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Recommendations for Studying *In Situ* Extracellular Vesicles From Solid Tissue

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ABSTRACT

Solid tissue-derived extracellular vesicles (ST-EVs) are extracellular vesicles (EVs) separated directly from solid tissues of both vertebrates and invertebrates. ST-EVs provide a physiologically relevant snapshot of tissue-specific molecular dynamics and can be enriched directly in situ, from tissues in their natural state, preserving the native characteristics of ST-EVs. However, their enrichment presents unique technical challenges compared to EVs derived from biofluids or cell culture media. The need for transparent reporting in ST-EV research is crucial to enhance the reproducibility, comparability, and reliability of research findings. The Solid Tissue Task Force, part of the Scientific Reproducibility Subcommittee of International Society for Extracellular Vesicles, aims to recommend reporting parameters and identify outstanding questions related to the pre-analytical and analytical handling of solid tissues, as well as ST-EV separation and characterization. These steps are essential for advancing the understanding of the biological roles of ST-EVs and their potential clinical applications.

Lucia Paolini and Laura J. Vella contributed equally to this work.

1 | Introduction

Extracellular vesicles (EVs) are nano- to micro-sized particles released by cells into the extracellular space, including body fluids and tissue microenvironments. EVs facilitate intercellular communication by transferring molecular cargo to recipient cells. Due to their involvement in physiological and pathological processes, EVs are being investigated for their therapeutic and diagnostic applications (Welsh et al. 2024).

Solid tissue-derived EVs (ST-EVs) refer to EVs separated from biological tissues rather than the more commonly studied sources of EVs, such as conditioned culture media or body fluids (e.g., blood, urine) (Théry et al. 2018; Welsh et al. 2024). ST-EVs can be harvested directly from solid tissue (in situ EVs) in their natural state (Perez-Gonzalez et al. 2012; Polanco et al. 2016; Jeurissen et al. 2017; Vella et al. 2017; Bottini et al. 2018; Crescitelli et al. 2020; Huang et al. 2020) or after culturing the tissue or tissue-derived cells *in vitro* (Jeurissen et al. 2017; Jingushi et al. 2018). This article will focus on the first strategy, which preserves the tissue's original characteristics without the need for culture or expansion, and will also address the unique challenges associated with it. ST-EVs are released by cells present in the organ, which include multiple cell types, such as epithelial cells, immune cells, or stromal cells. Notably, ST-EVs provide a direct representation of the originating tissue, providing a physiologically relevant snapshot of cellular and molecular dynamics that may be lost or diluted in biofluid-derived EVs.

A pioneering study published in 2012 was the first to enrich and characterize EVs from a solid tissue, in that case, mouse and human brain regions (Perez-Gonzalez et al. 2012) (Figure 1A1, A2). The authors developed an enzymatic digestion protocol to separate vesicles from both fresh and post-mortem frozen brain tissues. The method proved effective even with long-term frozen samples, as the structural and molecular integrity of the ST-EVs was preserved. Since its introduction, this original method (Perez-Gonzalez et al. 2012) has been widely adapted and modified in subsequent research (Hurwitz et al. 2018; Crescitelli et al. 2020, 2021; Lässer et al. 2021), focused mainly on brain tissue (Vella et al. 2017; Hurwitz et al. 2019). In 2019, a protocol for enriching ST-EVs from human melanoma tissue was published (Jang et al. 2019; Crescitelli et al. 2021). Using transmission electron microscopy, membrane vesicle structures were visualized in the interstitial fluid (extracellular space) of melanoma tissue (Figure 1B). A few years later, the same authors further improved the imaging of EVs within tissues with three-dimensional electron tomography to reconstruct the spatial arrangement of EVs (Olofsson Bagge et al. 2023) (Figure 1C1, C2), enhancing our understanding of the distribution and structure ST-EVs in their native environment.

ST-EVs have now been separated from both invertebrates (Russell, Kim, et al. 2020; Russell, Postupna, et al. 2020; Cvjetkovic et al. 2024) and vertebrates, with the majority of studies focusing on vertebrates such as humans and mice (Huang et al. 2020; Park et al. 2021; Crescitelli et al. 2022). Although this manuscript focuses on recommendations valid for ST-EVs from vertebrate tissues, these guidelines are also applicable to invertebrate-derived ST-EVs. Previous studies have utilized diverse tissue types, to explore the composition and functions of ST-EVs (Matejovič et al. 2021; Park et al. 2021; Brenna et al. 2020; Bub et al. 2022; Abdelmohsen et al. 2023; Matamoros-Angles et al. 2024). Papers published to date (June 2025) in the field are summarized in Table S1. The table outlines the types of tissues from vertebrates used as sources of ST-EVs, the condition of the tissues (fresh or frozen), the physiological and pathophysiological contexts, the key techniques employed for ST-EV separation and characterization and the subsequent experiments in which the ST-EVs were utilized.

