no effect on membrane integrity (assessed here as dipeptidyl peptidase IV activity), suggesting a degree of robustness in salivary EVs [196]. A recent study of urinary EVs evaluated the effects of storage by comparing freshly separated EVs and stored EVs (at -20 ºC compared to -80 ºC). It evaluated storage times of up to 4 years, the storage format of urine compared to separated EVs, and performed downstream transcriptomics analysis [197]. The study highlighted that the consequences of storage may be minimal or substantial depending on the parameters being evaluated and compared.

In relation to bacteria EVs, evidence supports the stability of OMVs at 37 °C for several hours [198]. However, as the properties and components of EVs from gram-negative, gram-positive and mycobacteria may be different, studies should be performed for each bacterial species rather than making generalized assumptions from the study of one or a few species. Bacterial EVs in bulk *in vitro* preparations can be stable at 4 °C for several weeks. Additives, such as sodium azide, to inhibit growth in bulk crude bacterial supernatants should be specified if used as a safeguard against the introduction of EVs from contaminant organisms [199].

Interestingly a straw poll of more than 100 attendees at a 2021 ISEV EV Club meeting that was dedicated to MISEV Guidelines and was held at two timepoints to maximize the opportunity for participation for those who wished, showed that the majority of people (almost 70% of responders) store their separated EVs before proceeding to perform any analysis. The EVs are typically stored at -80 °C (by approximately 75% of responders). Fewer than 50% of the

responders perform comparative studies pre- and post- storing to ensure that the EVs maintain the same characteristics and/or same functionality as they did when initially separated.

Consideration should be given to evaluating EVs freshly after separation from their matrix, if possible as these are least changed from how they were when released from their cells of origin. Then when considering storage, in the ideal world comparative pre- and post- storing studies would be done, rather than assume that the characteristics the EVs had following release from their cells of origin are unaffected with storage temperature and/or time, especially given that the recent comparative study -mentioned above- that showed negative impact of storage at -80 °C [194]. However, this is unlikely to be feasible. For example, if large quantities of EVs are needed to perform pre-clinical *in vivo* studies, this would not be possible. We strongly recommended that within a given study either freshly collected or previously stored EVs be used, but do not "mix-and-match" i.e., a somewhat random combination of both should be avoided. Also, we recommend that if freeze-thawing that the fundamental characterization of the EVs be performed after freeze-thawing rather than assuming that the EVs maintain the same characteristic they had when initial collected.

Whether or not optimal EV storage parameters have been confirmed for the EVs in question, EV isolates should be stored in appropriately sized aliquots to minimize potential detrimental impact that repeated freeze-thawing may have EV integrity and activity. It is highly recommended that the storage conditions (including temperature, snap-freezing or not, using a cryopreservative or not) and storage time be planned in advance and described when report research findings.

7 EV separation and concentration

EVs exist as membrane-bound particles dispersed within different biofluids. The efficiency and effectiveness by which the separation and concentration of EV subtypes from each other and/or non-EV components can be achieved is dependent on the methods employed and the properties of the original biofluid (**Section 7** reviews different biofluids). **Table 2** summarizes and builds on the points emphasized in MISEV2018 and the current guidelines. Sequential use of different separation techniques is often used to reduce 'contaminants' in the EV preparation: the experimental questions being addressed, or the ultimate application of the EVs, e.g., biodelivery, will dictate the standard required. The extent of EV separation and concentration should be assessed by the methods discussed in **Section 8**. A critical question in the EV field is whether specific proteins or other molecules, e.g., nucleic acids, sugars and lipids, which co-isolate with EVs, rather than being 'contaminants', are part of the dynamic EV 'corona' [200, 201] surrounding the vesicles and contribute to EV function.

Since MISEV2018, more work has been undertaken on the isolation and characterization of different types of EV, for example, density gradient fractionation, immunoaffinity and affinity capture [202, 203] have been used to characterize the protein, RNA and DNA content of specific EVs separated based on density or surface biomarker. Such studies have claimed to identify putative markers for different EV subtypes (e.g., Annexin A1 [172], SLC3A2 or BSG [204] for mEVs (microvesicles/ectosomes); Lamp1 [204] and Syntenin-1 for exosomes [205]). Different methods have also been employed to separate and characterize non-vesicular protein aggregates and other NVEPs, using density gradient fractionation for protein aggregates [172] and either asymmetric field flow fractionation [33] or ultracentrifugation at ultra-high-speeds for exomeres [34] or supermeres [36]. These additional studies have suggested that several proteins previously proposed to be sEV markers are equally, if not more, abundant in NVEPs.

For complex biological fluids, such as plasma or serum, contamination of enriched EV samples can be particularly problematic. Size-exclusion chromatography using commercially available columns may be a relatively easy way to separate EVs from soluble proteins, lipoproteins (although probably not LDLs) and nano-sized NVEPs etc., in order to determine whether EVs are the main carrier of the analyzed biomarker. Immunoaffinity isolation approaches may be most appropriate for strict EV subtype separation and subsequent biomarker analysis. Aptamers, short single-stranded DNA or RNA molecules that can bind a specific target, provide an alternative method for such EV separation from complex biofluids [206]. However, these methods entirely depend on the identification of appropriate surface biomarkers for immunocapture, and the subsets of EVs under study are, therefore, pre-selected. Sample size and scalability are also important considerations that may be possible to address using microfluidics [207] or array-based technologies[208]. In some cases, if the analyzed biomarker is abundant enough and/or

distributed equally between EV and non-EV circulating carriers, very crude separation approaches, leading to a mixture of EV- and non-EV-carriers, may be enough to identify these biomarkers.

8 Characterization of EVs

Measuring EV is non-trivial given their size, limited cargo, and the potential for measurement artifacts. If making claims about EVs, the extent to which an EVs will need to be quantified to justify the claims will depend on the source of the material, as different biofluids have different potentials for co-isolates e.g., plasma is an extremely heterogeneous sample that can differ between donors, whereas cell culture supernatants are less heterogeneous, and the reagents used within culture can be controlled thus having less potential for confounding co-isolates. Biofluids may also require additional reporting information due to influence of other preanalytical variables on EV number, see **Section 5**. In clinical studies, these may include metabolic parameters of the patients: time of the biopsies, fed or fasted state, BMI, and blood markers for metabolic diseases (e.g., VLDL, cholesterol) that can contaminate the EV preparation, along with donor health conditions such as obesity, diabetes, and liver diseases, which may modify the lipid composition of plasma and tissue EVs. For cell culture, the number of cells at the time of EV collection should be indicated. In the case of multiple collection points or bioreactor systems, the number of cells initially seeded, the expected doubling time, and the frequency of collection should be indicated. For tissue, the mass of the tissue used for EV isolation should be indicated.

EV composition between sources can be variable with respect to protein cargo, lipids, nucleic acids, and other biomolecules. While measurement of these individual components can be used as a method for inferring EV abundance, these values do not necessarily perfectly correlate with EV concentration or are maintained between source material and should not be solely relied upon as a measure of EV concentration. Currently, no generic marker or housekeeping protein exists that is capable identifying all EVs irrespective of source.

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 utilized EV characterization methods either not being EV specific or unable to detect all EVs, the reproducibility of EV data requires particular attention to transparent reporting of methods and results. A framework for reporting EV data has been previously developed and updated in the form of EV-TRACK [12, 13]. More recently, a concerted effort within the field has been driven on the standardization of EV characterization resulting in the ISEV workshops, the ISEV Rigor and Standardization Task Forces, and ISEV position papers [4, 15, 209].

MISEV continues to recommend that each EV preparation be 1) defined by quantitative measures of the source of EVs (e.g. number of secreting cells, volume of biofluid, mass of tissue); 2) characterized to the extent possible to determine abundance of EVs (total particle number and/or protein or lipid content); 3) tested for presence of components associated with EV subtypes or EVs generically, depending on the specificity one wishes to achieve; 4) tested for the presence of non-vesicular, co-isolated components; and in addition to 2018, 5) where EVs are being characterized with quantitative metrics provide a limit of detection.

8.1 Particle concentration

Despite the wide use of EV concentration (in particles mL^{-1}) as a reported metric for assay input standardization, assay output measurements, and translational purposes, among others, it is currently highly unreliable metric. Accurate measurement of EV concentration remains one of the most challenging metrics due to many techniques having a lack of sensitivity and specificity for EVs. Optical methods, such as flow cytometry, nanoparticle tracking analysis, and dynamic- and multi-angle light scattering are also dependent on other factors such as refractive index, and each have their own reporting considerations, **Section 9**. 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 characterized EV reference materials [210]. A key highlight of this work is the need for the field to adopt reporting their assay's limit of detection. The reporting of detection limit allows others to validate work irrespective of the sensitivity limit, and it is currently a

highly underutilized metric. This need is highlighted by the current literature, whose reporting of EV concentration in common biofluids such as plasma spans 6 orders of magnitude depending on the measurement method [211].

Greater confidence in EV concentration can be gained by comparing measurements using orthogonal methods and reporting their limits of detection. Orthogonal methods are those which do not have the same measurement limitations, such as an optical technique and a non-optical technique. For example, resistive pulse sensing (RPS) techniques that are calibrated with size-standards a limit of detection can be reported in diameter. The lower limit of detection for RPS will most likely be due to sensitivity limitations, while the upper limit of detection will be influenced by the pore size. For optical techniques such as single particle interferometric reflectance imaging sensing (SP-IRIS) and flow cytometry, the limit of detection may be reported in diameter from optical models or molecules of equivalent soluble fluorophore which result in concordant data across instruments and sensitivities [4, 212, 213]. Currently, there is no method to derive a traceable limit of detection for nanoparticle tracking analysis instrumentation or dynamic light scatter techniques, due to the number of variables involved in deriving particle diameter. Technique's that output concentration measurements without any phenotypic characterization, such as a membrane dye, can lead to over-estimation of the EVs concentration due to being unable to differentiate between EVs and other co-isolates. The efficacy of a membrane dye that universally stains all EVs irrespective of composition and derivation is yet to be demonstrated, which may further lead to underestimation of concentration if solely relied upon. Further instrument and assay specific recommendations can be found in **Section 9**.

8.2 Particle size

The measurement of EV size for all techniques requires assumptions to be made. An assumption made across many common high-throughput methods is that EVs are spherical. Techniques making this assumption include flow cytometry, nanoparticle tracking analysis, resistive pulse sensing, super-resolution microscopy, multi-angle light scattering, and dynamic light scattering. Currently, one of the most accurate methods to characterize EVs with varying morphologies is the use of high-resolution imaging methods, e.g., cryo-EM. The use of imaging methods to characterize the full diameter distribution of EVs is, however, limited by throughput and the relative abundance of different sized EVs. As imaging is a slow throughput method, many larger EVs that tend to be orders of magnitude less abundant may be ill quantified. The ability to quantify low contrast EVs below 100 nm may also be a limiting factor. Understanding the size distribution of EVs therefore will likely rely on collating multiple orthogonal measurements.

As more researchers begin utilizing a range of dedicated, small, single particle techniques with increased sensitivity, the diameter distribution of many EV derivations is beginning to show a trend in being a log-normal distribution, with the majority of EVs <100 nm [17-19, 91, 214, 215]. Due to many single particle analysis techniques being unable to resolve the full population of EVs, it is recommended that the EV diameter distribution of a population is shared and not summarized as a metric such as mean, mode, median, which can be easily skewed depending on the limit of detection and the asymmetric size distribution [210]. Researchers should be aware that the modal statistic from nanoparticle tracking analysis, for low refractive index particles in many cases, may in fact be a close approximation of the limit of detection for the instrument and not represent the true modal diameter of the EV population [214]. Techniques using software with proprietary algorithms to determine particle diameter may also result in variation between software versions or software platforms [17].The software used, and its version, should therefore be reported. Further instrument and assay specific recommendations can be found in **Section 9**.

8.3 Total protein quantification

EV sample protein quantification (in µg, µg mL^{-1}) can be approximated by colorimetric assays [Bradford or micro-bicinchonic acid (BCA)], fluorimetric assays, by global protein stain on SDS-PAGE, or absorbance readings, each with differing sensitivities and accuracies. The EV sample's protein concentration should be within the linear range of the reference curve; the reference curve used to derive protein concentration should preferably be shared, and the limit of detection of the protein quantification method used should be reported. Due to being a bulk analysis technique, protein quantification can result in overestimation due to co-isolated protein (e.g., albumin from culture medium or plasma/serum), especially when the less specific methods of EV separation are used or complex biofluids are used.

Colorimetric assays can also be influenced by amino acid composition of the proteins present e.g. BCA assays can be influenced by cysteine-cysteine, tyrosine and tryptophan residues, samples with a high proportion of these residues can result in overestimation of protein concentration [216]. Conversely, EV sample protein measurements can prove not sensitive enough if highly purified, low yield EV preparation methods are used. The measured protein concentration may also vary depending upon whether a method to disrupt the EV membrane have been used, exposing the entire protein content prior to performing the assay; the nature and concentration of any detergent used must be indicated. The use of protein concentration to estimate EV concentration should be used with caution as the enrichment of proteins per EV may occur with different cellular phenotypes or stimulations.

8.4 Quantification of total lipids

EV sample total lipid quantification can be achieved by measuring colorimetric assays [217], fluorescence of membrane intercalating dyes, total reflection Fourier-transform infrared spectroscopy (FTIS) , or chromatography [218]. However, the latter requires specialized equipment, and the former two types of assays may be insufficiently sensitive for small amount of EVs. In addition, whether these techniques equally detect all EVs independent of their specific lipid composition must still be established.

8.5 Quantification of total RNA

Quantification of total EV RNA can be obtained from global RNA assays by capillary electrophoresis instrument with consideration outlined in the 2017 ISEV position statement [8]. Due to extracellular RNAs (exRNAs) being derived from a variety of non-EV sources, combined with the current limitations in EV purifications methods, the use of total RNA quantification for assessing EV quantification or purity remains difficult to recommend. These co-isolates include ribonucleoproteins [219], exomeres [33], and lipoproteins [220]. The use of RNase to digest external RNA is therefore recommended in work claiming EVs to be the source of RNA cargo.

The most recent ISEV RNA position statement recommends 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 [8]. In samples containing very low total RNA quantity, highly sensitive RT-qPCR for certain transcripts may be used as a proxy for total RNA quantities. The use of pre-treatment DNAase is recommended for accurate RNA concentration due many techniques also being sensitive to DNA contamination.

8.6 Characterization of EV morphology

Many high-throughput methods characterize EV properties, such as diameter, indirectly as they are unable to assess morphology e.g., light scattering, fluorescence, or displacement. EV morphology is currently best assessed using high-resolution imaging techniques such as: scanning electron microscopy (SEM) [221] , transmission electron microscopy (TEM) [222], cryo-EM [223-225]; and scanning-probe microscopy (SPM) including atomic force microscopy (AFM) [226, 227]. These techniques are not necessarily interchangeable or capable of comparable image quality. For example, cryo-EM can clearly show the lipid-bilayer, preserve the EV size better 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). It is conceivable for the morphology of larger EVs to be assessed using conventional microscopy techniques where resolution is diffraction limited ($\gtrsim$200 nm). Imaging techniques also allow the assessment of EV purity as they can visualize co-isolated particles.

A current limitation of all imaging methods is the reduced throughput and potential to bias areas of image analysis [228]. Irrespective of imaging technique, all experimental details must be reported. These include the instrument brand, instrument and software version, the settings used for acquisition and, for analysis, the precise process for EM or fluorescence microscopy and how the imaged areas were selected, as well as controls and calibration information where relevant. Further details can be found in **Section 9**.

8.7 Characterization of EVs by their protein composition

The existence of multiple EV types is recognized by the field. Due to the heterogeneity between EV types and their sources of derivation, MISEV2022 continues not to propose specific molecular markers to differentiate between EV

subtypes across all cell derivations. MISEV2022 recommends the five-component framework introduced in MISEV2018 for reporting claims related to the protein content of EVs. It should be noted that these categories are of primary utility to bulk analysis methods, rather than single EV analysis methods which have their own controls (see **Section 9**).

Category 1: Transmembrane or GPI-anchored proteins localized at the external membrane of prokaryotic cells, and plasma membrane and/or endosomes of eukaryotic cells represent hallmarks of any type of EVs: their presence demonstrates the lipid-bilayer structure specific of EVs, whether they bud directly off the plasma membrane or after transit through the endosomal pathway. Like MISEV2018, MISEV2022 does not highlight tetraspanins (CD9, CD63, CD81 in particular) as the best or unique markers of EVs, since any full-length transmembrane or GPI-containing protein validates as well the lipid-bilayer-enclosed EV structure. However, for the latter markers, the detection technique must also demonstrate the intact nature of the analyte, since single-pass or GPI-anchored proteins can be cleaved from the membrane, resulting in a soluble form which could be present as a "contaminant" in an EV preparation. Multi-pass transmembrane proteins are more strongly associated to lipid bilayers and thus more reliable markers of intact EVs.

Category 2: Presence of cytosolic proteins (eukaryotic cells and Gram-positive bacteria) or periplasmic proteins (Gram-negative bacteria) demonstrates that the analyzed preparation displays the structure of lipid bilayers enclosing intracellular material, as expected for any EV. Proteins presumably actively incorporated into EVs are those with ability to bind to membranes or to cytosolic sequences of transmembrane proteins. Others, like cytosolic enzymes or cytoskeletal proteins are more promiscuous EV components.

Category 3: Some proteins are major constituents of non-EV structures often co-isolate with EVs. Evaluating the presence of such proteins helps to assess the degree of purity of the EV preparation. Identifying non-EV constituents as controls will depend on the source of EVs and isolation methods used.

In biofluids like blood plasma, EVs may co-isolate with lipoproteins [229] and non-integral proteins, such as albumin or soluble acetylcholinesterase [230], amongst other. For plasma/serum and EVs from cells cultured in the presence of animal serum, or liver cells that secrete lipoproteins, we propose apolipoproteins A1/2 and B (***APOA1/2, APOB***), and albumin (***ALB***) as the current best negative markers to exclude their presence [231]. However, it cannot be ruled out that a fraction of such markers may be specifically associated with some EVs [232]. There is some evidence to suggest a protein corona may exist around EVs making it difficult to identify unbound vs. bound protein co-isolates when using bulk assays[200]. In urine, Tamm-Horsfall protein (uromodulin/***UMOD***) forms aggregates that co-precipitate with EVs unless the fluid is chemically treated [233]. Overall, however, since we cannot propose a threshold of abundance of these proteins in EV preparations below which acceptable purity is reached, we stress that it may be more appropriate to measure and report the efficacy of depletion rather than to expect a binary presence/absence of proposed negative markers.

Category 4: These proteins should be evaluated if authors want to claim specificity of their study to the small EV subtype(s): Proteins localized in/on intracellular compartments of eukaryotic secreting cells other than the plasma membrane and endosomes (i.e. components of the nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, autophagosomes, peroxisomes) are found in some types of EVs, but a priori not enriched in the smaller EVs (approximately < 200 nm diameter) of plasma membrane or endosomal origin.

Category 5: Covers secreted or luminal proteins that can associate with EVs by binding to specific (e.g. growth factor receptors) or to promiscuous (e.g. proteoglycan, lipid) receptors on the EV surface: their identification in EV preparations should be accompanied by exploration of the cognate EV-associated receptor(s).

MISEV2022 continues to recommend that three categories of markers be analyzed for all bulk EV preparations in order to demonstrate the existence of EVs (Categories 1 & 2) and the purity of EVs from common contaminates (Category 3), Table 3. There continue to be no recommended universal 'negative controls' to specific EV subtypes.

A variety of methods exist to do determine the presence of proteins markers. The sensitivity and reliability of these methods can vary. The current assay and instrument specific reporting considerations are outlined in **Section 9**.

8.8 Identifying EVs based on non-protein component markers

While protein markers for EVs phenotyping is common, non-protein markers also have utility in identifying EVs. The analysis of non-protein markers is typically done directly with techniques such as lipid mass spectrometry or Raman spectroscopy (**Section 9.8**), 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 9.3**.

The utility of detecting non-protein targets, such as phosphatidylserine, intracellular esterases, nucleic acids, or membrane intercalation is dependent upon the source and composition of EVs. The combination of protein and non-protein markers when colocalized can provide greater evidence of EV specificity in labelling for single particle measurements, when compared to staining of one component alone e.g. a membrane intercalating dye and tetraspanin label positive event.

Due to most non-protein component markers not being EV-specific, caution should be taken when relying upon only a single non-protein marker. Membrane dyes when used with source materials that do not contain lipid structures, such as lipoproteins, have utility in separating vesicular from non-vesicular particles. When used with complex biofluids such as plasma/serum their ability to separate EVs may be limited due to also binding to lipoproteins also. Dyes that are activated or accumulated using intraluminal enzymes such as esterases may not be present in all EV derivations and may represent only a subset of EVs from heterogeneous sample sources. Nucleic acid dyes have been used in EVs, however more research is required for recommendations on controls and identifying the specificity of the dyes for intraluminal vs. extracellular DNA/RNA [234].

8.9 Topology of EV-associated components

The topology of EV-associated cargo, such as proteins, nucleic acids, glycans, etc. is important to understand when characterizing EVs along with ruling out the existence of potential of co-isolates or remnants of cell debris when performing bulk analysis methods, such as Western Blots. Studies have reported the sensitivity of proteins [235], RNA [236], and DNA [237] on the surface of EVs to digestion methods. Currently, it is unclear whether this observation in unexpected topology is due to debris from active secreted components, dead or dying cells sticking to the surface of EVs, a result of isolation method such as ultracentrifugation, or is an outcome of an as-yet unknown transport mechanism across membranes.

The location of EV cargo is not only important in characterizing EV structure but also in understanding function as luminally active components of EVs require membrane fusions or membrane transport events to occur to achieve a recipient cell response. Exposed components may, by contrast, affect recipient cells without EV-cell fusion events occurring. The topology of putative active components should therefore be determined by performing mild digestions, permeabilizations, or antibody studies by adopting or adapting previously published methods [8, 235, 238-243].

An example of topology determination using a bulk analysis method such as SDS-PAGE would compare an untreated sample to a) sample with a degrading enzyme such as a protease, b) sample with lysis detergent that doesn't affect downstream analysis c) sample treated with both degrading enzyme and lysis detergent. The cargo of interest for each sample is then analyzed upon neutralization of enzyme activity. The loss of signal from enzyme-treated, detergent-untreated samples indicates that cargo is exposed on the surface of EVs. The loss of signal only upon detergent + enzyme treatment indicates intraluminal cargo. However, a positive control of efficient enzymatic digestion in the absence of detergent should be performed by analyzing, in parallel with the cargo of interest, a known surface-exposed molecule (non-exhaustive examples include: MFGE8, which binds to phosphatidylserine on the surface of EVs, or a large transmembrane protein, such as an integrin). Alternatively, single particle analysis methods can use antibodies targeted towards external or cytoplasmic epitopes on EV membranes.

It is recommended that nucleases are used in combination with proteinase to determine the topology of nucleic acids.

8.10 Bulk and Single EV characterization

The use of bulk analysis methods to quantify EV sample composition are useful. This is due to bulk analysis methods typically being more accessible and many methods having sufficient sensitivity to characterize the presence of protein or non-protein contents in a bulk sample. However, in order to demonstrate the subtypes or heterogeneity of particles present within the sample from a bulk preparations, quantification at an individual EV level or selective enrichment must be done.

Single EV analysis methods using high-resolution visualization techniques can allow characterization of EV structure and composition. However, in some cases it may be difficult to utilize in a high-throughput or quantitative manner to obtain statistical power. Single EV analysis techniques without imaging capabilities, such as flow cytometry, RPS, or NTA may provide higher throughput but in some cases slightly lower sensitivities than hist-sensitivity imaging methods. The information provided can, however, still contain relevant information regarding diameter distribution, protein or non-protein marker abundance above the limit of detection.

Where possible it is recommended that the use of non-visualization techniques use orthogonal analyses to provide reassurance of the characterization data where artefacts or biases may occur. For example, the determination of diameter distribution from NTA or flow cytometry typically relies on enough light being scattered or fluoresced from a particle for it to be tracked/detected, whereas methods such as resistive pulse sensing are not dependent on optical properties such as refractive index or fluorescence, but it is currently unable to differentiate EVs from non-EV particles. When setting up orthogonal analyses, the detection ranges of the used techniques should be considered. For example, phenotyping of EVs using electron microscopy vs. conventional flow cytometry will likely be visualizing different portions of an EV population due to statistical abundance of smaller EVs (readily detected by electron microscopy but not conventional flow cytometry) as compared to larger EVs (which are detected predominantly with conventional flow cytometry but are relatively rare in a field of view using electron microscopy)[244].

9 Technique specific reporting considerations

While some controls e.g., positive and negative controls, are often applicable across assays and instrumentation to provide reassurance that the target of interest is in fact being detected, many controls and reporting considerations can be subjective depending upon the assay or instrumentation used. 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. Here a collated list of minimal assay and instrument specific reporting considerations are detailed. These are generally applicable irrespective of experiment design. The techniques listed in the follow 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.

9.1 Atomic Force Microscopy

Atomic Force Microscopy (AFM) provides access to the label- and stain-free imaging of individual EVs and co-isolated nanoparticles [245, 246]. 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, including saline buffer and cell culture media. AFM morphometry can be used to obtain EV size distribution, EV ultrastructural details and to check for the presence and relative amounts of contaminants [247-250]. In addition, AFM is one of the very few techniques capable of measuring single vesicle nanomechanical properties [251, 252], which were found to correlate with EV identity, circulation and function [253-259]. The unique mechanical fingerprint of EVs can be also leveraged to discriminate them from non-EV nano-objects of similar size and shape [260].

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 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. In addition, EV mechanical studies should describe the assumed contact mechanic model [261-263], and, ideally, provide enough data for the reader to be able to test alternative models.

9.2 Bead-based flow cytometry

Bead-based flow cytometry has been used widely by the field primarily to interrogate the presence of surface proteins on EVs. The technique is based on using large beads activated to capture any protein (e.g. surfactant-free aldehyde/sulfate beads), hence which capture particles regardless of their surface composition [222], or beads that have been conjugated to antibodies that capture particle exposing the corresponding antigen. More recently, commercially available EV multiplex kits have become available allowing the interrogation of 30+ unique capture antibodies [264, 265]. Once captured in each of these scenarios, the particles are then labeled with a fluorescently conjugated antibody for detection.

When reporting bead-based approaches, controls should be implemented which include isotypes as detection antibodies (for pan-EV capture beads), or isotype-conjugated capture beads, and capture beads with detection antibody alone (for antibody-coated capture beads). The reporting of stained beads as a percentage is not a valid statistic, reporting normalized bead median fluorescence intensities is recommended [266]. The reporting and sharing of data and median fluorescent intensity statistics in molecules of equivalent soluble fluorophore (as with single EV flow cytometry) is recommended to allow standardization of data across instrument platforms and settings. If making beads, reagents, and conjugation should be reported, while commercial reagent catalogue and lot numbers should be reported. Other reporting parameters include: the sample incubation time with beads, post-bead incubation wash methodology, detection antibody staining time, and post-staining wash methodology.

9.3 Considerations for EV labelling & tagging

Two broad approaches for EV detection are possible. EVs can be labelled with reagents, such as antibodies or dyes, or alternatively, fluorescent, or bioluminescent fusions of specific EV proteins can be expressed in EV secreting cells.

9.3.1 Protein- and non-protein labelling

While the most available and commonly used EV labelled reagents are those that are fluorescently active, in principle any conjugated reagent, such as radioactively labelled, should share similar controls. Due to EVs small size and limited cargo the detection of protein and non-protein markers is difficult and can easily be confounded by unbound reagents from the labelling process or co-isolates from the isolation method used. 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, the presence of unbound dye will deprecate the sensitivity of EV detection, and more easily lead to false positive events e.g., super-resolution microscopy, SP-IRIS, single EV flow cytometry.

The utilization of protein and non-protein labels may in some cases pose challenges of specificity and unbound reagent removal creating artefacts. Where possible it is recommended to use a buffer with reagent control in any assay to rule out detection artefacts arising from unbound label being present and resulting in false positive events. In cases where antibodies are used, manufacturer-matched isotype controls at the same concentration as the stained controls are recommended to demonstrate specificity.

One labelling strategy involves using lipid dyes that bind to the membrane component of EVs [223, 267-270]. Lipid-specificity does not guarantee EV specificity as particles, such as lipoproteins, may be co-isolated and stained from certain biofluids. The localization of lipid-labelling strategies with a protein marker is therefore recommended where possible. Other scenarios to consider and control for are the potential for lipid-labels having the ability to aggregate [269, 271], or vary in their lipid affinity between EVs sources with differing membrane composition. Thus, a control condition with dye but no EVs can provide information on the prevalence of dye aggregates [271, 272].

Labels that bind to reactive groups of proteins can be used to label the EV surface [273-275]. However, these dyes will also label free protein contaminants present in an EV preparation and so it is important to report the exact

method of EV isolation used. When protein artefacts are a possibility a low concentration detergent is recommended to demonstrate the lability of membrane and reduction of signal.

In assays where purification is required after staining, the use of procedural controls should be used where possible to demonstrate that the EV population before and after the purification is consistent, that the purification procedure did not introduce artefacts into the downstream sample analysis, and that the removal of dye has in fact occurred. For example, if a post-staining isolation is performed to remove unbound reagent from stained EVs, the following controls would be recommended.

1. Analyze a buffer with reagent control before and after label depletion method (e.g. size-exclusion chromatograph) to demonstrate that the reagents removal can be achieved.

2. Analyze unstained EVs before and after the label depletion method to demonstrate that it does not change or selectively enrich the EV population.

3. Analyze the reagent-stained sample after purification where unbound/free reagent has been removed. The use of purification methods before and after staining EVs may alter their results, particularly for small EVs where staining may noticeably increase their diameter or density.

9.3.2 Protein-tagging

Labelling EV specific proteins can be done by constructing fusion proteins connecting the protein of interest to a fluorescent protein, such as GFP [276-279]. However, this is only feasible for EV-donor cells/tissues that can be genetically modified. A key issue is the selection of the tagged protein and its suitability as an EV or EV subtype marker (**Section 8.7)**.

It should be kept in mind that alterations in expression of a tagged protein may also affect EV biogenesis pathways [29]. There is considerable scope for labelling a range of EV markers, particularly those with membrane association [279, 280], both to explore possibilities for enhancing the labelling of single EVs and to distinguish different EV subtypes, for which secretory and uptake pathways can then be analyzed [204, 281]. Importantly, fluorescence of GFP-based protein fusions is quenched by low pH. When these molecules reside within more acidic intracellular compartments, such as late endosomes and lysosomes, they can only be tracked using antibody staining [2].

Potential controls include over-expression or knockdown/knockout of the target protein or blocking/pre-adsorption of the antibody with the immunogen. In either case, fluorescently tagged or labelled EV approaches may affect EV secretion, loading or function, therefore, where possible, the content or functionality of labelled EVs should be compared to unlabeled EVs to assess the consistency of approaches. The map of the plasmids use for chimeric protein over-expression should be provided and possibly deposited in Addgene or other repositories. Correct localization of the chimeric protein should also be tested by immunofluorescence.

9.4 Diffraction-limited fluorescence microscopy

Applications of fluorescence microscopy techniques can range from live cell imaging to single molecule localization. EVs 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 [240, 267, 276-278, 282, 283], as well as the composition of single EVs [279, 284]. 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 is the most suitable system for analyzing EV content [284] (See also **Section 9.13**). Confocal and light sheet microscopes, especially the most recent models, are capable of single molecule detection for calibration [285] and dynamic studies, but are more suitable for live cell imaging experiments [276, 283]. These methods and potential drawbacks have been extensively reviewed [286-289].

In microscopy experiments it is essential to report the type of microscope, magnification, laser power and exposure time because fluorescently labelled samples have a limited number of labelled molecules. Therefore, each labelled sample can provide only a finite number of photons before photobleaching. Therefore, each experiment must be optimized to use this limited “photon budget” to obtain the maximum amount of information [290]. Consequently, the sample is exposed for short time using minimal excitation to perform live cell experiments [291, 292] or higher

excitation power and longer camera exposure to favor single molecule detection [283]. While calibration of the system is mandatory for quantitative microscopy experiments [285, 293], 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 [294, 295], 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. If the code is developed by the authors, it should be deposited and made accessible to the community. Available algorithms [283, 296, 297] 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.

9.5 Dynamic Light Scattering (DLS)

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 [298-301]. The hydrodynamic diameter is defined as the diameter of a solid sphere that would exhibit the same diffusion coefficient as the measured particles 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, is based on the polynomial expansion of the natural logarithm of the autocorrelation function and yields the average hydrodynamic diameter and the polydispersity index (PI), which is a dimensionless measure of the broadness of the size distribution. However, 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[302], but for polydisperse size distributions of EV samples [17], 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. 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 [303]. In either case, please follow the recommendations on nomenclature and reporting of DLS measurements from the international standard ISO 22412:2017 [304].

9.6 Electron microscopy (EM)

Electron microscopy and its variations are one of the few techniques capable of analyzing the majority of EVs. There exist a number of examples of EV analysis by SEM [221, 224], TEM [17], and cryo-EM [225, 226, 305]. While each of these methods are 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 e.g. contrasting and embedding in a mixture of uranyl compounds and methylcellulose to maintain the bilayer morphology, 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 bilayer morphology [222]. SEM shows the surface aspect of EVs of any size, but the images at the highest magnification required to visualize the smallest EVs may be more difficult to analyze.

There have been limited standardization studies across EM methods to determine minimal reporting requirements. From the research that has been conducted within TEM it is recommended that three major criteria be reported: fixation, adsorption and negative staining methods [228]. Fixation includes: the fixative used, its concentration and incubation time be reported. 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. Reported images should include both low and high magnification and state how reported image locations were selected.

9.7 Nanoparticle tracking analysis (NTA)

NTA, known as single particle tracking in other fields, is a widely utilized optical technique in the EV field primarily to report particle size and concentration. The use of NTA to determine effective refractive index and epitope existence has also been demonstrated [306, 307]. NTA derives diameter by measuring a particle's diffusion coefficient and 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 [17, 308]. Currently, there is no method of determining or reporting a set limit of detection for NTA. Several standardization studies have been conducted comparing results between users and instruments [214, 309, 310]. The use of NTA to measure the diameter distributions and concentration of complex biofluids should be interpreted with caution due to co-isolates such as lipoproteins, large protein complexes, also being counted and EVs larger than a few hundred nanometers are difficult to quantify. Detection of particles with NTA can be done using light scattering, which is reliant on a particle's refractive index and diameter, or fluorescence. The use of fluorescence NTA is dependent on unbound labelling being removed, using a dye that is resistant to photobleaching, and having enough dye per particle to be detectable.

When reporting NTA data is recommended that the instrument model, camera type, camera settings, laser wavelength, laser power, software version, analysis settings, and particles per frame be reported. As outlined in **Section 8.2**, it is preferable to report NTA diameter distributions rather than a single diameter statistic for EV data, due to NTA statistics being easily skewed by the limit of detection. If known, the algorithm used to produce diameter distributions should be reported due to potential for differing results depending on the algorithm used [308, 311]. When using fluorescent NTA, it is recommended to report the number of total particles in light scatter mode along with the number of labelled particles in fluorescence mode.

9.8 Raman Spectroscopy

Raman spectroscopy (RS) is a label-free analytical optical technique capable of qualitatively and quantitatively resolving the chemical composition of a sample, based on inelastically scattered photons impinging from the sample upon irradiation with a narrow-linewidth laser [312]. Therefore, a Raman spectrum is essentially a chemical fingerprint of a sample. 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 [312]. 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 [313, 314]. This method utilizes 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 [315-324].

Inter- and intra-device variability in Raman spectra can arise for several reasons, including laser variations and non-uniform response of each the optical elements, including the detector, to different light energies (known as spectral response). Raman systems should therefore be carefully calibrated [325]. Modern commercial Raman systems have automatic routines to perform such calibrations, 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, e.g., 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 that all the pertinent parameters associated with the sample preparation are denoted. As EV samples are typically suspended in aqueous solutions with varying concentrations of different dissolved compounds, and thus osmotic pressures, there is a need to consider and report the EV sample

preparation steps. For instance, whether the EVs were measured in aqueous state using SERS nanoprobes, or dried on a quartz glass slide followed by RS spectra acquisition [326]. The correlation and conceivable differences of wet vs dried RS measurements of biofluids is still being investigated to elucidate if there is a preferable state [327]. Currently, both approaches are considered feasible provided the EV sample preparation steps are detailed, and that the sample preparation or the measurement parameters have not altered the sample in any way.

Along with instrument and sample considerations, the chosen 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 of other researchers. After the acquisition (and before the downstream analyses) the spectra should be postprocessed using strictly concise data manipulation parameters. For example, if baseline correction and/or background subtraction is implemented, all the related parameters need to 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 ideally openly available.

9.9 Real-Time qPCR RNA Analysis

Due to its ability to detect and measure small amounts of nucleic acids RT-qPCR is a widely utilized technique across research areas, including the EV field. As it's use and technical understanding has increased, areas of caution and the importance of reporting transparency have been highlighted, culminating in the development of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [11]. The MIQE guidelines are written broadly for all RT-qPCR experiments and includes a checklist intended to accompany the submission of manuscripts.