1.1 | Challenges in Separation of EVs From Solid Tissue

Separation of ST-EVs is challenging. The process of separating EVs from tissue involves multiple steps and requires expert handling. The first step is, usually, tissue dissociation, which is typically achieved via mechanical and/or enzymatic processing of the tissue (Perez-Gonzalez et al. 2012; Polanco et al. 2016; Jeurissen et al. 2017; Vella et al. 2017; Crescitelli et al. 2020; Huang et al. 2020). Dissociation of tissue increases the risks of cell lysis and co-isolation of non-ST-EV particles, formation of EV-mimetics (Welsh et al. 2024), and isolation of other non-EV cellular components (i.e., intracellular vesicles or organelles from cells (Vella et al. 2017) considered to be contaminants in EV preparations (Shimizu et al. 2003; Vilcaes et al. 2021; Solana-Balaguer et al. 2023). Factors such as species, tissue type, storage, post-mortem interval and the physiological or pathological state of the tissue impact the yield and integrity of ST-EV preparation (Matejovič et al. 2021; Abdelmohsen et al. 2023; Lou et al. 2023). Furthermore, improper handling can degrade the tissue and EVs, altering ST-EV content and affecting analyses (Huang et al. 2020; Shen et al. 2023).

Although the steps following tissue dissociation share similarities with the separation of EVs from fluids, a more rigorous and thorough approach is required to address additional confounders that can co-isolate with ST-EVs. Multiple separation and characterization steps are essential for effectively distinguishing ST-EVs from non-EV contaminants and non-vesicular components, thereby accurately determining the composition and function of ST-EVs. These factors explain why ST-EV preparations from solid tissues are referred to as 'enriched' in EVs, rather than 'purified.'

The need for transparent reporting in EV research is crucial to enhance the reproducibility, comparability, and reliability of research findings. Transparent reporting involves clearly defining the methods and protocols used in EV studies, including how EVs are separated and characterized, as well as the source of the EVs, and the specific experimental conditions. This need is underscored by the establishment of the Minimal Information for Studies of Extracellular Vesicles (MISEV) 2023 guidelines by the International Society for Extracellular Vesicles (ISEV) (Welsh et al. 2024), with the support of the ISEV Scientific Reproducibility Task Forces (Welsh et al. 2020; Erdbrügger et al. 2021; Fernandez-Becerra et al. 2023; Welsh et al. 2024; Crescitelli et al. 2025).

To address these challenges and ensure consistency in ST-EV research, these guidelines aim:

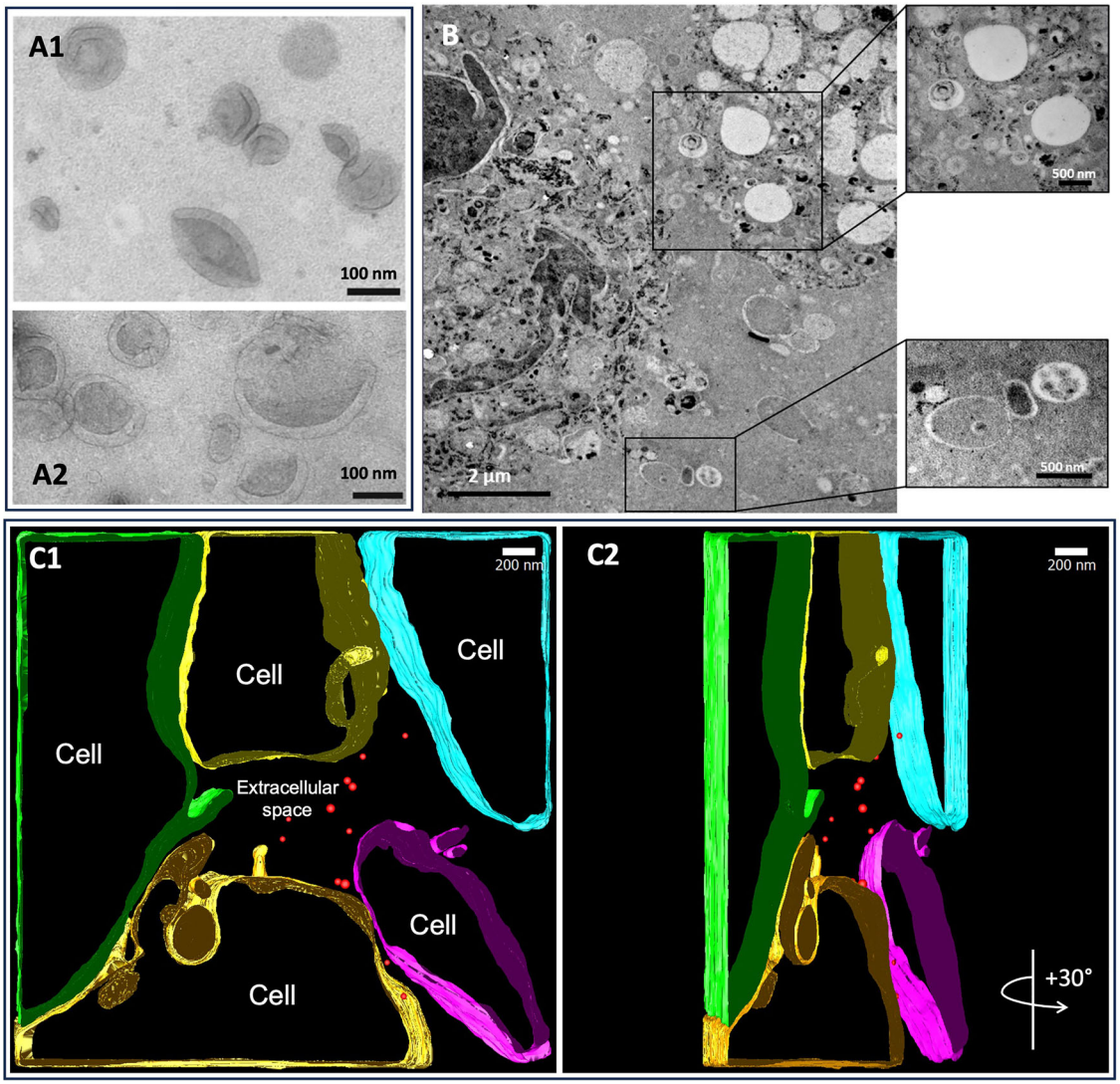

FIGURE 1 | History of ST-EV visualization. First published micrograph of ST-EVs isolated from (A1) murine hemi-brains and (A2) frozen human brain. (B) Representative electron microscopy section of human melanoma metastatic tissue showing cells and ST-EVs. Higher magnification images show different types of ST-EVs in the extracellular space. (C1) The first 3D reconstruction of a tissue focusing on ST-EVs. The cells are visualized by computer-added colours (green, yellow, turquoise, magenta and orange) in the human healthy liver, and the smaller spherical elements in the extracellular space are indicated in red. (C2) 3D model shown rotated at +30° along the y-axis. A1 and A2 are modified, with permission, from Perez-Gonzalez R. et al., J Biol Chem 2012 (Perez-Gonzalez et al. 2012); B1 is modified, with permission, from Jang SC. and Crescitelli R. et al., J Extracell Vesicles 2019 (Jang et al. 2019); C1 and C2 are modified, with permission, from Olofsson Bagge R. et al., J Extracell Vesicles 2023 (Olofsson Bagge et al. 2023).

- to make recommendations on best practice for tissue selection and dissociation, and ST-EV separation.
- to provide standardized reporting guidelines to improve reproducibility.
- to highlight outstanding questions unique to the separation and characterization of ST-EVs.

The manuscript is structured to highlight key recommendations which are summarized in tables (Tables 1–4) at the end of each section. By standardizing how researchers dissociate tissue and subsequently separate, analyse and report on ST-EVs, the field will improve reproducibility, paving the way for significant advancements in understanding ST-EV composition, function, and the application of ST-EVs.

2 | Recommendations and Reporting Guidelines

2.1 | Solid Tissue Handling and Pre-Analytical Variables

Pre-analytical variables in tissue collection and storage can influence the characteristics of tissue specimens. These factors could impact the integrity, molecular composition and functional characteristics of ST-EVs (Ascierto et al. 2019). Ideally, ST-EVs should be enriched from freshly obtained tissues, as this minimizes degradation and preserves ST-EV integrity. However, practical limitations, including feasibility, logistics and accessibility to fresh tissue samples, often necessitate alternative approaches (Huang et al. 2020). Currently, ST-EVs are most commonly

TABLE 1 | Recommendations and reporting guidelines for the tissue specimens.