When analyzing EV-associated RNA further considerations are required to ensure that the measurement is not an artefact or co-isolate. The standardization of EV-RNA analysis and bioinformatics are topics that are continuing to develop and addressed in ISEV position papers [6, 8]. An EV-specific EV-RNA experiment considerations and reporting checklist were developed in the 2017 ISEV position paper [8]. MISEV2022 recommends utilizing both the MIQE checklist and ISEV EV-RNA checklist for the reporting EV-RNA experiments utilizing RT-qPCR, with the ISEV EV-RNA checklist also being applicable to reporting other RNA analysis techniques.

9.10 Resistive pulse sensing (RPS)

RPS is a non-optical technique utilizing the Coulter principle to determine the concentration and diameter of particles [328], 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 and the capability measure particles up to several microns. The use of RPS to measure the diameter distributions and concentration of complex biofluids should be interpreted with caution due to co-isolates such as lipoproteins, large protein complexes, also being counted and being undifferentiable from EVs. RPS measurements do however have very high concordance with TEM data [17].

When reporting RPS data it is recommended that instrument model, pore size, calibration bead diameter and source, software version be reported. For stretchable pores, the applied voltage, applied stretch, and procedure to optimize settings should be shared[329]. For microfluidic RPS, appropriate dilution buffer to lower the surface tension of water should be considered and reported [330]. As outlined in **Section 8.2**, 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 limit of detection. 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 [242].

9.11 Single-EV flow cytometry

The detection of single EVs using flow cytometry has been utilized for several decades and resulted in the development of many considerations. This optical technique can be utilized to detect EVs down to ~40 nm in specialized cases [18], and ~100 nm using many modern conventional cytometers by light scatter and fluorescence [223, 270, 331, 332]. Through calibration of data, flow cytometry has been demonstrated to be capable of

characterizing particle diameter [18, 223, 333, 334], epitope abundance [213, 335], epitope density [213], effective refractive index [333], and number concentration within standardized size ranges[212].

Recently, a comprehensive experiment and reporting framework was developed (MIFlowCyt-EV) and published as a position paper as part of 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 [4, 244, 336]. 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 EV Flow Cytometry Working Group website (www.evflowcytometry.org). It is recommended the MIFlowCyt-EV framework be completed and attached as supplementary material for manuscripts using single EV flow cytometry.

9.12 Single Particle – Interferometric Reflectance Imaging Sensing (SP-IRIS)

Combined interferometric imaging/fluorescence imaging is a recently introduced technique in the EV field to assess protein expression of single EVs [167, 214, 337, 338]. This technique captures particles by biorecognition agents (e.g., antibodies, peptides) on to a multiplexed array of micron-sized spots. In interference reflectance imaging sensor (IRIS) mode, interference patterns from scattered light are used to detect, the size and count of captured particles. The lower limit of size detection with current platforms is estimated to be 50 nm at a refractive index of 1.41, though the theoretical detection limit is much lower [339]. A key aspect with regards to converting interference to nominal size is the dependence on refractive index, which varies within EVs [340]. Current SP-IRIS platforms assume a constant refractive index which may result in variation across orthogonal measurements. It is recommended that software version and estimated refractive index parameter should be reported.

In fluorescence mode, captured particles can be detected with fluorescent probes in three color channels. Some aspects of this mode require careful consideration of calibrations and control experiments to obtain rigorous results. It is recommended that, for particles smaller than the diffraction limit, e.g., <~250 nm in diameter for visible light, that the detected events be validated to confirm single particles were in fact detected. This can be achieved by a dilution series to ensure that fluorescent intensity per particle scales with solution concentration. To confirm that fluorescence is associated specifically with EVs, surfactant treatment to disrupt vesicles can be utilized. For fluorophore-antibody detection, antibody clones, conjugated fluorophore type, incubation concentration, light-source wavelength, bandpass filter cut-offs, analysis software version, and fluorescence cut-offs/method of choosing these cut-offs should be included to increase repeatability and comparability of studies. Utilizing negative controls such as a mouse IgG capture spot or chips incubated with EV depleted fluids is recommended for choosing these cut-offs.

9.13 Super-resolution microscopy

To break the diffraction barrier, fluorescent super-resolution microscopy methods modulate the light to ensure that neighboring molecules do not emit simultaneously. A resolution 10-fold higher than the diffraction limit can be achieved using two main approaches: 1) stimulated emission depletion (STED) [341, 342] spatially regulates activation of an ensemble of fluorophores using synchronized two laser system with a phase plate; 2) single molecule localization microscopy (SMLM) techniques, such as (d)STORM [343, 344] and (f)PALM [345, 346], temporally regulate stochastic activation of single fluorophores. The nanometer scale resolution of STED and SMLM is well suited for detecting and characterizing individual EVs. For example, in isolated EVs, these techniques have been used to image EV membranes [347-349], proteins [348, 350-356], DNA fragments [356], and miRNAs [357, 358]. Using quantitative analysis, these methods have been further used to define EV size [347-350, 352]; and to quantify the detected molecular content of proteins [352], number of localizations of miRNA [358], and number of localizations of DNA fragments in EVs [356]. Additionally, STED and SMLM have been used to image EVs in cellular environments. Specifically, these methods assessed the uptake of EVs in cells [351, 357, 359-361] and detected EVs [362] or EV clusters [363] secreted by cells.

To rigorously characterize EVs, super-resolution microscopy methods comprise tailored approaches for sample preparation, sample imaging, and data analysis. *Sample preparation:* 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. Given the diversity of the analytical preparation of EVs, the reporting should include details about the protocol for labelling EVs (e.g., EV purification method, appropriate reagent controls and/or references, reagent concentration, incubation times/buffers, 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 onto coverslips, fixation protocol, and controls for affinity isolation (e.g., isotype or non-fouling surface). *Sample imaging:* For both commercial and home-built systems, reporting should describe the major microscope components: laser lines, camera, filters, objectives, and other relevant optical path components. Descriptions of protocols should include laser powers, relevant microscope configuration, imaging conditions (including buffer for SMLM), and imaging parameters. Reports on multicolor imaging should detail the alignment between channels and any applied correction for chromatic aberration [364, 365].

In STED, the resulting images consist of intensity maps, and analysis typically relies on approaches established in confocal microscopy [366]; 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 [367]. To quantify detected molecular densities and molecular organization with SMLM, it is important to define the photophysical priorities of fluorescent reporters (e.g., average number of localizations per molecule, maximal dark time) [367]. 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.

9.14 Western Blotting

Western blotting is a commonly used method for identifying the presence of proteins in EV containing preparations. It can be performed by loading side-by-side EV samples either in specified protein amount, or in specified EV derivation source (biofluid volume, or cultured cell number): the former allows detection of differences in EV cargos distribution, the latter allows detection of differences in total amount of EV cargos. For cell culture EVs, source material lysates either in specified protein amount or in cell-equivalent amounts must be loaded on the same gel, to determine if the analyzed proteins are enriched in EVs as compared with their producing cells. This comparison, however, can be easily performed only for analysis of EVs from cell culture conditioned medium; it is more difficult for biofluids where there is a heterogeneous source of EV derivation.

Where possible appropriate antibody positive and negative control samples should be included beside the experimental samples. Controls for assessing the purity of sample prep should also be included if claiming the protein is present on or in the EVs, see **Section 8.7**. The antibody information (specificity, clone, source), sample denaturing conditions, membrane type, buffers, and imaging equipment and parameters should all be reported.

10 Evaluation of EV release and uptake

10.1 EV release

EV release can be visualized by using a range of methods, including those employing fluorescent tags and dyes (**Section 9.3**), which permit real-time imaging, reviewed in [368]. In MISEV2018, approaches used to analyze the functions of specific EVs involving blocking or stimulating EV secretion with a range of genetic manipulations and drugs were discussed, e.g. *Rab27a* knockdown and neutral sphingomyelinase inhibition [369]. While these treatments are often used to inhibit exosome secretion and determine exosome function, they almost invariably also impact the secretion of other EVs and/or cellular processes. MISEV2018 highlighted the importance of identifying biogenesis machinery that is confined to specific EV subtypes and this remains a priority, with very few additional regulators

identified in the last four years. An alternative approach is to use at least two different methods affecting independent processes to block the production of specific EVs to confirm their function. The resulting EVs and control preparations should be analyzed using the physical and molecular methods described in **Section 8**, i.e., with particular attention given to normalization methods (eg based on the protein mass of secreting cells, EV number, EV protein mass), 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.

10.2 EV uptake and signaling

MISEV2018 briefly discussed EV uptake, highlighting some of the caveats to this analysis. For example, the long-lived nature of EV dyes and other labelling substances, which can be incorporated into cellular membranes, may not reflect the presence of EVs. In addition, as discussed above (**Section 9.3**), labelling with lipophilic or other surface-coating fluorophores may modify EVs, thereby affecting biogenesis and/or uptake.

Bearing in mind these limitations, recent work using cell and subcellular fractionation approaches suggests that EV uptake occurs at a low rate, but about 30% of proteins present in these EVs are transferred into the cytosol, via a mechanism involving acidification and membrane fusion [370]. By labelling specific EV subtypes or blocking their biogenesis in secreting cells, it may be possible to use these methodologies to determine whether uptake mechanisms vary between different subtypes.

It is also important to consider to what extent uptake relates to function. Major sites of EV accumulation in target cells are typically late endosomes and lysosomes [370]. However, particularly in examples where EV surface ligands play important roles in signaling [34], these compartments would not be expected to be involved in these signaling events, which might take place at the cell surface (and not require EV uptake) or in early endosomes. Furthermore, EVs may fuse to the plasma membrane and donate their contents to the cell. In some cases, inhibition of EV uptake by drugs in clinical use has been reported to disrupt EV-induced pre-metastatic niche formation [371]. Going forward, selective blockade of specific intracellular trafficking pathways may be required to identify which pathways are critical for EV function and whether these are dependent on EV subtype and target cell type.

11 Functional studies

MISEV2018 recommended a series of considerations in relation to functional studies of EVs, which continue to hold in MISEV2022. Firstly, dose-response studies (with some consideration of likely physiological range), and if possible, comparative analysis of the sample before and after EV subtype removal should be included. Secondly, negative controls should be used, for example, for functional analysis in cell culture, media not conditioned by cells and for patient disease-associated functions, biofluids from healthy, matched, or untreated donors. Functional analysis of EVs from biofluid samples, however, remains challenging, because of the increased level of contamination in EV preparations. Thirdly, further controls should be employed to rigorously determine whether a specific function is attributed to EVs versus soluble or non-EV co-isolating macromolecular components.

The more detailed justification and experimental suggestions for functional analysis from MISEV2018, together with further updates are summarized in **Table 4**. The latter include recommendations concerning EV normalization and EV enrichment protocols, as well as consideration of co-isolated components and the physiological relevance of the assay methods employed. Overall, these recommendations are made to reduce over-interpretation of functional studies, where it is frequently difficult to confirm that the activities identified are EV-specific or even EV-dependent, although there are exceptions, e.g. tissue factor [372]. In cases where it is not possible to perform these confirmatory studies, MISEV strongly encourages the authors to report why this is not possible, e.g., due to lack of available material, and propose a conservative conclusion, such as “EV-containing or EV-enriched material displays the relevant activity”, rather than claiming that the activity is EV-specific or -dependent. In cases where it is not possible to perform these confirmatory studies, e.g., lack of available material, MISEV2022 strongly encourages authors to propose a conservative conclusion, such as “EV-containing or EV-enriched material display the relevant activity”, rather than claiming that the activity is EV-specific or -dependent.

There is increasing interest in employing EVs as tools for bio-delivery (**Section 12**). Improved understanding of EV uptake mechanisms and their relevance to function (**Section 10.2**), as well as careful characterization of EV subtype functions is likely to be a key requirement in optimizing these approaches over the coming years.

12 EV analysis using model organisms

9.1 Benefits and challenges

MISEV2014 and MISEV2018 primarily focused on the analysis of EVs separated from cells cultured *in vitro* or concentrated from biofluids, permitting the bulk separation and characterization of EVs. EVs from human plasma and serum are studied extensively and represent the secretory products of cells under physiological or disease conditions. These EVs are, however, typically derived from a complex mixture of cell types, e.g., blood, endothelial and multiple other cells from different tissues, hence complicating the analysis.

In MISEV2022, complementary analysis using model organisms, which can, for example, focus in on EVs released from a specific cell type, is discussed. These *in vivo* approaches can provide fundamental insights into EV biology and function in physiological settings, mirroring those in humans. These studies, which may involve a range of different cell types, can reveal processes and sub-populations of EVs that have not previously been recognized. Imaging of EVs in living animals and tissues also allows dynamic processes to be visualized, an area that has recently been comprehensively reviewed in [368]. This review highlights the maturation of imaging techniques to a stage where the different stages of the lifespan of an EV in its physiological environment can be studied. This has been facilitated by the development of diverse tags and reporter systems, see also **Section 12.2**. These *in vivo* studies, therefore, can provide mechanistic insights into EV secretion, biodistribution, persistence and function [373], to which *in vitro* work may add mechanistic detail. Due to limitations on both the amounts EVs produced and current methodologies to separate and concentrate them, it is typically not possible to isolate EVs from model organisms. Methods have, however, been developed to purify and perform proteomic analysis of EVs released from the nematode worms, *Caenorhabditis elegans* (e.g., [374, 375]). Although there is still debate about the details of the EV isolation procedure, e.g., differential centrifugation speed [376], this could prove a powerful route to investigate the impact of genetic changes and environmental challenges on the EV secretome at a whole organism level.

The forms of analysis, which can be undertaken in model organisms, but not *in vitro*, are particular strengths, with specific *in vivo* models best suited to probing different aspects of EV biology (**Table 6**). Simpler organisms are often more genetically tractable and therefore often better suited to addressing more fundamental questions concerning EV biology. While models sharing more similarity with humans, such as mouse and zebrafish, are more likely to reveal mechanisms directly relevant to human health and disease.

Some models and cell types studied *in vivo* may have features that make them particularly relevant for the study of specific aspects of EV biology. For example, the transparent zebrafish embryo is ideal for real-time biodistribution and uptake studies [377]. Cells of the yolk syncytial layer in zebrafish secrete very high numbers of EVs, which can be tracked throughout the circulation and distant tissues. Secondary cells in the fruit fly, have enlarged endosomal compartments, allowing the visualization of ILV biogenesis in secreting cells (Corrigan et al., 2014; Fan et al.., 2020). These examples highlight an important point that is relevant to *in vitro*, as well as *in vivo*, studies: EV secretion from or delivery to any cell may reflect specializations of that cell type, and so mechanistic findings ultimately need to be confirmed in other cell types.

Several of the challenges in the analysis of EV biology *in vitro* are also encountered in model organisms, as briefly considered below. Studies in simpler organisms, however, provide some of the best opportunities to screen for novel regulators of EV biology using genetic approaches and to test their specificity over an extended time course. The MISEV2022 guidelines for EV analysis in model organisms have been kept broad, rather than too prescriptive to provide a platform to enable innovative new approaches in a diverse range of models to thrive and to help the field to move forward. It is, however, important to increase rigor and reproducibility of EV analysis using model organisms

by careful reporting, for instance, describing the cell types under investigation, e.g. secreting and/or recipient cells as clearly as possible.

12.1 Cellular release of different EV subtypes

Typically, EVs are labelled *in vivo* using proteins with fluorescent tags, most commonly CD63-GFP. The caveats associated with this approach are discussed in **Section 9.3.2** and the literature [368]. They include the potential disruption of cellular or EV biology through fusion protein overexpression, which may be required to provide sufficient signal, and the potential labelling of only specific EV subtypes in imaging experiments. Identification of gene traps in which EV markers are labelled through genetic manipulation at their endogenous locus or the use of multiple EV markers provides possible solutions to this problem.

The non-specific effects of genetic manipulations that inhibit EV or EV subtype secretion (**Section 10.1**) also limits the interpretation of experiments designed to identify secretory pathways and EV functionality, although the ability to readily undertake multiple genetic manipulations allows hypotheses to be tested in several different ways [29, 378-380], and may even permit identification of subtype-specific mechanisms [381]. Several studies using these types of manipulation, particularly in *Drosophila*, have supported the role of EVs or exosomes in a specific biological activity, providing some of the best evidence to date that EVs have functions in normal physiology.

Recommendations - Careful reporting is needed, for example, strategies used to label exosomes or other EVs, levels of expression of tagged proteins, subtype specificity and microscopy technologies utilized. It is important to critically assess evidence of disruptions to, for example, the endosomal system or EV content, which is afforded by expressing fluorescent reporters in specific *in vivo* systems and to consider these issues in making conclusions. When employing genetic manipulations to suppress EV or exosome secretion, data should be interpreted conservatively taking in to account the limited selectivity of many approaches. Consideration of the specificity of knocking down a regulator or bioactive cargo is needed, since this may well alter the secretory ability of a cell as well as its cargo. Conclusions should take in to account these caveats.

12.2 EV biodistribution, uptake and function

Studies in zebrafish have been particularly helpful in informing our understanding of the biodistribution and uptake of circulating EVs [377], though like other *in vivo* studies, they typically rely on fluorescent labelling of EVs with the same limitations as discussed in **Section 10.1**. In live imaging, EVs are typically scored as puncta, which cannot be resolved as single vesicles, except via super-resolution techniques, and so may in some cases represent EV clusters.

When fluorescent EVs are endocytosed, over time they are trafficked to late endosomes and lysosomes, where EVs degrade or back-fuse with the limiting membrane [377]. There is also more limited evidence for interactions between EVs and the cell surface [382], which might be required for ligand-receptor interactions. Analysis of the role of EVs in paracrine signaling in the *Drosophila* wing disc has highlighted an association with cellular projections called cytonemes that bring the surfaces of two distant cells into close 'synapse-like' contact prior to EV transfer [383]. Vertebrate cells also make cytonemes [384], but it remains to be seen whether these specialized forms of cell-cell interactions are involved in other types of paracrine EV signaling. In summary, there are multiple mechanisms by which EVs might interact with target cells, and these may be relevant when assessing EV uptake and function *in vivo*.

Direct demonstrations of physiological EV functions *in vivo* are still limited by the genetic tools available for blocking EV secretion, as discussed in **Section 10.1**. It is also unclear which EVs have physiological activity, those EVs at the cell surface or those that are endocytosed, and if the latter, in which compartments these EVs reside or function. These latter questions can be addressed in *in vivo* systems by genetically manipulating target cells, and this promises to be an exciting area of development going forward.

In mice, it is not possible to visualize circulating EVs in real-time and studies of EV function from normal tissue have been limited to *ex* vivo detection to date. However, pre-clinical studies with xenograft models, using human cancer cell lines, have allowed tumor EVs to be specifically labelled and their distribution traced [35].

Furthermore, with the caveats already discussed in **Section 10.1**, it has been possible to assign functions to these EVs, such as roles in metastasis, by inhibiting their secretion through genetic manipulation of EV biogenesis regulators [385, 386]. In one particularly innovative study where tumor EVs carried the mRNA for the DNA recombinase Cre to target cells, it was possible to show correlation between delivery of this cargo to the cytoplasm and change in cellular behavior [387]. A key consideration with all these experiments is that they assess tumor EV secretion and function, which may be different from physiological EV biology.

A complementary approach that has been used in mice and zebrafish is to inject labelled EVs into the circulation (or tissues) [388]. A significant advantage of this approach is that it is much more straightforward to manipulate the injected EVs before they are administered, though the biological effects of EV labelling, for example, still need to be considered. However, the major disadvantage of this approach mirrors one of the problems with functional studies of EVs *in vitro*: the injection and ultimate delivery of EVs to target cells inevitably does not mirror endogenous mechanisms, e.g. timing (bolus/continuous), dose (limited by detection method), site of injection, and therefore it may be difficult to relate any findings to physiological functions (**Section 10**).

Finally, the pathological roles of EVs secreted by parasites have also been studied, **Table 7** [389]. Although this work focuses on a specialized form of EV secretion, it nicely illustrates the potential functions of inter-species EV exchange in biology, also reported for plant EVs (**Section 6.4**). These studies have provided both evolutionary breadth and new thinking to the EV field.

Recommendations - EV uptake should be clearly distinguished from function, as it does not necessarily lead to an effect on the recipient cell. Imaging technologies employed should be fully reported and their ability to distinguish individual EVs versus EV clusters considered. The specificity of genetic manipulations used to block exosome and EV production and hence function needs to be reported and discussed. It is important to consider the likely impact of these manipulations on other secretory processes in drawing conclusions (**Section 12.1**).

13 Conclusions

14 References

1. Lotvall, J., et al., *Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles.* J Extracell Vesicles, 2014. **3**: p. 26913.
2. Corrigan, L., et al., *BMP-regulated exosomes from Drosophila male reproductive glands reprogram female behavior.* J Cell Biol, 2014. **206**(5): p. 671-88.
3. Thery, C., et al., *Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines.* J Extracell Vesicles, 2018. **7**(1): p. 1535750.
4. Welsh, J.A., et al., *MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments.* J Extracell Vesicles, 2020. **9**(1): p. 1713526.
5. Witwer, K.W., et al., *Standardization of sample collection, isolation and analysis methods in extracellular vesicle research.* J Extracell Vesicles, 2013. **2**(1): p. 20360.
6. Hill, A.F., et al., *ISEV position paper: extracellular vesicle RNA analysis and bioinformatics.* J Extracell Vesicles, 2013. **2**.
7. Lener, T., et al., *Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper.* J Extracell Vesicles, 2015. **4**: p. 30087.
8. Mateescu, B., et al., *Obstacles and opportunities in the functional analysis of extracellular vesicle RNA - an ISEV position paper.* J Extracell Vesicles, 2017. **6**(1): p. 1286095.

9. Russell, A.E., et al., *Biological membranes in EV biogenesis, stability, uptake, and cargo transfer: an ISEV position paper arising from the ISEV membranes and EVs workshop.* J Extracell Vesicles, 2019. **8**(1): p. 1684862.
10. Erdbrügger, U., et al., *Urinary extracellular vesicles: A position paper by the Urine Task Force of the International Society for Extracellular Vesicles.* J Extracell Vesicles, 2021. **10**(7): p. e12093.
11. Bustin, S.A., et al., *The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments.* Clinical Chemistry, 2009. **55**(4): p. 611-622.
12. Roux, Q., et al., *The EV-TRACK summary add-on: integration of experimental information in databases to ensure comprehensive interpretation of biological knowledge on extracellular vesicles.* J Extracell Vesicles, 2020. **9**(1): p. 1699367.
13. Consortium, E.-T., et al., *EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research.* Nat Methods, 2017. **14**(3): p. 228-232.
14. Kalra, H., et al., *Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation.* PLoS Biol, 2012. **10**(12): p. e1001450.
15. Nieuwland, R., et al., *Rigor and standardization of extracellular vesicle research: Paving the road towards robustness.* J Extracell Vesicles, 2020. **10**(2): p. e12037.
16. Witwer, K.W. and C. Théry, *Extracellular vesicles or exosomes? On primacy, precision, and popularity influencing a choice of nomenclature.* J Extracell Vesicles, 2019. **8**(1): p. 1648167.
17. van der Pol, E., et al., *Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing.* J Thromb Haemost, 2014. **12**(7): p. 1182-92.
18. Tian, Y., et al., *Quality and efficiency assessment of six extracellular vesicle isolation methods by nano-flow cytometry.* J Extracell Vesicles, 2020. **9**(1): p. 1697028.
19. Lennon, K.M., et al., *Single molecule characterization of individual extracellular vesicles from pancreatic cancer.* J Extracell Vesicles, 2019. **8**(1): p. 1685634.
20. Melentijevic, I., et al., *C. elegans neurons jettison protein aggregates and mitochondria under neurotoxic stress.* Nature, 2017. **542**(7641): p. 367-371.
21. Nicolás-Ávila, J.A., et al., *A Network of Macrophages Supports Mitochondrial Homeostasis in the Heart.* Cell, 2020. **183**(1): p. 94-109.e23.
22. Turek, M., et al., *Muscle-derived exophers promote reproductive fitness.* EMBO Rep, 2021. **22**(8): p. e52071.
23. Ikegami, K. and F. Ijaz, *Current understandings of the relationship between extracellular vesicles and cilia.* J Biochem, 2021. **169**(2): p. 139-145.
24. Akella, J.S. and M.M. Barr, *The tubulin code specializes neuronal cilia for extracellular vesicle release.* Dev Neurobiol, 2021. **81**(3): p. 231-252.
25. Nishimura, T., et al., *Filopodium-derived vesicles produced by MIM enhance the migration of recipient cells.* Dev Cell, 2021. **56**(6): p. 842-859.e8.
26. Ma, L., et al., *Discovery of the migrasome, an organelle mediating release of cytoplasmic contents during cell migration.* Cell Res, 2015. **25**(1): p. 24-38.
27. Hu, X., et al., *Release of cholesterol-rich particles from the macrophage plasma membrane during movement of filopodia and lamellipodia.* Elife, 2019. **8**.
28. Valcz, G., et al., *En bloc release of MVB-like small extracellular vesicle clusters by colorectal carcinoma cells.* J Extracell Vesicles, 2019. **8**(1): p. 1596668.
29. Fan, S.J., et al., *Glutamine deprivation alters the origin and function of cancer cell exosomes.* Embo j, 2020. **39**(16): p. e103009.
30. Thon, J.N. and J.E. Italiano, *Platelet formation.* Semin Hematol, 2010. **47**(3): p. 220-6.
31. Marki, A., et al., *Elongated neutrophil-derived structures are blood-borne microparticles formed by rolling neutrophils during sepsis.* J Exp Med, 2021. **218**(3).
32. Tersteeg, C., et al., *FLow-induced PRotrusions (FLIPRs): a platelet-derived platform for the retrieval of microparticles by monocytes and neutrophils.* Circ Res, 2014. **114**(5): p. 780-91.

33. Zhang, H., et al., *Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation.* Nat Cell Biol, 2018. **20**(3): p. 332-343.
34. Zhang, Q., et al., *Transfer of Functional Cargo in Exomeres.* Cell Rep, 2019. **27**(3): p. 940-954.e6.
35. Hoshino, A., et al., *Extracellular Vesicle and Particle Biomarkers Define Multiple Human Cancers.* Cell, 2020. **182**(4): p. 1044-1061.e18.
36. Zhang, Q., et al., *Supermeres are functional extracellular nanoparticles replete with disease biomarkers and therapeutic targets.* Nat Cell Biol, 2021. **23**(12): p. 1240-1254.
37. Bálint, Š., et al., *Supramolecular attack particles are autonomous killing entities released from cytotoxic T cells.* Science, 2020. **368**(6493): p. 897-901.
38. Wainwright, S.M., et al., *Drosophila Sex Peptide controls the assembly of lipid microcarriers in seminal fluid.* Proc Natl Acad Sci U S A, 2021. **118**(5).
39. Lozano-Andrés, E., et al., *Tetraspanin-decorated extracellular vesicle-mimetics as a novel adaptable reference material.* J Extracell Vesicles, 2019. **8**(1): p. 1573052.
40. Garcia-Manrique, P., et al., *Selected Tetraspanins Functionalized Niosomes as Potential Standards for Exosome Immunoassays.* Nanomaterials (Basel), 2020. **10**(5).
41. Vázquez-Ríos, A.J., et al., *Exosome-mimetic nanoplatforms for targeted cancer drug delivery.* J Nanobiotechnology, 2019. **17**(1): p. 85.
42. Staufer, O., et al., *Bottom-up assembly of biomedical relevant fully synthetic extracellular vesicles.* Sci Adv, 2021. **7**(36): p. eabg6666.
43. Lane, R.E., et al., *Analysis of exosome purification methods using a model liposome system and tunable-resistive pulse sensing.* Sci Rep, 2015. **5**: p. 7639.
44. Welsh, J.A., et al., *Towards defining reference materials for measuring extracellular vesicle refractive index, epitope abundance, size and concentration.* J Extracell Vesicles, 2020. **9**(1): p. 1816641.
45. Zheng, J.J., et al., *Isolation of HDL by sequential flotation ultracentrifugation followed by size exclusion chromatography reveals size-based enrichment of HDL-associated proteins.* Sci Rep, 2021. **11**(1): p. 16086.
46. Gyorgy, B., et al., *Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters.* Blood, 2011. **117**(4): p. e39-48.
47. Börger, V., et al., *International Society for Extracellular Vesicles and International Society for Cell and Gene Therapy statement on extracellular vesicles from mesenchymal stromal cells and other cells: considerations for potential therapeutic agents to suppress coronavirus disease-19.* Cytotherapy, 2020. **22**(9): p. 482-485.
48. Zhang, J., et al., *Quantitative proteomic analysis of age-related changes in human cerebrospinal fluid.* Neurobiol Aging, 2005. **26**(2): p. 207-27.
49. Shah, S.S., et al., *Age-specific reference values for cerebrospinal fluid protein concentration in neonates and young infants.* J Hosp Med, 2011. **6**(1): p. 22-7.
50. Wong, M., et al., *Cerebrospinal fluid protein concentration in pediatric patients: defining clinically relevant reference values.* Arch Pediatr Adolesc Med, 2000. **154**(8): p. 827-31.
51. Sjögren, M., et al., *Tau and Abeta42 in cerebrospinal fluid from healthy adults 21-93 years of age: establishment of reference values.* Clin Chem, 2001. **47**(10): p. 1776-81.
52. Li, G., et al., *Cerebrospinal fluid biomarkers for Alzheimer's and vascular disease vary by age, gender, and APOE genotype in cognitively normal adults.* Alzheimers Res Ther, 2017. **9**(1): p. 48.
53. Riekse, R.G., et al., *Effect of statins on Alzheimer's disease biomarkers in cerebrospinal fluid.* J Alzheimers Dis, 2006. **10**(4): p. 399-406.
54. Wong, V., *Medication use as a confounding factor in the use of the cerebrospinal fluid tau/beta-amyloid42 ratio.* Arch Neurol, 2007. **64**(9): p. 1357; author reply 1357-9.
55. Liu, Y., et al., *Association of Cigarette Smoking With Cerebrospinal Fluid Biomarkers of Neurodegeneration, Neuroinflammation, and Oxidation.* JAMA Netw Open, 2020. **3**(10): p. e2018777.

56. Wang, Z.T., et al., *Associations of Alcohol Consumption with Cerebrospinal Fluid Biomarkers of Alzheimer's Disease Pathology in Cognitively Intact Older Adults: The CABLE Study.* J Alzheimers Dis, 2021. **82**(3): p. 1045-1054.
57. Howell, J.C., et al., *Race modifies the relationship between cognition and Alzheimer's disease cerebrospinal fluid biomarkers.* Alzheimers Res Ther, 2017. **9**(1): p. 88.
58. Lucey, B.P., et al., *Diurnal oscillation of CSF Aβ and other AD biomarkers.* Mol Neurodegener, 2017. **12**(1): p. 36.
59. Cameron, S., et al., *Collection and Analyses of Cerebrospinal Fluid for Pediatric Translational Research.* Pediatr Neurol, 2019. **98**: p. 3-17.
60. Patel, D.B., et al., *Towards rationally designed biomanufacturing of therapeutic extracellular vesicles: impact of the bioproduction microenvironment.* Biotechnol Adv, 2018. **36**(8): p. 2051-2059.
61. Gurunathan, S., M.H. Kang, and J.H. Kim, *A Comprehensive Review on Factors Influences Biogenesis, Functions, Therapeutic and Clinical Implications of Exosomes.* Int J Nanomedicine, 2021. **16**: p. 1281-1312.
62. Witwer, K.W., et al., *Defining mesenchymal stromal cell (MSC)-derived small extracellular vesicles for therapeutic applications.* J Extracell Vesicles, 2019. **8**(1): p. 1609206.
63. Tracy, S.A., et al., *A comparison of clinically relevant sources of mesenchymal stem cell-derived exosomes: Bone marrow and amniotic fluid.* J Pediatr Surg, 2019. **54**(1): p. 86-90.
64. Palviainen, M., et al., *Metabolic signature of extracellular vesicles depends on the cell culture conditions.* J Extracell Vesicles, 2019. **8**(1): p. 1596669.
65. Ludwig, N., et al., *Optimization of cell culture conditions for exosome isolation using mini-size exclusion chromatography (mini-SEC).* Exp Cell Res, 2019. **378**(2): p. 149-157.
66. Zhu, Q.J., et al., *Exosomes from high glucose-treated macrophages activate glomerular mesangial cells via TGF-β1/Smad3 pathway in vivo and in vitro.* Faseb j, 2019. **33**(8): p. 9279-9290.
67. Rocha, S., et al., *3D Cellular Architecture Affects MicroRNA and Protein Cargo of Extracellular Vesicles.* Adv Sci (Weinh), 2019. **6**(4): p. 1800948.
68. Patel, D.B., et al., *Enhanced extracellular vesicle production and ethanol-mediated vascularization bioactivity via a 3D-printed scaffold-perfusion bioreactor system.* Acta Biomater, 2019. **95**: p. 236-244.
69. Nakase, I., et al., *Environmental pH stress influences cellular secretion and uptake of extracellular vesicles.* FEBS Open Bio, 2021. **11**(3): p. 753-767.
70. Maeshige, N., et al., *High-intensity ultrasound irradiation promotes the release of extracellular vesicles from C2C12 myotubes.* Ultrasonics, 2021. **110**: p. 106243.
71. Fukuta, T., A. Nishikawa, and K. Kogure, *Low level electricity increases the secretion of extracellular vesicles from cultured cells.* Biochem Biophys Rep, 2020. **21**: p. 100713.
72. Eichholz, K.F., et al., *Human bone marrow stem/stromal cell osteogenesis is regulated via mechanically activated osteocyte-derived extracellular vesicles.* Stem Cells Transl Med, 2020. **9**(11): p. 1431-1447.
73. Zhu, D., et al., *Impact of chemically defined culture media formulations on extracellular vesicle production by amniotic epithelial cells.* Proteomics, 2021. **21**(13-14): p. e2000080.
74. Lehrich, B.M., Y. Liang, and M.S. Fiandaca, *Foetal bovine serum influence on in vitro extracellular vesicle analyses.* J Extracell Vesicles, 2021. **10**(3): p. e12061.
75. Yang, C., et al., *Exosomes released from Mycoplasma infected tumor cells activate inhibitory B cells.* PLoS One, 2012. **7**(4): p. e36138.
76. Arigony, A.L., et al., *The influence of micronutrients in cell culture: a reflection on viability and genomic stability.* Biomed Res Int, 2013. **2013**: p. 597282.
77. Lehrich, B.M., et al., *Fetal Bovine Serum-Derived Extracellular Vesicles Persist within Vesicle-Depleted Culture Media.* Int J Mol Sci, 2018. **19**(11).
78. Auber, M., et al., *Serum-free media supplements carry miRNAs that co-purify with extracellular vesicles.* J Extracell Vesicles, 2019. **8**(1): p. 1656042.