Recommendations
Human biospecimens · Biospecimens collected from surgery: consider removing blood surrounding the tissue/biopsy by rinsing in saline or PBS. Consider freezing the tissue in liquid nitrogen or at -80°C as soon as possible after excision. Alternatively, store the tissue on ice if proceeding immediately to ST-EV isolation (Moore et al. 2011; Mucci et al. 2013). · Biobanked specimens: use specimens from tissue biobanks with standardized collection, processing and storing procedures, such as those recommended in the BRISQ or NCI guidelines (Moore et al. 2011; Mucci et al. 2013). Where tissues have been stored at -80°C, avoid repeat freeze-thawing. *Animal tissues* · If perfusion is required to clear blood, for example, in the brain (Huang et al. 2020), use an automated perfusion (with pressure and controlled flow rates) over manual perfusion to improve reproducibility and to ensure that artifacts are not generated due to excessive pressure. In the case of the brain, removing the meninges may help reduce blood-derived EV contamination, note that; however, it can cause loss of ST-EVs from the interstitial space (Pérez-González et al. 2017). · Always keep the tissue on ice if proceeding immediately to ST-EV separation to avoid protein degradation (Moore et al. 2011; Mucci et al. 2013)
Reporting guidelines
When applicable report the following: · Species (human or mouse, e.g.), anatomical site and region, tissue weight, collection process (i.e., sterile vs. non-sterile collection, time between tissue collection and preservation/processing, time of warm and/or cold ischemia), type or long-time preservation, whether the tissue is fresh or frozen, number of animals or donors, whether the samples are pooled tissues and/or ST-EVs, In the case of human samples, also patient information (age, gender and diagnosis) and post-mortem interval, name and location of the biobank or clinical organization where specimens were obtained. · Animal specimens: report the number of animals used, sex, strain, age and if perfusion was performed, specify protocol and buffer used. · Any parameters that could have impacted integrity of the tissue (freeze thaw cycles, blood contamination as visible by eye, additives or the presence of visible freeze fracture). · Ethical approval number and approving authority.
Outstanding questions
· What is the impact of collection, processing, and storage conditions on ST-EV recovery, and could pre-analytical variables, such as storage buffers, improve ST-EV yield and integrity? Can ST-EVs be separated from chemically preserved tissues (e.g., formalin-fixed tissues [Yang et al. 2022]) · Does perfusion, as commonly performed in animal models, improve the isolation of ST-EVs from specific tissues? If perfusion is not feasible, what methods can be employed to minimize contamination from blood or lymph

extracted from human and mouse post-mortem tissue that has been fresh frozen at −80°C.

Recommendations for the handling and preservation of human biospecimens are available in the form of Biospecimen Reporting for Improved Study Quality (BRISQ) guidelines (Moore et al. 2011) or U.S. National Cancer Institute's (NCI) Recommendations for Postmortem Recovery of Normal Human Biospecimens for Research (Mucci et al. 2013). However, these recommendations were not specifically designed for the analysis of ST-EVs. The recommendations in (Table 1) are based on BRISQ and NCI guidelines for organ handling and storage and serve as a framework to guide and enable the best quality ST-EVs possible.

2.2 | Solid Tissue Dissociation

ST-EVs are found both as freely diffusing in the interstitial space and as interacting with components of the extracellular matrix (ECM) (Wang et al. 2022; Arif and Moulin 2023; Debnath et al. 2023; Cominal et al. 2024; Smirnova et al. 2024; Tassinari et al. 2024). The dissociation methods for the isolation of ST-EVs enrich for free EVs and likely co-recover ECM or matrix-associated vesicles. The proportion is unclear and likely varies across tissues, depending on the composition of the ECM.

To enrich ST-EVs from intact tissues, several methods have been employed, using either enzymatic or physical procedures. The most commonly used and widely accepted approach involves slicing the tissue to increase the surface area, followed by a mild and brief enzymatic digestion treatment. The enzymatic activity then needs to be stopped by the addition of a protease inhibitor or by enzyme dilution. The enzyme should target the dominant ECM components and be optimized for tissue type, species, and buffer conditions. Although several studies (e.g., Crescitelli et al. 2021) show that enzymatic dissociation can preserve EV surface markers, the enzyme type, concentration and exposure time should be optimized for each tissue, as over-digestion can damage EV epitopes (Martínez-Greene et al. 2023; Matamoros-Angles et al. 2024).