79. Zhang, S., D.J. Wear, and S. Lo, *Mycoplasmal infections alter gene expression in cultured human prostatic and cervical epithelial cells.* FEMS Immunol Med Microbiol, 2000. **27**(1): p. 43-50.
80. Gaurivaud, P., et al., *Mycoplasmas are no exception to extracellular vesicles release: Revisiting old concepts.* PLoS One, 2018. **13**(11): p. e0208160.
81. Wang, B., et al., *High glucose suppresses autophagy through the AMPK pathway while it induces autophagy via oxidative stress in chondrocytes.* Cell Death Dis, 2021. **12**(6): p. 506.
82. Xu, J., R. Camfield, and S.M. Gorski, *The interplay between exosomes and autophagy - partners in crime.* J Cell Sci, 2018. **131**(15).
83. Zou, W., et al., *Exosome Release Is Regulated by mTORC1.* Adv Sci (Weinh), 2019. **6**(3): p. 1801313.
84. Stegmayr, B. and G. Ronquist, *Promotive effect on human sperm progressive motility by prostasomes.* Urol Res, 1982. **10**(5): p. 253-7.
85. Sullivan, R., G. Frenette, and J. Girouard, *Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit.* Asian J Androl, 2007. **9**(4): p. 483-91.
86. Simon, C., et al., *Extracellular Vesicles in Human Reproduction in Health and Disease.* Endocr Rev, 2018. **39**(3): p. 292-332.
87. Dobhal, G., et al., *Isolation, characterisation and detection of breath-derived extracellular vesicles.* Sci Rep, 2020. **10**(1): p. 17381.
88. An, J., et al., *Extracellular vesicle-derived microbiome obtained from exhaled breath condensate in patients with asthma.* Ann Allergy Asthma Immunol, 2021. **126**(6): p. 729-731.
89. Clayton, A., et al., *Considerations towards a roadmap for collection, handling and storage of blood extracellular vesicles.* J Extracell Vesicles, 2019. **8**(1): p. 1647027.
90. Berckmans, R.J., et al., *Extracellular vesicles and coagulation in blood from healthy humans revisited.* J Extracell Vesicles, 2019. **8**(1): p. 1688936.
91. Dong, L., et al., *Comprehensive evaluation of methods for small extracellular vesicles separation from human plasma, urine and cell culture medium.* J Extracell Vesicles, 2020. **10**(2): p. e12044.
92. Ramirez-Garrastacho, M., et al., *Extracellular vesicles as a source of prostate cancer biomarkers in liquid biopsies: a decade of research.* Br J Cancer, 2022. **126**(3): p. 331-350.
93. Burger, D., et al., *Urinary podocyte microparticles identify prealbuminuric diabetic glomerular injury.* J Am Soc Nephrol, 2014. **25**(7): p. 1401-7.
94. Carreras-Planella, L., et al., *Urinary vitronectin identifies patients with high levels of fibrosis in kidney grafts.* J Nephrol, 2021. **34**(3): p. 861-874.
95. Morikawa, Y., et al., *Elevated Levels of Urinary Extracellular Vesicle Fibroblast-Specific Protein 1 in Patients with Active Crescentic Glomerulonephritis.* Nephron, 2019. **141**(3): p. 177-187.
96. Tutrone, R., et al., *Clinical utility of the exosome based ExoDx Prostate(IntelliScore) EPI test in men presenting for initial Biopsy with a PSA 2-10 ng/mL.* Prostate Cancer Prostatic Dis, 2020. **23**(4): p. 607-614.
97. Oosthuyzen, W., et al., *Vasopressin Regulates Extracellular Vesicle Uptake by Kidney Collecting Duct Cells.* J Am Soc Nephrol, 2016. **27**(11): p. 3345-3355.
98. Grange, C., et al., *Urinary Extracellular Vesicles Carrying Klotho Improve the Recovery of Renal Function in an Acute Tubular Injury Model.* Mol Ther, 2020. **28**(2): p. 490-502.
99. Barreiro, K. and H. Holthofer, *Urinary extracellular vesicles. A promising shortcut to novel biomarker discoveries.* Cell Tissue Res, 2017. **369**(1): p. 217-227.
100. Hühmer, A.F., et al., *Protein analysis in human cerebrospinal fluid: Physiological aspects, current progress and future challenges.* Dis Markers, 2006. **22**(1-2): p. 3-26.
101. Jack, C.R., Jr., et al., *NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease.* Alzheimers Dement, 2018. **14**(4): p. 535-562.
102. Gaetani, L., et al., *Neurofilament light chain as a biomarker in neurological disorders.* J Neurol Neurosurg Psychiatry, 2019. **90**(8): p. 870-881.
103. Rao, P., E. Benito, and A. Fischer, *MicroRNAs as biomarkers for CNS disease.* Front Mol Neurosci, 2013. **6**: p. 39.

104. Gui, Y., et al., *Altered microRNA profiles in cerebrospinal fluid exosome in Parkinson disease and Alzheimer disease.* Oncotarget, 2015. **6**(35): p. 37043-53.
105. Saman, S., et al., *Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease.* J Biol Chem, 2012. **287**(6): p. 3842-9.
106. Yang, Y., et al., *Cerebrospinal Fluid Particles in Alzheimer Disease and Parkinson Disease.* J Neuropathol Exp Neurol, 2015. **74**(7): p. 672-87.
107. Guo, M., et al., *Microglial exosomes facilitate α-synuclein transmission in Parkinson's disease.* Brain, 2020. **143**(5): p. 1476-1497.
108. Manek, R., et al., *Protein Biomarkers and Neuroproteomics Characterization of Microvesicles/Exosomes from Human Cerebrospinal Fluid Following Traumatic Brain Injury.* Mol Neurobiol, 2018. **55**(7): p. 6112-6128.
109. Guha, D., et al., *Proteomic analysis of cerebrospinal fluid extracellular vesicles reveals synaptic injury, inflammation, and stress response markers in HIV patients with cognitive impairment.* J Neuroinflammation, 2019. **16**(1): p. 254.
110. Guha, D., et al., *Cerebrospinal fluid extracellular vesicles and neurofilament light protein as biomarkers of central nervous system injury in HIV-infected patients on antiretroviral therapy.* Aids, 2019. **33**(4): p. 615-625.
111. Pieragostino, D., et al., *Enhanced release of acid sphingomyelinase-enriched exosomes generates a lipidomics signature in CSF of Multiple Sclerosis patients.* Sci Rep, 2018. **8**(1): p. 3071.
112. Akers, J.C., et al., *A cerebrospinal fluid microRNA signature as biomarker for glioblastoma.* Oncotarget, 2017. **8**(40): p. 68769-68779.
113. Wang, M., et al., *Exosomal LGALS9 in the cerebrospinal fluid of glioblastoma patients suppressed dendritic cell antigen presentation and cytotoxic T-cell immunity.* Cell Death Dis, 2020. **11**(10): p. 896.
114. Hayashi, N., et al., *Proteomic analysis of exosome-enriched fractions derived from cerebrospinal fluid of amyotrophic lateral sclerosis patients.* Neurosci Res, 2020. **160**: p. 43-49.
115. Yelick, J., et al., *Elevated exosomal secretion of miR-124-3p from spinal neurons positively associates with disease severity in ALS.* Exp Neurol, 2020. **333**: p. 113414.
116. Lewczuk, P., et al., *International quality control survey of neurochemical dementia diagnostics.* Neurosci Lett, 2006. **409**(1): p. 1-4.
117. Klener, J., et al., *Instability of cerebrospinal fluid after delayed storage and repeated freezing: a holistic study by drop coating deposition Raman spectroscopy.* Clin Chem Lab Med, 2014. **52**(5): p. 657-64.
118. Mattsson, N., et al., *The Alzheimer's Association external quality control program for cerebrospinal fluid biomarkers.* Alzheimers Dement, 2011. **7**(4): p. 386-395.e6.
119. Aasebø, E., et al., *Effects of blood contamination and the rostro-caudal gradient on the human cerebrospinal fluid proteome.* PLoS One, 2014. **9**(3): p. e90429.
120. Reiber, H., *Proteins in cerebrospinal fluid and blood: barriers, CSF flow rate and source-related dynamics.* Restor Neurol Neurosci, 2003. **21**(3-4): p. 79-96.
121. Brandner, S., et al., *Ventricular and lumbar cerebrospinal fluid concentrations of Alzheimer's disease biomarkers in patients with normal pressure hydrocephalus and posttraumatic hydrocephalus.* J Alzheimers Dis, 2014. **41**(4): p. 1057-62.
122. Teunissen, C.E., et al., *A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking.* Neurology, 2009. **73**(22): p. 1914-22.
123. You, J.S., et al., *The impact of blood contamination on the proteome of cerebrospinal fluid.* Proteomics, 2005. **5**(1): p. 290-6.
124. Ogawa, Y., et al., *Exosome-like vesicles with dipeptidyl peptidase IV in human saliva.* Biol Pharm Bull, 2008. **31**(6): p. 1059-62.

125. Chiappin, S., et al., *Saliva specimen: a new laboratory tool for diagnostic and basic investigation.* Clin Chim Acta, 2007. **383**(1-2): p. 30-40.
126. Kaczor-Urbanowicz, K.E., et al., *Clinical validity of saliva and novel technology for cancer detection.* Biochim Biophys Acta Rev Cancer, 2019. **1872**(1): p. 49-59.
127. Aps, J.K. and L.C. Martens, *Review: The physiology of saliva and transfer of drugs into saliva.* Forensic Sci Int, 2005. **150**(2-3): p. 119-31.
128. Han, P., et al., *Salivary Outer Membrane Vesicles and DNA Methylation of Small Extracellular Vesicles as Biomarkers for Periodontal Status: A Pilot Study.* Int J Mol Sci, 2021. **22**(5).
129. Ngamchuea, K., et al., *Chemical analysis in saliva and the search for salivary biomarkers - a tutorial review.* Analyst, 2017. **143**(1): p. 81-99.
130. Khurshid, Z., et al., *Human Saliva Collection Devices for Proteomics: An Update.* Int J Mol Sci, 2016. **17**(6).
131. Beale, D.J., et al., *A Review of Analytical Techniques and Their Application in Disease Diagnosis in Breathomics and Salivaomics Research.* Int J Mol Sci, 2016. **18**(1).
132. Navazesh, M., *Methods for collecting saliva.* Ann N Y Acad Sci, 1993. **694**: p. 72-7.
133. Gomar-Vercher, S., et al., *Stimulated and unstimulated saliva samples have significantly different bacterial profiles.* PLoS One, 2018. **13**(6): p. e0198021.
134. Xu, F., L. Laguna, and A. Sarkar, *Aging-related changes in quantity and quality of saliva: Where do we stand in our understanding?* J Texture Stud, 2019. **50**(1): p. 27-35.
135. Li-Hui, W., et al., *Gender differences in the saliva of young healthy subjects before and after citric acid stimulation.* Clin Chim Acta, 2016. **460**: p. 142-5.
136. Rad, M., et al., *Effect of Long-term Smoking on Whole-mouth Salivary Flow Rate and Oral Health.* J Dent Res Dent Clin Dent Prospects, 2010. **4**(4): p. 110-4.
137. Keremi, B., et al., *Stress and Salivary Glands.* Curr Pharm Des, 2017. **23**(27): p. 4057-4065.
138. Ligtenberg, A.J., et al., *The Effect of Exercise on Salivary Viscosity.* Diagnostics (Basel), 2016. **6**(4).
139. Bhattarai, K.R., H.R. Kim, and H.J. Chae, *Compliance with Saliva Collection Protocol in Healthy Volunteers: Strategies for Managing Risk and Errors.* Int J Med Sci, 2018. **15**(8): p. 823-831.
140. Boere, J., et al., *Extracellular Vesicles in Joint Disease and Therapy.* Front Immunol, 2018. **9**: p. 2575.
141. Michael, B.N.R., et al., *Characterization of cell-derived microparticles in synovial fluid and plasma of patients with rheumatoid arthritis.* Rheumatol Int, 2019. **39**(8): p. 1377-1387.
142. Boere, J., et al., *Extracellular vesicles in synovial fluid from juvenile horses: No age-related changes in the quantitative profile.* Vet J, 2019. **244**: p. 91-93.
143. Gao, K., et al., *Association between cytokines and exosomes in synovial fluid of individuals with knee osteoarthritis.* Mod Rheumatol, 2020. **30**(4): p. 758-764.
144. Rüwald, J.M., et al., *Extracellular Vesicle Isolation and Characterization from Periprosthetic Joint Synovial Fluid in Revision Total Joint Arthroplasty.* J Clin Med, 2020. **9**(2).
145. Mustonen, A.M., et al., *Characterization of hyaluronan-coated extracellular vesicles in synovial fluid of patients with osteoarthritis and rheumatoid arthritis.* BMC Musculoskelet Disord, 2021. **22**(1): p. 247.
146. Foers, A.D., et al., *Enrichment of extracellular vesicles from human synovial fluid using size exclusion chromatography.* J Extracell Vesicles, 2018. **7**(1): p. 1490145.
147. Kolhe, R., et al., *Sex-Specific Differences in Extracellular Vesicle Protein Cargo in Synovial Fluid of Patients with Osteoarthritis.* Life (Basel), 2020. **10**(12).
148. Schioppo, T., et al., *The role of extracellular vesicles in rheumatoid arthritis: a systematic review.* Clin Rheumatol, 2021. **40**(9): p. 3481-3497.
149. Foers, A.D., et al., *Proteomic analysis of extracellular vesicles reveals an immunogenic cargo in rheumatoid arthritis synovial fluid.* Clin Transl Immunology, 2020. **9**(11): p. e1185.
150. Ballard, O. and A.L. Morrow, *Human milk composition: nutrients and bioactive factors.* Pediatr Clin North Am, 2013. **60**(1): p. 49-74.
151. Busatto, S., et al., *The nanostructured secretome.* Biomater Sci, 2019. **8**(1): p. 39-63.

152. Zonneveld, M.I., et al., *Recovery of extracellular vesicles from human breast milk is influenced by sample collection and vesicle isolation procedures.* J Extracell Vesicles, 2014. **3**.
153. Mukhopadhya, A., et al., *Optimisation and comparison of orthogonal methods for separation and characterisation of extracellular vesicles to investigate how representative infant milk formula is of milk.* Food Chem, 2021. **353**: p. 129309.
154. Rahman, M.M., et al., *Acidification effects on isolation of extracellular vesicles from bovine milk.* PLoS One, 2019. **14**(9): p. e0222613.
155. Somiya, M., Y. Yoshioka, and T. Ochiya, *Biocompatibility of highly purified bovine milk-derived extracellular vesicles.* J Extracell Vesicles, 2018. **7**(1): p. 1440132.
156. Gao, H.N., et al., *Yak-milk-derived exosomes promote proliferation of intestinal epithelial cells in an hypoxic environment.* J Dairy Sci, 2019. **102**(2): p. 985-996.
157. Benmoussa, A., et al., *Isolating Multiple Extracellular Vesicles Subsets, Including Exosomes and Membrane Vesicles, from Bovine Milk Using Sodium Citrate and Differential Ultracentrifugation.* Bio Protoc, 2020. **10**(11): p. e3636.
158. Mukhopadhya, A., J. Santoro, and L. O'Driscoll, *Extracellular vesicle separation from milk and infant milk formula using acid precipitation and ultracentrifugation.* STAR Protoc, 2021. **2**(4): p. 100821.
159. Bose, S., et al., *Extracellular vesicles: An emerging platform in gram-positive bacteria.* Microb Cell, 2020. **7**(12): p. 312-322.
160. Brown, L., et al., *Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi.* Nat Rev Microbiol, 2015. **13**(10): p. 620-30.
161. Kuehn, M.J. and N.C. Kesty, *Bacterial outer membrane vesicles and the host-pathogen interaction.* Genes Dev, 2005. **19**(22): p. 2645-55.
162. Tulkens, J., O. De Wever, and A. Hendrix, *Analyzing bacterial extracellular vesicles in human body fluids by orthogonal biophysical separation and biochemical characterization.* Nat Protoc, 2020. **15**(1): p. 40-67.
163. Yoo, J.Y., et al., *16S rRNA gene-based metagenomic analysis reveals differences in bacteria-derived extracellular vesicles in the urine of pregnant and non-pregnant women.* Exp Mol Med, 2016. **48**(2): p. e208.
164. Dauros Singorenko, P., et al., *Isolation of membrane vesicles from prokaryotes: a technical and biological comparison reveals heterogeneity.* J Extracell Vesicles, 2017. **6**(1): p. 1324731.
165. Lunavat, T.R., et al., *BRAF(V600) inhibition alters the microRNA cargo in the vesicular secretome of malignant melanoma cells.* Proc Natl Acad Sci U S A, 2017. **114**(29): p. E5930-e5939.
166. Jang, S.C., et al., *Mitochondrial protein enriched extracellular vesicles discovered in human melanoma tissues can be detected in patient plasma.* J Extracell Vesicles, 2019. **8**(1): p. 1635420.
167. Crescitelli, R., C. Lasser, and J. Lotvall, *Isolation and characterization of extracellular vesicle subpopulations from tissues.* Nat Protoc, 2021. **16**(3): p. 1548-1580.
168. Hurwitz, S.N., J.M. Olcese, and D.G. Meckes, Jr., *Extraction of Extracellular Vesicles from Whole Tissue.* J Vis Exp, 2019(144).
169. Steenbeek, S.C., et al., *Cancer cells copy migratory behavior and exchange signaling networks via extracellular vesicles.* Embo j, 2018. **37**(15).
170. Cianciaruso, C., et al., *Molecular Profiling and Functional Analysis of Macrophage-Derived Tumor Extracellular Vesicles.* Cell Rep, 2019. **27**(10): p. 3062-3080.e11.
171. Jingushi, K., et al., *Extracellular vesicles isolated from human renal cell carcinoma tissues disrupt vascular endothelial cell morphology via azurocidin.* Int J Cancer, 2018. **142**(3): p. 607-617.
172. Jeppesen, D.K., et al., *Reassessment of Exosome Composition.* Cell, 2019. **177**(2): p. 428-445.e18.
173. Perez-Gonzalez, R., et al., *The exosome secretory pathway transports amyloid precursor protein carboxyl-terminal fragments from the cell into the brain extracellular space.* J Biol Chem, 2012. **287**(51): p. 43108-15.
174. Vella, L.J., et al., *A rigorous method to enrich for exosomes from brain tissue.* J Extracell Vesicles, 2017. **6**(1): p. 1348885.

175. Huang, Y., et al., *Influence of species and processing parameters on recovery and content of brain tissue-derived extracellular vesicles.* J Extracell Vesicles, 2020. **9**(1): p. 1785746.
176. Yelamanchili, S.V., et al., *MiR-21 in Extracellular Vesicles Leads to Neurotoxicity via TLR7 Signaling in SIV Neurological Disease.* PLoS Pathog, 2015. **11**(7): p. e1005032.
177. Hurwitz, S.N., et al., *An optimized method for enrichment of whole brain-derived extracellular vesicles reveals insight into neurodegenerative processes in a mouse model of Alzheimer's disease.* J Neurosci Methods, 2018. **307**: p. 210-220.
178. Jeurissen, S., et al., *The isolation of morphologically intact and biologically active extracellular vesicles from the secretome of cancer-associated adipose tissue.* Cell Adh Migr, 2017. **11**(2): p. 196-204.
179. Gallart-Palau, X., A. Serra, and S.K. Sze, *Enrichment of extracellular vesicles from tissues of the central nervous system by PROSPR.* Mol Neurodegener, 2016. **11**(1): p. 41.
180. Crescitelli, R., et al., *Subpopulations of extracellular vesicles from human metastatic melanoma tissue identified by quantitative proteomics after optimized isolation.* J Extracell Vesicles, 2020. **9**(1): p. 1722433.
181. Banigan, M.G., et al., *Differential expression of exosomal microRNAs in prefrontal cortices of schizophrenia and bipolar disorder patients.* PLoS One, 2013. **8**(1): p. e48814.
182. Polanco, J.C., et al., *Extracellular Vesicles Isolated from the Brains of rTg4510 Mice Seed Tau Protein Aggregation in a Threshold-dependent Manner.* J Biol Chem, 2016. **291**(24): p. 12445-12466.
183. Rutter, B.D. and R.W. Innes, *Extracellular Vesicles Isolated from the Leaf Apoplast Carry Stress-Response Proteins.* Plant Physiol, 2017. **173**(1): p. 728-741.
184. Chen, A., B. He, and H. Jin, *Isolation of Extracellular Vesicles from Arabidopsis.* Curr Protoc, 2022. **2**(1): p. e352.
185. Rutter, B.D. and R.W. Innes, *Growing pains: addressing the pitfalls of plant extracellular vesicle research.* New Phytol, 2020. **228**(5): p. 1505-1510.
186. Cai, Q., et al., *Message in a Bubble: Shuttling Small RNAs and Proteins Between Cells and Interacting Organisms Using Extracellular Vesicles.* Annu Rev Plant Biol, 2021. **72**: p. 497-524.
187. Karimi, H.Z., et al., *Arabidopsis apoplastic fluid contains sRNA- and circular RNA-protein complexes that are located outside extracellular vesicles.* Plant Cell, 2022.
188. De Bellis, D., et al., *Extracellular vesiculo-tubular structures associated with suberin deposition in plant cell walls.* Nat Commun, 2022. **13**(1): p. 1489.
189. Cong, M., et al., *Technology insight: Plant-derived vesicles-How far from the clinical biotherapeutics and therapeutic drug carriers?* Adv Drug Deliv Rev, 2022. **182**: p. 114108.
190. Leiferman, A., et al., *Storage of Extracellular Vesicles in Human Milk, and MicroRNA Profiles in Human Milk Exosomes and Infant Formulas.* J Pediatr Gastroenterol Nutr, 2019. **69**(2): p. 235-238.
191. Evtushenko, E.G., et al., *Adsorption of extracellular vesicles onto the tube walls during storage in solution.* PLoS One, 2020. **15**(12): p. e0243738.
192. Lőrincz Á, M., et al., *Effect of storage on physical and functional properties of extracellular vesicles derived from neutrophilic granulocytes.* J Extracell Vesicles, 2014. **3**: p. 25465.
193. Le Saux, S., et al., *Post-production modifications of murine mesenchymal stem cell (mMSC) derived extracellular vesicles (EVs) and impact on their cellular interaction.* Biomaterials, 2020. **231**: p. 119675.
194. Gelibter, S., et al., *The impact of storage on extracellular vesicles: A systematic study.* J Extracell Vesicles, 2022. **11**(2): p. e12162.
195. Trenkenschuh, E., et al., *Enhancing the Stabilization Potential of Lyophilization for Extracellular Vesicles.* Adv Healthc Mater, 2021: p. e2100538.
196. Kumeda, N., et al., *Characterization of Membrane Integrity and Morphological Stability of Human Salivary Exosomes.* Biol Pharm Bull, 2017. **40**(8): p. 1183-1191.

197. Barreiro, K., et al., *Urinary extracellular vesicles: Assessment of pre-analytical variables and development of a quality control with focus on transcriptomic biomarker research.* J Extracell Vesicles, 2021. **10**(12): p. e12158.
198. Schulz, E., et al., *Hot EVs - How temperature affects extracellular vesicles.* Eur J Pharm Biopharm, 2020. **146**: p. 55-63.
199. Lucas, M., et al., *Extraction and Separation of Mycobacterial Proteins.* Methods Mol Biol, 2021. **2314**: p. 77-107.
200. Tóth, E., et al., *Formation of a protein corona on the surface of extracellular vesicles in blood plasma.* J Extracell Vesicles, 2021. **10**(11): p. e12140.
201. Palviainen, M., et al., *Extracellular vesicles from human plasma and serum are carriers of extravesicular cargo-Implications for biomarker discovery.* PLoS One, 2020. **15**(8): p. e0236439.
202. Muraoka, S., et al., *Comprehensive proteomic profiling of plasma and serum phosphatidylserine-positive extracellular vesicles reveals tissue-specific proteins.* iScience, 2022. **25**(4): p. 104012.
203. Martins Á, M., et al., *Glycosylation of Cancer Extracellular Vesicles: Capture Strategies, Functional Roles and Potential Clinical Applications.* Cells, 2021. **10**(1).
204. Mathieu, M., et al., *Specificities of exosome versus small ectosome secretion revealed by live intracellular tracking of CD63 and CD9.* Nat Commun, 2021. **12**(1): p. 4389.
205. Kugeratski, F.G., et al., *Quantitative proteomics identifies the core proteome of exosomes with syntenin-1 as the highest abundant protein and a putative universal biomarker.* Nat Cell Biol, 2021. **23**(6): p. 631-641.
206. Liu, C., et al., *Low-cost thermophoretic profiling of extracellular-vesicle surface proteins for the early detection and classification of cancers.* Nat Biomed Eng, 2019. **3**(3): p. 183-193.
207. Shirejini, S.Z. and F. Inci, *The Yin and Yang of exosome isolation methods: conventional practice, microfluidics, and commercial kits.* Biotechnol Adv, 2021: p. 107814.
208. Xue, V.W., et al., *Proteomic profiling in extracellular vesicles for cancer detection and monitoring.* Proteomics, 2021. **21**(13-14): p. e2000094.
209. Clayton, A., et al., *Summary of the ISEV workshop on extracellular vesicles as disease biomarkers, held in Birmingham, UK, during December 2017.* J Extracell Vesicles, 2018. **7**(1): p. 1473707.
210. Welsh, J.A., et al., *Towards defining reference materials for extracellular vesicle size, concentration, refractive index and epitope abundance.* arXiv: Quantitative Methods, 2020.
211. Johnsen, K.B., et al., *What is the blood concentration of extracellular vesicles? Implications for the use of extracellular vesicles as blood-borne biomarkers of cancer.* Biochim Biophys Acta Rev Cancer, 2019. **1871**(1): p. 109-116.
212. van der Pol, E., et al., *Standardization of extracellular vesicle measurements by flow cytometry through vesicle diameter approximation.* J Thromb Haemost, 2018. **16**(6): p. 1236-1245.
213. Welsh, J.A., J.C. Jones, and V.A. Tang, *Fluorescence and Light Scatter Calibration Allow Comparisons of Small Particle Data in Standard Units across Different Flow Cytometry Platforms and Detector Settings.* Cytometry A, 2020. **97**(6): p. 592-601.
214. Bachurski, D., et al., *Extracellular vesicle measurements with nanoparticle tracking analysis - An accuracy and repeatability comparison between NanoSight NS300 and ZetaView.* J Extracell Vesicles, 2019. **8**(1): p. 1596016.
215. Tian, Y., et al., *Protein Profiling and Sizing of Extracellular Vesicles from Colorectal Cancer Patients via Flow Cytometry.* ACS Nano, 2018. **12**(1): p. 671-680.
216. Brady, P.N. and M.A. Macnaughtan, *Evaluation of colorimetric assays for analyzing reductively methylated proteins: Biases and mechanistic insights.* Anal Biochem, 2015. **491**: p. 43-51.
217. Visnovitz, T., et al., *An improved 96 well plate format lipid quantification assay for standardisation of experiments with extracellular vesicles.* J Extracell Vesicles, 2019. **8**(1): p. 1565263.
218. Mihaly, J., et al., *Characterization of extracellular vesicles by IR spectroscopy: Fast and simple classification based on amide and CH stretching vibrations.* Biochim Biophys Acta Biomembr, 2017. **1859**(3): p. 459-466.

219. Turchinovich, A., et al., *Characterization of extracellular circulating microRNA.* Nucleic Acids Res, 2011. **39**(16): p. 7223-33.
220. Vickers, K.C., et al., *MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins.* Nat Cell Biol, 2011. **13**(4): p. 423-33.
221. Cavallaro, S., et al., *Comparison and optimization of nanoscale extracellular vesicle imaging by scanning electron microscopy for accurate size-based profiling and morphological analysis.* Nanoscale Advances, 2021.
222. Théry, C., et al., *Isolation and characterization of exosomes from cell culture supernatants and biological fluids.* Curr Protoc Cell Biol, 2006. **Chapter 3**: p. Unit 3.22.
223. Stoner, S.A., et al., *High sensitivity flow cytometry of membrane vesicles.* Cytometry A, 2016. **89**(2): p. 196-206.
224. Wu, Y., W. Deng, and D.J. Klinke, 2nd, *Exosomes: improved methods to characterize their morphology, RNA content, and surface protein biomarkers.* Analyst, 2015. **140**(19): p. 6631-42.
225. Linares, R., et al., *High-speed centrifugation induces aggregation of extracellular vesicles.* J Extracell Vesicles, 2015. **4**: p. 29509.
226. Hoog, J.L. and J. Lotvall, *Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy.* J Extracell Vesicles, 2015. **4**: p. 28680.
227. Sharma, S., et al., *Quantitative nanostructural and single-molecule force spectroscopy biomolecular analysis of human-saliva-derived exosomes.* Langmuir, 2011. **27**(23): p. 14394-400.
228. Rikkert, L.G., et al., *Quality of extracellular vesicle images by transmission electron microscopy is operator and protocol dependent.* J Extracell Vesicles, 2019. **8**(1): p. 1555419.
229. Sódar, B.W., et al., *Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection.* Sci Rep, 2016. **6**: p. 24316.
230. Liao, Z., et al., *Acetylcholinesterase is not a generic marker of extracellular vesicles.* J Extracell Vesicles, 2019. **8**(1): p. 1628592.
231. Karimi, N., et al., *Detailed analysis of the plasma extracellular vesicle proteome after separation from lipoproteins.* Cell Mol Life Sci, 2018. **75**(15): p. 2873-2886.
232. Østergaard, O., et al., *Quantitative proteome profiling of normal human circulating microparticles.* J Proteome Res, 2012. **11**(4): p. 2154-63.
233. Musante, L., et al., *Biochemical and physical characterisation of urinary nanovesicles following CHAPS treatment.* PLoS One, 2012. **7**(7): p. e37279.
234. Liu, H., et al., *Analysis of extracellular vesicle DNA at the single-vesicle level by nano-flow cytometry.* J Extracell Vesicles, 2022. **11**(4): p. e12206.
235. Cvjetkovic, A., et al., *Detailed Analysis of Protein Topology of Extracellular Vesicles-Evidence of Unconventional Membrane Protein Orientation.* Sci Rep, 2016. **6**: p. 36338.
236. Deregibus, M.C., et al., *Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA.* Blood, 2007. **110**(7): p. 2440-8.
237. Németh, A., et al., *Antibiotic-induced release of small extracellular vesicles (exosomes) with surface-associated DNA.* Sci Rep, 2017. **7**(1): p. 8202.
238. Sharma, A., et al., *In vitro dissection of protein translocation into the mammalian endoplasmic reticulum.* Methods Mol Biol, 2010. **619**: p. 339-63.
239. McKenzie, A.J., et al., *KRAS-MEK Signaling Controls Ago2 Sorting into Exosomes.* Cell Rep, 2016. **15**(5): p. 978-987.
240. Lai, C.P., et al., *Visualization and tracking of tumour extracellular vesicle delivery and RNA translation using multiplexed reporters.* Nat Commun, 2015. **6**: p. 7029.
241. Sung, B.H. and A.M. Weaver, *Exosome secretion promotes chemotaxis of cancer cells.* Cell Adh Migr, 2017. **11**(2): p. 187-195.
242. Osteikoetxea, X., et al., *Differential detergent sensitivity of extracellular vesicle subpopulations.* Org Biomol Chem, 2015. **13**(38): p. 9775-82.

243. Bonsergent, E. and G. Lavieu, *Content release of extracellular vesicles in a cell-free extract.* FEBS Lett, 2019. **593**(15): p. 1983-1992.
244. van der Pol, E., J.A. Welsh, and R. Nieuwland, *Minimum information to report about a flow cytometry experiment on extracellular vesicles: Communication from the ISTH SSC subcommittee on vascular biology.* J Thromb Haemost, 2022. **20**(1): p. 245-251.
245. Sharma, S., M. LeClaire, and J.K. Gimzewski, *Ascent of atomic force microscopy as a nanoanalytical tool for exosomes and other extracellular vesicles.* Nanotechnology, 2018. **29**(13): p. 132001.
246. Bordanaba-Florit, G., et al., *Using single-vesicle technologies to unravel the heterogeneity of extracellular vesicles.* Nat Protoc, 2021. **16**(7): p. 3163-3185.
247. Paolini, L., et al., *Residual matrix from different separation techniques impacts exosome biological activity.* Sci Rep, 2016. **6**: p. 23550.
248. Parisse, P., et al., *Atomic force microscopy analysis of extracellular vesicles.* Eur Biophys J, 2017. **46**(8): p. 813-820.
249. Cavallaro, S., et al., *Multiparametric Profiling of Single Nanoscale Extracellular Vesicles by Combined Atomic Force and Fluorescence Microscopy: Correlation and Heterogeneity in Their Molecular and Biophysical Features.* Small, 2021. **17**(14): p. e2008155.
250. Paolini, L., et al., *Fourier-transform Infrared (FT-IR) spectroscopy fingerprints subpopulations of extracellular vesicles of different sizes and cellular origin.* J Extracell Vesicles, 2020. **9**(1): p. 1741174.
251. Gautron, J., et al., *Avian eggshell biomineralization: an update on its structure, mineralogy and protein tool kit.* BMC Mol Cell Biol, 2021. **22**(1): p. 11.
252. Piontek, M.C., R.B. Lira, and W.H. Roos, *Active probing of the mechanical properties of biological and synthetic vesicles.* Biochim Biophys Acta Gen Subj, 2021. **1865**(4): p. 129486.
253. Whitehead, B., et al., *Tumour exosomes display differential mechanical and complement activation properties dependent on malignant state: implications in endothelial leakiness.* J Extracell Vesicles, 2015. **4**: p. 29685.
254. Vorselen, D., et al., *The fluid membrane determines mechanics of erythrocyte extracellular vesicles and is softened in hereditary spherocytosis.* Nat Commun, 2018. **9**(1): p. 4960.
255. Sorkin, R., et al., *Nanomechanics of Extracellular Vesicles Reveals Vesiculation Pathways.* Small, 2018. **14**(39): p. e1801650.
256. LeClaire, M., et al., *Nanoscale Extracellular Vesicles Carry the Mechanobiology Signatures of Breast Cancer Cells.* ACS Applied Nano Materials, 2021. **4**(9): p. 9876-9885.
257. Bortot, B., et al., *Small extracellular vesicles from malignant ascites of patients with advanced ovarian cancer provide insights into the dynamics of the extracellular matrix.* Mol Oncol, 2021. **15**(12): p. 3596-3614.
258. Ye, S., et al., *Quantitative Nanomechanical Analysis of Small Extracellular Vesicles for Tumor Malignancy Indication.* Adv Sci (Weinh), 2021. **8**(18): p. e2100825.
259. Romanò, S., et al., *Label-free spectroscopic characterization of exosomes reveals cancer cell differentiation.* Anal Chim Acta, 2022. **1192**: p. 339359.
260. Ridolfi, A., et al., *AFM-Based High-Throughput Nanomechanical Screening of Single Extracellular Vesicles.* Anal Chem, 2020. **92**(15): p. 10274-10282.
261. Calò, A., et al., *Force measurements on natural membrane nanovesicles reveal a composition-independent, high Young's modulus.* Nanoscale, 2014. **6**(4): p. 2275-85.
262. Vorselen, D., et al., *Competition between Bending and Internal Pressure Governs the Mechanics of Fluid Nanovesicles.* ACS Nano, 2017. **11**(3): p. 2628-2636.
263. Ridolfi, A., et al., *Stiffness of Fluid and Gel Phase Lipid Nanovesicles: Weighting the Contributions of Membrane Bending Modulus and Luminal Pressurization.* Langmuir, 2021. **37**(41): p. 12027-12037.
264. Wiklander, O.P.B., et al., *Systematic Methodological Evaluation of a Multiplex Bead-Based Flow Cytometry Assay for Detection of Extracellular Vesicle Surface Signatures.* Front Immunol, 2018. **9**: p. 1326.

265. Koliha, N., et al., *A novel multiplex bead-based platform highlights the diversity of extracellular vesicles.* J Extracell Vesicles, 2016. **5**: p. 29975.
266. Welsh, J.A., et al., *MPAPASS software enables stitched multiplex, multidimensional EV repertoire analysis and a standard framework for reporting bead-based assays.* Cell Reports Methods, 2022. **2**(1): p. 100136.
267. Feng, D., et al., *Cellular internalization of exosomes occurs through phagocytosis.* Traffic, 2010. **11**(5): p. 675-87.
268. Lundy, S.K., M.W. Klinker, and D.A. Fox, *Killer B lymphocytes and their fas ligand positive exosomes as inducers of immune tolerance.* Front Immunol, 2015. **6**: p. 122.
269. de Rond, L., et al., *Comparison of Generic Fluorescent Markers for Detection of Extracellular Vesicles by Flow Cytometry.* Clin Chem, 2018. **64**(4): p. 680-689.
270. Sandau, U.S., et al., *Methamphetamine use alters human plasma extracellular vesicles and their microRNA cargo: An exploratory study.* J Extracell Vesicles, 2020. **10**(1): p. e12028.
271. Pužar Dominkuš, P., et al., *PKH26 labeling of extracellular vesicles: Characterization and cellular internalization of contaminating PKH26 nanoparticles.* Biochim Biophys Acta Biomembr, 2018. **1860**(6): p. 1350-1361.
272. Simonsen, J.B., *Pitfalls associated with lipophilic fluorophore staining of extracellular vesicles for uptake studies.* J Extracell Vesicles, 2019. **8**(1): p. 1582237.
273. Lim, G.T., et al., *Bioorthogonally surface-edited extracellular vesicles based on metabolic glycoengineering for CD44-mediated targeting of inflammatory diseases.* J Extracell Vesicles, 2021. **10**(5): p. e12077.
274. Roberts-Dalton, H.D., et al., *Fluorescence labelling of extracellular vesicles using a novel thiol-based strategy for quantitative analysis of cellular delivery and intracellular traffic.* Nanoscale, 2017. **9**(36): p. 13693-13706.
275. Tian, T., et al., *Visualizing of the cellular uptake and intracellular trafficking of exosomes by live-cell microscopy.* J Cell Biochem, 2010. **111**(2): p. 488-96.
276. Mittelbrunn, M., et al., *Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells.* Nat Commun, 2011. **2**: p. 282.
277. Joshi, B.S., et al., *Endocytosis of Extracellular Vesicles and Release of Their Cargo from Endosomes.* ACS Nano, 2020. **14**(4): p. 4444-4455.
278. Heusermann, W., et al., *Exosomes surf on filopodia to enter cells at endocytic hot spots, traffic within endosomes, and are targeted to the ER.* J Cell Biol, 2016. **213**(2): p. 173-84.
279. Corso, G., et al., *Systematic characterization of extracellular vesicle sorting domains and quantification at the single molecule - single vesicle level by fluorescence correlation spectroscopy and single particle imaging.* J Extracell Vesicles, 2019. **8**(1): p. 1663043.
280. Dooley, K., et al., *A versatile platform for generating engineered extracellular vesicles with defined therapeutic properties.* Mol Ther, 2021. **29**(5): p. 1729-1743.
281. Mathieu, M., et al., *Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication.* Nat Cell Biol, 2019. **21**(1): p. 9-17.
282. Christianson, H.C., et al., *Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity.* Proc Natl Acad Sci U S A, 2013. **110**(43): p. 17380-5.
283. Elgamal, S., et al., *Imaging intercellular interaction and extracellular vesicle exchange in a co-culture model of chronic lymphocytic leukemia and stromal cells by lattice light-sheet fluorescence microscopy.* Methods Enzymol, 2020. **645**: p. 79-107.
284. Han, C., et al., *Single-vesicle imaging and co-localization analysis for tetraspanin profiling of individual extracellular vesicles.* J Extracell Vesicles, 2021. **10**(3): p. e12047.
285. Willy, N.M., et al., *CALM supports clathrin-coated vesicle completion upon membrane tension increase.* Proc Natl Acad Sci U S A, 2021. **118**(25).
286. Colombo, F., E.G. Norton, and E. Cocucci, *Microscopy approaches to study extracellular vesicles.* Biochim Biophys Acta Gen Subj, 2021. **1865**(4): p. 129752.

287. Chuo, S.T., J.C. Chien, and C.P. Lai, *Imaging extracellular vesicles: current and emerging methods.* J Biomed Sci, 2018. **25**(1): p. 91.
288. Gallego-Perez, D., et al., *On-Chip Clonal Analysis of Glioma-Stem-Cell Motility and Therapy Resistance.* Nano Lett, 2016. **16**(9): p. 5326-32.
289. Panagopoulou, M.S., et al., *Phenotypic analysis of extracellular vesicles: a review on the applications of fluorescence.* J Extracell Vesicles, 2020. **9**(1): p. 1710020.
290. Li, D., et al., *ADVANCED IMAGING. Extended-resolution structured illumination imaging of endocytic and cytoskeletal dynamics.* Science, 2015. **349**(6251): p. aab3500.
291. Coffman, V.C. and J.Q. Wu, *Every laboratory with a fluorescence microscope should consider counting molecules.* Mol Biol Cell, 2014. **25**(10): p. 1545-8.
292. Heddleston, J.M., et al., *A guide to accurate reporting in digital image acquisition - can anyone replicate your microscopy data?* J Cell Sci, 2021. **134**(6).
293. Montero Llopis, P., et al., *Best practices and tools for reporting reproducible fluorescence microscopy methods.* Nat Methods, 2021.
294. Higginbotham, J.N., et al., *Amphiregulin exosomes increase cancer cell invasion.* Curr Biol, 2011. **21**(9): p. 779-86.
295. de Voogt, W.S., M.E. Tanenbaum, and P. Vader, *Illuminating RNA trafficking and functional delivery by extracellular vesicles.* Adv Drug Deliv Rev, 2021. **174**: p. 250-264.
296. Jaqaman, K., et al., *Robust single-particle tracking in live-cell time-lapse sequences.* Nat Methods, 2008. **5**(8): p. 695-702.
297. Aguet, F., et al., *Advances in analysis of low signal-to-noise images link dynamin and AP2 to the functions of an endocytic checkpoint.* Dev Cell, 2013. **26**(3): p. 279-91.
298. Berne, B.J. and R. Pecora, *Dynamic light scattering : with applications to chemistry, biology, and physics*. 1976: Wiley.
299. ISO, *Particle size analysis — Dynamic light scattering (DLS)*. 2017.
300. Hackley, V.A. and J.D. Clogston, *Measuring the hydrodynamic size of nanoparticles in aqueous media using batch-mode dynamic light scattering.* Methods Mol Biol, 2011. **697**: p. 35-52.
301. Stetefeld, J., S.A. McKenna, and T.R. Patel, *Dynamic light scattering: a practical guide and applications in biomedical sciences.* Biophys Rev, 2016. **8**(4): p. 409-427.
302. Provencher, S.W., *A constrained regularization method for inverting data represented by linear algebraic or integral equations.* Computer Physics Communications, 1982. **27**(3): p. 213-227.
303. Palmieri, V., et al., *Dynamic light scattering for the characterization and counting of extracellular vesicles: a powerful noninvasive tool.* Journal of Nanoparticle Research, 2014. **16**(9): p. 2583.
304. ISO, *Particle size analysis — Dynamic light scattering (DLS)*. 2017.
305. de Vrij, J., et al., *Quantification of nanosized extracellular membrane vesicles with scanning ion occlusion sensing.* Nanomedicine (Lond), 2013. **8**(9): p. 1443-58.
306. Gardiner, C., et al., *Measurement of refractive index by nanoparticle tracking analysis reveals heterogeneity in extracellular vesicles.* J Extracell Vesicles, 2014. **3**: p. 25361.
307. van der Pol, E., et al., *Refractive index determination of nanoparticles in suspension using nanoparticle tracking analysis.* Nano Lett, 2014. **14**(11): p. 6195-201.
308. Walker, J.G., *Improved nano-particle tracking analysis.* Measurement Science and Technology, 2012. **23**(6): p. 065605.
309. Hole, P., et al., *Interlaboratory comparison of size measurements on nanoparticles using nanoparticle tracking analysis (NTA).* J Nanopart Res, 2013. **15**: p. 2101.
310. Vestad, B., et al., *Size and concentration analyses of extracellular vesicles by nanoparticle tracking analysis: a variation study.* J Extracell Vesicles, 2017. **6**(1): p. 1344087.
311. Kestens, V., et al., *Validation of a particle tracking analysis method for the size determination of nano- and microparticles.* J Nanopart Res, 2017. **19**(8): p. 271.
312. Smith, E. and G. Dent, *Modern Raman Spectroscopy: A Practical Approach.* Modern Raman Spectroscopy: A Practical Approach, 2005: p. 1-210.

313. Jones, R.R., et al., *Raman Techniques: Fundamentals and Frontiers.* Nanoscale Res Lett, 2019. **14**(1): p. 231.
314. Langer, J., et al., *Present and Future of Surface-Enhanced Raman Scattering.* ACS Nano, 2020. **14**(1): p. 28-117.
315. Smith, Z.J., et al., *Single exosome study reveals subpopulations distributed among cell lines with variability related to membrane content.* J Extracell Vesicles, 2015. **4**: p. 28533.
316. Gualerzi, A., et al., *Raman spectroscopy uncovers biochemical tissue-related features of extracellular vesicles from mesenchymal stromal cells.* Sci Rep, 2017. **7**(1): p. 9820.
317. Kwizera, E.A., et al., *Molecular Detection and Analysis of Exosomes Using Surface-Enhanced Raman Scattering Gold Nanorods and a Miniaturized Device.* Theranostics, 2018. **8**(10): p. 2722-2738.
318. Ma, D., et al., *Quantitative detection of exosomal microRNA extracted from human blood based on surface-enhanced Raman scattering.* Biosens Bioelectron, 2018. **101**: p. 167-173.
319. Lee, W., et al., *Label-Free Prostate Cancer Detection by Characterization of Extracellular Vesicles Using Raman Spectroscopy.* Anal Chem, 2018. **90**(19): p. 11290-11296.
320. Carlomagno, C., et al., *Raman Fingerprint of Extracellular Vesicles and Conditioned Media for the Reproducibility Assessment of Cell-Free Therapeutics.* Front Bioeng Biotechnol, 2021. **9**: p. 640617.
321. Gualerzi, A., et al., *Raman spectroscopy as a quick tool to assess purity of extracellular vesicle preparations and predict their functionality.* J Extracell Vesicles, 2019. **8**(1): p. 1568780.
322. Rojalin, T., et al., *Hybrid Nanoplasmonic Porous Biomaterial Scaffold for Liquid Biopsy Diagnostics Using Extracellular Vesicles.* ACS Sens, 2020. **5**(9): p. 2820-2833.
323. Park, J., et al., *Exosome Classification by Pattern Analysis of Surface-Enhanced Raman Spectroscopy Data for Lung Cancer Diagnosis.* Anal Chem, 2017. **89**(12): p. 6695-6701.
324. Enciso-Martinez, A., et al., *Synchronized Rayleigh and Raman scattering for the characterization of single optically trapped extracellular vesicles.* Nanomedicine, 2020. **24**: p. 102109.
325. Raj, A., et al., *Toward standardization of Raman spectroscopy: Accurate wavenumber and intensity calibration using rotational Raman spectra of H-2, HD, D-2, and vibration-rotation spectrum of O-2.* Journal of Raman Spectroscopy, 2020. **51**(10): p. 2066-2082.
326. Cameron, J.M., et al., *Biofluid spectroscopic disease diagnostics: A review on the processes and spectral impact of drying.* J Biophotonics, 2018. **11**(4): p. e201700299.
327. Butler, H.J., et al., *Using Raman spectroscopy to characterize biological materials.* Nat Protoc, 2016. **11**(4): p. 664-87.
328. Hogg, W.R. and W.H. Coulter, *Apparatus and method for measuring a dividing particle size of a particulate system*. 1967: United States.
329. Coumans, F.A., et al., *Reproducible extracellular vesicle size and concentration determination with tunable resistive pulse sensing.* J Extracell Vesicles, 2014. **3**: p. 25922.
330. Cimorelli, M., et al., *Standardized procedure to measure the size distribution of extracellular vesicles together with other particles in biofluids with microfluidic resistive pulse sensing.* PLoS One, 2021. **16**(4): p. e0249603.
331. Welsh, J.A., et al., *A simple, high-throughput method of protein and label removal from extracellular vesicle samples.* Nanoscale, 2021. **13**(6): p. 3737-3745.
332. Morales-Kastresana, A., et al., *High-fidelity detection and sorting of nanoscale vesicles in viral disease and cancer.* J Extracell Vesicles, 2019. **8**(1): p. 1597603.
333. van der Pol, E., et al., *Absolute sizing and label-free identification of extracellular vesicles by flow cytometry.* Nanomedicine, 2018. **14**(3): p. 801-810.
334. Welsh, J.A., et al., *FCMPASS Software Aids Extracellular Vesicle Light Scatter Standardization.* Cytometry A, 2020. **97**(6): p. 569-581.
335. Gorgens, A., et al., *Optimisation of imaging flow cytometry for the analysis of single extracellular vesicles by using fluorescence-tagged vesicles as biological reference material.* J Extracell Vesicles, 2019. **8**(1): p. 1587567.

336. Welsh, J.A., et al., *MIFlowCyt-EV: The Next Chapter in the Reporting and Reliability of Single Extracellular Vesicle Flow Cytometry Experiments.* Cytometry A, 2020.
337. Daaboul, G.G., et al., *Digital Detection of Exosomes by Interferometric Imaging.* Sci Rep, 2016. **6**(1): p. 37246.
338. Dogrammatzis, C., et al., *Diverse Populations of Extracellular Vesicles with Opposite Functions during Herpes Simplex Virus 1 Infection.* J Virol, 2021. **95**(6).
339. Young, G., et al., *Quantitative mass imaging of single biological macromolecules.* Science, 2018. **360**(6387): p. 423-427.
340. de Rond, L., et al., *Deriving Extracellular Vesicle Size From Scatter Intensities Measured by Flow Cytometry.* Curr Protoc Cytom, 2018. **86**(1): p. e43.
341. Hell, S.W. and J. Wichmann, *Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy.* Opt Lett, 1994. **19**(11): p. 780-2.
342. Klar, T.A. and S.W. Hell, *Subdiffraction resolution in far-field fluorescence microscopy.* Opt Lett, 1999. **24**(14): p. 954-6.
343. Rust, M.J., M. Bates, and X.W. Zhuang, *Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM).* Nature Methods, 2006. **3**(10): p. 793-795.
344. Wombacher, R., et al., *Live-cell super-resolution imaging with trimethoprim conjugates.* Nature Methods, 2010. **7**(9): p. 717-719.
345. Betzig, E., et al., *Imaging intracellular fluorescent proteins at nanometer resolution.* Science, 2006. **313**(5793): p. 1642-1645.
346. Hess, S.T., T.P.K. Girirajan, and M.D. Mason, *Ultra-high resolution imaging by fluorescence photoactivation localization microscopy.* Biophysical Journal, 2006. **91**(11): p. 4258-4272.
347. Nizamudeen, Z., et al., *Rapid and accurate analysis of stem cell-derived extracellular vesicles with super resolution microscopy and live imaging.* Biochim Biophys Acta Mol Cell Res, 2018. **1865**(12): p. 1891-1900.
348. Zong, S., et al., *Single molecule localization imaging of exosomes using blinking silicon quantum dots.* Nanotechnology, 2018. **29**(6): p. 065705.
349. Sharma, S., et al., *Impact of isolation methods on the biophysical heterogeneity of single extracellular vesicles.* Sci Rep, 2020. **10**(1): p. 13327.
350. Mondal, A., et al., *Effective Visualization and Easy Tracking of Extracellular Vesicles in Glioma Cells.* Biol Proced Online, 2019. **21**: p. 4.
351. Chen, C., et al., *Imaging and Intracellular Tracking of Cancer-Derived Exosomes Using Single-Molecule Localization-Based Super-Resolution Microscope.* ACS Appl Mater Interfaces, 2016. **8**(39): p. 25825-25833.
352. Lennon, K.M., et al., *Single molecule characterization of individual extracellular vesicles from pancreatic cancer.* Journal of extracellular vesicles, 2019. **8**(1): p. 1685634-1685634.
353. Sanada, T., et al., *Transmission of HBV DNA Mediated by Ceramide-Triggered Extracellular Vesicles.* Cell Mol Gastroenterol Hepatol, 2017. **3**(2): p. 272-283.
354. Wang, X., et al., *14-3-3zeta delivered by hepatocellular carcinoma-derived exosomes impaired anti-tumor function of tumor-infiltrating T lymphocytes.* Cell Death Dis, 2018. **9**(2): p. 159.
355. Avalos-Padilla, Y., et al., *The ESCRT-III machinery participates in the production of extracellular vesicles and protein export during Plasmodium falciparum infection.* PLoS Pathog, 2021. **17**(4): p. e1009455.
356. Maire, C.L., et al., *Genome-wide methylation profiling of glioblastoma cell-derived extracellular vesicle DNA allows tumor classification.* Neuro Oncol, 2021. **23**(7): p. 1087-1099.
357. Chen, C., et al., *Visualization and intracellular dynamic tracking of exosomes and exosomal miRNAs using single molecule localization microscopy.* Nanoscale, 2018. **10**(11): p. 5154-5162.
358. Oleksiuk, O., et al., *Single-Molecule Localization Microscopy allows for the analysis of cancer metastasis-specific miRNA distribution on the nanoscale.* Oncotarget, 2015. **6**(42): p. 44745-57.

359. Polanco, J.C., et al., *Exosomes taken up by neurons hijack the endosomal pathway to spread to interconnected neurons.* Acta Neuropathol Commun, 2018. **6**(1): p. 10.
360. Pfeiler, S., et al., *CD36-triggered cell invasion and persistent tissue colonization by tumor microvesicles during metastasis.* Faseb j, 2019. **33**(2): p. 1860-1872.
361. Toda, H., et al., *Plasma-derived extracellular vesicles from Plasmodium vivax patients signal spleen fibroblasts via NF-kB facilitating parasite cytoadherence.* Nature Communications, 2020. **11**(1).
362. Ambrose, A.R., et al., *Corrected Super-Resolution Microscopy Enables Nanoscale Imaging of Autofluorescent Lung Macrophages.* Biophys J, 2020. **119**(12): p. 2403-2417.
363. Valcz, G., et al., *En bloc release of MVB-like small extracellular vesicle clusters by colorectal carcinoma cells.* Journal of Extracellular Vesicles, 2019. **8**(1).
364. Hebisch, E., et al., *A protocol for registration and correction of multicolour STED superresolution images.* J Microsc, 2017. **267**(2): p. 160-175.
365. Churchman, L.S. and J.A. Spudich, *Colocalization of fluorescent probes: accurate and precise registration with nanometer resolution.* Cold Spring Harbor protocols, 2012. **2012**(2): p. 141-149.
366. Gould, T.J., S.T. Hess, and J. Bewersdorf, *Optical nanoscopy: from acquisition to analysis.* Annu Rev Biomed Eng, 2012. **14**: p. 231-54.
367. Khater, I.M., I.R. Nabi, and G. Hamarneh, *A Review of Super-Resolution Single-Molecule Localization Microscopy Cluster Analysis and Quantification Methods.* Patterns (N Y), 2020. **1**(3): p. 100038.
368. Verweij, F.J., et al., *The power of imaging to understand extracellular vesicle biology in vivo.* Nat Methods, 2021.
369. Trajkovic, K., et al., *Ceramide triggers budding of exosome vesicles into multivesicular endosomes.* Science, 2008. **319**(5867): p. 1244-7.
370. Bonsergent, E., et al., *Quantitative characterization of extracellular vesicle uptake and content delivery within mammalian cells.* Nat Commun, 2021. **12**(1): p. 1864.
371. Ortiz, A., et al., *An Interferon-Driven Oxysterol-Based Defense against Tumor-Derived Extracellular Vesicles.* Cancer Cell, 2019. **35**(1): p. 33-45.e6.
372. Kleinjan, A., et al., *Microparticles in vascular disorders: how tissue factor-exposing vesicles contribute to pathology and physiology.* Thromb Res, 2012. **130 Suppl 1**: p. S71-3.
373. Verweij, F.J., et al., *Quantifying exosome secretion from single cells reveals a modulatory role for GPCR signaling.* J Cell Biol, 2018. **217**(3): p. 1129-1142.
374. Russell, J.C., et al., *Composition of Caenorhabditis elegans extracellular vesicles suggests roles in metabolism, immunity, and aging.* Geroscience, 2020. **42**(4): p. 1133-1145.
375. Nikonorova, I.A., et al., *Isolation, profiling, and tracking of extracellular vesicle cargo in Caenorhabditis elegans.* Curr Biol, 2022.
376. Russell, J.C., et al., *Purification and Analysis of Caenorhabditis elegans Extracellular Vesicles.* J Vis Exp, 2020(157).
377. Verweij, F.J., et al., *Live Tracking of Inter-organ Communication by Endogenous Exosomes In Vivo.* Dev Cell, 2019. **48**(4): p. 573-589.e4.
378. Gross, J.C., et al., *Active Wnt proteins are secreted on exosomes.* Nat Cell Biol, 2012. **14**(10): p. 1036-45.
379. Beckett, K., et al., *Drosophila S2 cells secrete wingless on exosome-like vesicles but the wingless gradient forms independently of exosomes.* Traffic, 2013. **14**(1): p. 82-96.
380. Budnik, V., C. Ruiz-Cañada, and F. Wendler, *Extracellular vesicles round off communication in the nervous system.* Nat Rev Neurosci, 2016. **17**(3): p. 160-72.
381. Beer, K.B., et al., *Extracellular vesicle budding is inhibited by redundant regulators of TAT-5 flippase localization and phospholipid asymmetry.* Proc Natl Acad Sci U S A, 2018. **115**(6): p. E1127-e1136.
382. Hyenne, V., et al., *RAL-1 controls multivesicular body biogenesis and exosome secretion.* J Cell Biol, 2015. **211**(1): p. 27-37.
383. Gradilla, A.C., et al., *Exosomes as Hedgehog carriers in cytoneme-mediated transport and secretion.* Nat Commun, 2014. **5**: p. 5649.

384. Mattes, B., et al., *Wnt/PCP controls spreading of Wnt/β-catenin signals by cytonemes in vertebrates.* Elife, 2018. **7**.
385. Peinado, H., et al., *Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET.* Nat Med, 2012. **18**(6): p. 883-91.
386. Costa-Silva, B., et al., *Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver.* Nat Cell Biol, 2015. **17**(6): p. 816-26.
387. Zomer, A., et al., *In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior.* Cell, 2015. **161**(5): p. 1046-1057.
388. Hoshino, A., et al., *Tumour exosome integrins determine organotropic metastasis.* Nature, 2015. **527**(7578): p. 329-35.
389. Mardahl, M., A. Borup, and P. Nejsum, *A new level of complexity in parasite-host interaction: The role of extracellular vesicles.* Adv Parasitol, 2019. **104**: p. 39-112.
390. Oliveira, D.L., et al., *Characterization of yeast extracellular vesicles: evidence for the participation of different pathways of cellular traffic in vesicle biogenesis.* PLoS One, 2010. **5**(6): p. e11113.
391. Zhao, K., et al., *Extracellular vesicles secreted by Saccharomyces cerevisiae are involved in cell wall remodelling.* Commun Biol, 2019. **2**: p. 305.
392. Wood, C.R., et al., *The cilium secretes bioactive ectosomes.* Curr Biol, 2013. **23**(10): p. 906-11.
393. Baldrich, P., et al., *Plant Extracellular Vesicles Contain Diverse Small RNA Species and Are Enriched in 10- to 17-Nucleotide "Tiny" RNAs.* Plant Cell, 2019. **31**(2): p. 315-324.
394. He, B., et al., *RNA-binding proteins contribute to small RNA loading in plant extracellular vesicles.* Nat Plants, 2021. **7**(3): p. 342-352.
395. Liégeois, S., et al., *The V0-ATPase mediates apical secretion of exosomes containing Hedgehog-related proteins in Caenorhabditis elegans.* J Cell Biol, 2006. **173**(6): p. 949-61.
396. Wehman, A.M., et al., *The P4-ATPase TAT-5 inhibits the budding of extracellular vesicles in C. elegans embryos.* Curr Biol, 2011. **21**(23): p. 1951-9.
397. Hyenne, V., et al., *Studying the Fate of Tumor Extracellular Vesicles at High Spatiotemporal Resolution Using the Zebrafish Embryo.* Dev Cell, 2019. **48**(4): p. 554-572.e7.
398. Matusek, T., et al., *The ESCRT machinery regulates the secretion and long-range activity of Hedgehog.* Nature, 2014. **516**(7529): p. 99-103.
399. Koles, K., et al., *Mechanism of evenness interrupted (Evi)-exosome release at synaptic boutons.* J Biol Chem, 2012. **287**(20): p. 16820-34.
400. Korkut, C., et al., *Regulation of postsynaptic retrograde signaling by presynaptic exosome release.* Neuron, 2013. **77**(6): p. 1039-46.
401. Tassetto, M., M. Kunitomi, and R. Andino, *Circulating Immune Cells Mediate a Systemic RNAi-Based Adaptive Antiviral Response in Drosophila.* Cell, 2017. **169**(2): p. 314-325.e13.
402. Jewett, K.A., et al., *Glucocerebrosidase reduces the spread of protein aggregation in a Drosophila melanogaster model of neurodegeneration by regulating proteins trafficked by extracellular vesicles.* PLoS Genet, 2021. **17**(2): p. e1008859.
403. Yang, T., et al., *Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in Danio rerio.* Pharm Res, 2015. **32**(6): p. 2003-14.
404. Shtam, T., et al., *Plasma exosomes stimulate breast cancer metastasis through surface interactions and activation of FAK signaling.* Breast Cancer Res Treat, 2019. **174**(1): p. 129-141.
405. Kobayashi-Sun, J., et al., *Uptake of osteoblast-derived extracellular vesicles promotes the differentiation of osteoclasts in the zebrafish scale.* Commun Biol, 2020. **3**(1): p. 190.
406. Scott, A., et al., *In Vivo Characterization of Endogenous Cardiovascular Extracellular Vesicles in Larval and Adult Zebrafish.* Arterioscler Thromb Vasc Biol, 2021. **41**(9): p. 2454-2468.
407. Sung, B.H., et al., *Directional cell movement through tissues is controlled by exosome secretion.* Nat Commun, 2015. **6**: p. 7164.
408. Zomer, A., et al., *Studying extracellular vesicle transfer by a Cre-loxP method.* Nat Protoc, 2016. **11**(1): p. 87-101.

409. Ghoroghi, S., et al., *Ral GTPases promote breast cancer metastasis by controlling biogenesis and organ targeting of exosomes.* Elife, 2021. **10**.
410. de Oliveira, H.C., et al., *Biogenesis of Fungal Extracellular Vesicles: What Do We Know?* Curr Top Microbiol Immunol, 2021. **432**: p. 1-11.
411. de Oliveira, H.C., et al., *Pathogenic Delivery: The Biological Roles of Cryptococcal Extracellular Vesicles.* Pathogens, 2020. **9**(9).
412. Buck, A.H., et al., *Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity.* Nat Commun, 2014. **5**: p. 5488.
413. Coakley, G., et al., *Extracellular Vesicles from a Helminth Parasite Suppress Macrophage Activation and Constitute an Effective Vaccine for Protective Immunity.* Cell Rep, 2017. **19**(8): p. 1545-1557.

15 Tables

Table 1 Classification of extracellular vesicles (EV) subtypes

EV classification	MISEV2018 recommendations	MISEV2022
1. EV class markers	No generic markers for either exosomes or microvesicles	Still valid, no universally recognized markers for specific EV subtypes.
2. EV subtype operational terms	Encouraged, based on either physical or biochemical criteria. (a) Physical characteristics, e.g. diameter: small extracellular vesicles (sEVs) < 200 nm medium/large EVs (m/lEVs) > 200 nm density: low, medium, high (defined ranges) (b) Biochemical composition, e.g., contain a specific protein (c) Cellular origin and/or conditions generated	The use of operational terms and size limitations is still recommended but recognized as subjective depending on the isolation method used i.e., within a study small and large EVs could be defined by filter cutoff, or centrifugation steps. In any case, provide definition of EV subset within the context of its isolation.
3. Other EV names	Define clearly and prominently	Still valid, but criteria updated
3. Other names	N.A.	Extracellular particles (EPs), particles for which EV identity cannot be confirmed by MISEV2022 criteria. Non-vesicular extracellular particles (NVEP), particle which do not contain a lipid bilayer.

Table 2 EV separation considerations. Abbreviations used: PEG (polyethylene glycol), MW (molecular weight), SEC (size exclusion chromatography, dUC (differential ultracentrifugation), small extracellular vesicles (sEVs).

EV separation and enrichment	MISEV2018 recommendations	MISEV2022 update
1. Method choice	Consider EV recovery vs specificity, which is biofluid-dependent: high recovery, low specificity: all or most of concentrated secretome, e.g., PEG-based kits, low MW cut off filtration. Intermediate recovery, specificity: mixed EVs plus protein, e.g., SEC, dUC, low recovery, high specificity: EV subtypes, plus protein; but high recovery for specific EV subtypes, e.g., affinity isolation, density gradient. high recovery, specificity, suggested may not have been currently possible.	Still valid Additional methods involving new principles. Other extracellular particles are being identified and/or more clearly defined, with new methods to specifically isolate them emerging.
2. Comprehensive reporting to aid reproducibility	Use of EV-TRACK knowledgebase to facilitate.	Still valid. Key additional information for new techniques, included in revised checklist??
3. Procedural controls	Some procedures modify EVs, e.g., by binding to EVs, so particularly important to include controls for such processes.	Still valid. While affinity isolation methods may modify EVs and impact on functional analysis, they may provide powerful approaches for biomarker analysis.

Table 3 Protein content-based EV characterization. At least one protein of categories 1a or 1b, 2a (optionally 2b), 3a or 3b must be analyzed to demonstrate the EV nature and the degree of purity of an EV preparation. Analysis of proteins of category 4 is required when claiming specific analysis of small EVs, and of category 5 to document functional activities. Examples of proteins commonly found in mammalian cell-derived EVs are provided, but other proteins that fall into the provided categories can be used, particularly for analysis of EVs from prokaryotic (bacteria) or non-mammalian eukaryotic sources (including parasites and plants). *XX* = human gene names. *XX** or *XX*** used for families of multiple proteins, for example for integrins: *ITGA** indicates any integrin alpha chain.

Category	**1- Transmembrane or GPI-anchored proteins associated to plasma membrane and/or endosomes**	**2- Cytosolic proteins recovered in EVs**	**3- Major components of non-EV co-isolated structures**	**4- Transmembrane, lipid-bound and soluble proteins associated to other intracellular compartments than PM/endosomes**	**5- Secreted proteins recovered with EVs**
Use for	All EVs	All EVs	All EVs as purity control	Subtypes of EVs (e.g. large oncosomes, large EVs) and/or pathologic/atypical state	Functional component of EVs: need to determine the mode of association with EVs
	1a: non-tissue specific. Tetraspanins (CD63, CD81, CD82); other multi-pass membrane proteins (CD47; heterotrimeric G proteins GNA*) MHC class I (HLA-A/B/C, H2-K/D/Q), Integrins (ITGA*/ITGB*), transferrin receptor (TFR2); LAMP1/2; heparan sulfate proteoglycans including syndecans (SDC*); EMMPRIN (BSG); ADAM10; GPI-anchored 5′nucleotidase CD73 (NT5E), complement-binding proteins CD55 and CD59; sonic hedgehog (SHH)	2a: with lipid or membrane protein-binding ability. ESCRT-I/II/III (TSG101, CHMP*) and accessory proteins: ALIX (PDCD6IP), VPS4A/B; ARRDC1; Flotillins-1 and 2 (FLOT1/2); caveolins (CAV*); EHD*; RHOA; annexins (ANXA*); Heat shock proteins HSC70 (HSPA8), and HSP84 (HSP90AB1) note that both are abundant also in exomeres; ARF6; syntenin (SDCBP); microtubule-associated Tau (MAPT, neurons)	3a: lipoproteins (produced by liver, abundant in plasma, serum). ApolipoproteinsA1/2 and B APOA1/2, APOB; APOB100; albumin (ALB)	4a: nucleus. Histones (HIST1H**); Lamin A/C (LMNA)	5a: Cytokines and growth factors. e.g. TGFB1/2; IFNG, VEGFA, FGF1/2, PDGF*, EGF, interleukins (IL*)…
	1b: cell/tissue specific. Some TSPANs: TSPAN8 (epithelial cell), CD37 and CD53 (leukocytes), CD9 (absent from NK, B and some MSC); PECAM1 (endothelial cells); ERBB2 (breast cancer); EPCAM (epithelial); CD90 (THY1) (MSCs); CD45 (PTPRC) (immune cells), CD41 (ITGA2B) or CD42a (GP9) (platelets); Glycophorin A (GYPA) (RBC); CD14 (monocytes), MHC class II (HLA-DR/DP/DQ, H2-A*); CD3* (T cells); Acetylcholinesterase/AChE-	2b: promiscuous incorporation in EVs (and possibly exomeres). Heat shock protein HSP70 (HSPA1A), cytoskeleton: actin (ACT*), tubulin (TUB*); enzymes (GAPDH)	3b: protein and protein/nucleic acid aggregates. Tamm-Horsfall protein (Uromodulin/UMOD) (urine); ribosomal proteins	4b: mitochondria IMMT, cytochrome C (CYC1); TOMM20	5b: adhesion and extracellular matrix proteins. Fibronectin (FN1), Collagen (COL**), MFGE8; galectin3-binding protein (LGALS3BP), CD5L; fetuin-A (AHSG)

	S (neurons), AChE-E (erythrocytes); amyloid beta A4/APP (neurons); multidrug resistance-associated protein (ABCC1)	
		4c: secretory pathway (endoplasmic reticulum, Golgi apparatus) calnexin (CANX); Grp94 (HSP90B1); BIP (HSPA5), GM130 (GOLGA2)
		4d: others (autophagosomes, cytoskeleton…). ATG9A, Actinin1/4 (ACTN1/4), cytokeratin 18 (KRT18)

Table 4 Experimental analysis of EV activity.

EV function	MISEV2018 recommendations	MISEV2022 update
1. EV subtype specificity	Analysis of all fractions of source material to avoid missing EV subtype activity.	Comparative analysis of EVs from the same cell type +/- drug, stress or kd, can help to highlight specific functions
2. EV normalization	Dependent on scientific question, normalize EVs relative to a source material or EV characteristic, e.g. cell or EV protein levels, or by co-isolation standards, e.g. added tracer. Report and justify normalization strategy, but no other specific recommendation, survey also suggested no consensus.	There is still a lack of clarity about the most biologically relevant factor to normalize against. To circumvent this one option is to use at least two different and relatively independent normalization methods, e.g., relative to EV-secreting cell (number or protein content) and an EV-specific measure (number or protein content).
3. No cell-cell contact	An EV-associated function can be reinforced if transfer can be shown between two cells that are not in contact, e.g. via trans-well co-culture. It may, however, be difficult to mimic physiological conditions or cell-cell contact may also be required.	EV-mediated transfer may require signaling structures, eg cytonemes, tunnelling nanotubes, filopodia surfing, see **Section 12.2**.
4. EVs vs soluble factors	To argue an activity is EV-borne, show it is enriched in EVs versus same amount of EV-depleted biofluid. It is, however, important to note that EVs and soluble mediators may have synergistic activities.	For EV enrichment, SEC or DGC may be preferable to dUC. These methods can be combined two approaches used to bolster the link between EVs and an activity. To strengthen conclusions both EV dose response and depletion studies are encouraged.
5. EVs vs co-isolates	Determine if activity is due to EVs, or co-isolated components or both, and explain if too little material to do this. Co-isolates include lipoproteins ribonucleoprotein aggregates, and exomeres.	Some co-isolated components have been separated by combining isolation methods (see **4** above) or new methodologies, e.g., by extended high-speed ultracentrifugation, allowing more extensive analysis (see **Section 2**).
6. Exosome-specific	Preparations include exosomes and other types of sEV potentially with different functions. There are important caveats to approaches suggested to selectively reduce or enhance exosome secretion, including genetic manipulation and pharmacological treatments (Section 6.1)	Immunocapture may provide a complementary method for blocking specific EV subtypes. The urgent need for more specific inhibitory genetic or pharmacological treatments remains.
7. EV components	Knockdown or knockout often used to conclude specific protein or RNA is responsible. There is, however, a need to characterize EVs released from modified cells to confirm these are otherwise unaffected. If not possible, limitations need to be explained.	For surface molecules, compare inhibition of specific protein expression in secreting cells with blockade using a neutralizing antibody. Careful consideration of synergistic functions – could blockade of multiple components give the same result.

8. EV source specific	Care is required in claiming specificity or generality of different EVs in the absence of molecular characterization and analysis of other secreting cell types	Remains valid
9. Application of EVs		Consider whether EVs should be added acutely or chronically, and whether they should be added as a bolus or by changing culture medium. All methods are non-physiological, since they do not mirror normal tissue EV release processes.

Table 5 Journal of extracellular vesicles published positions papers

Title	Year	Ref
Standardization of sample collection, isolation and analysis methods in extracellular vesicle research	2013	[5]
ISEV position paper: extracellular vesicle RNA analysis and bioinformatics	2013	[6]
Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles	2014	[1]
Applying extracellular vesicles-based therapeutics in clinical trials – an ISEV position paper	2015	[7]
Obstacles and opportunities in the functional analysis of extracellular vesicle RNA – an ISEV position paper	2017	[8]
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	[3]
Biological membranes in EV biogenesis, stability, uptake, and cargo transfer: an ISEV position paper arising from the ISEV membranes and EVs workshop	2019	[9]
MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments	2020	[4]
Urinary extracellular vesicles: A position paper by the Urine Task Force of the International Society for Extracellular Vesicles	2021	[10]

Table 6 Studying EV biology using model organisms.
Abbreviations: genetic tractability and human similarity are rated from: weak ("+") to strong ("++++").

***In vivo* models**	**EV secreting cells**	**Other specific strengths**	**Genetic tractability**	**Human similarity**
Budding yeast ***Saccharomyces cerevisiae***	Unicellular yeast, [390, 391]	Whole organism analysis i*n vivo*	++++	+
Green alga ***Chlamydomonas reinhardtii***	Flagellated unicellular algae [392]	Cilia biology	++++	+
Flowering plant ***Arabadopsis thaliana***	Leaf cells [393, 394]	Plant immunity	++++	+
Worm ***Caenorhabditis elegans***	Embryonic cells [395-397]	Whole organism analysis *in vivo*	++++	++
	Ciliated sensory neurons [375]	Cilia biology		
Fly ***Drosophila melanogaster***	Wing imaginal disc [379, 383, 398] [378]	Wnt/Hedgehog morphogen signaling	++++	++
	Synaptic bouton of neuronal axons [399, 400]	Synaptic function		
	Hemocytes [401]	Adaptive immune system model		
	Male secondary cells [2, 29]	Large MVBs: exosome subtype biogenesis. Mated ♀♀: function		
	Muscle cells [402]	Neurodegeneration model		
Zebrafish ***Dario rerio***	Yolk syncytial layer [377, 397, 403]	Transparent animals: EV imaging in bloodstream; target cell biodistribution	+++	+++
	Plasma [404]	Metastasis model		
	Osteoblasts [405]	Fracture healing model		
	Cardiomyocytes [406]	Cardiovascular disease model		
Chicken ***Gallus gallus***	Chorioallantoic membrane (CAM) [407]	High resolution live imaging of cell migration	+	+++

Mouse ***Mus musculus***	Syngeneic grafts, human tumor xenografts [385-388, 408, 409]	Whole organism, pre-clinical metastasis model	++	++++

Table 7 Regulation and functions of EVs from pathological microorganisms

Pathological organisms	**Examples**	**Role of EVs in disease**
Fungi [410]	Yeast *Cryptococcus neoformans [411]*	Suppress host immune response
Protozoa	Trypanosomatids *Trypanosoma cruzi*	
Helminths	Nematodes *Heliogmosomoides polygyrus* [412, 413]	