Among the enzymes used, papain was the first to be employed for isolating ST-EVs from brain tissue (Perez-Gonzalez et al. 2012). Later collagenases, such as type III (brain tissues, [Vella et al.

TABLE 2 | Recommendations and reporting guidelines for tissue dissociation.

Recommendations

· Enzymatic digestion is preferred over physical dissociation methods like homogenization or vortexing to reduce artifact formation (Crescitelli et al. 2021).
· To minimize non-specific digestion of cells and ST-EVs, enzyme concentration and digestion duration should be optimized for each tissue type, species and experiment (Matamoros-Angles et al. 2024).
· Keep the tissue on ice at all times (except when required for enzymatic digestion), including using buffers that have been kept on ice or at 4°C (Moore et al. 2011; Mucci et al. 2013).
· Handle the tissue and dissociation products for the minimum time necessary at each step (Moore et al. 2011; Mucci et al. 2013).
· Following enzymatic digestion, inhibit the specific protease used (Vella et al. 2017; Matamoros-Angles et al. 2024).
· Use the same dissociation and digestion protocol for all samples to enable accurate comparison. Ideally, ST-EV separation should be performed simultaneously on all samples, though this may not always be practical.

Reporting guidelines

· Thawing method (on ice/room temperature).
· Preparation steps (slicing parameters, if any).
· Method of dissociation.
· If enzymatic digestion is used, provide details of the enzyme (company, batch, units, concentration, duration and temperature).
· Provide details of the protease inhibitors, when used (company, units, concentration, and temperature).
· Describe temperature and time of each step of the procedure.

Outstanding questions

· How to measure/quantify degree of digestion: have all ST-EVs been released and are the cells intact?
· What are tissue (digestion) parameters that prohibit successful ST-EV separation? What is the optimal balance between sufficient tissue dissociation and preservation of ST-EV integrity?

2017]), type IV (tumours [Fu et al. 2024]) and type D (Crescitelli et al. 2022; Matamoros-Angles et al. 2024), and collagenase with dispase II (adipose tissues [Sabio and Crewe 2023]) or liberase with DNase I (cardiac tissue [Liang, Sagar et al. 2023]) have been demonstrated to be effective for dissociating solid tissues (Sabio and Crewe 2023).

In addition to enzymatic methods, non-enzymatic approaches, such as direct homogenization, tissue teasing with a small spatula, vortexing or crushing tissue between two pieces of ground glass, have been described in the literature (Yelamanchili et al. 2015; Hurwitz et al. 2018; Gallart-Palau et al. 2019; Hurwitz et al. 2019; Abdelmohsen et al. 2023).

Each of these treatments offers specific advantages and limitations. However, it is important to note that harsh physical treatment can damage the plasma membrane, leading to the release of intracellular compartments, microsome formation, and the creation of EV-mimetics (Crescitelli et al. 2021).

Enzymatic treatment is not always necessary (Crescitelli et al. 2021). A recent study demonstrates that an enzyme-free protocol can also achieve effective EV separation (Matamoros-Angles et al. 2024), with potentially reduced impact on EV surface proteins and proteomic profiles. Therefore, the choice of protocol should consider the specific experimental goals, tissue type, and downstream applications. Table 2 summarizes the recommended guidelines for tissue dissociation.

2.3 | Current Practices for ST-EV Separation

ST-EV enrichment after tissue dissociation involves a multi-step procedure. This typically begins with an optional filtration step using a cell strainer, low speed centrifugation for pre-clearing, followed by crude EV isolation, typically by ultracentrifugation (UC) and or separation with either a single or combination of methods including immunoselection (Zhang, Yu, et al. 2023), size exclusion chromatography (SEC) (Huang et al. 2020; Zhang, Yu, et al. 2023), ultrafiltration (UF) (Huang et al. 2020), and density-gradient ultracentrifugation (DG) (Deng et al. 2009; Vella et al. 2017; Hurwitz et al. 2019; Hoshino et al. 2020; Huang et al. 2020; Crescitelli et al. 2021; Zhang, Yu, et al. 2023). Each strategy has unique trade-offs, including processing time, purity, yield and required technical expertise (Hendrix et al. 2023). Due to the diverse range of tissues and species involved in ST-EV separation, a universal standard of practice is not available; however, we want to provide some recommendations for best practices as described below.

Following tissue dissociation, filtration through a 40–100 μm mesh filter (commonly 70 μm) may be performed to remove residual, non-digested tissue fragments or cell clumps. The utility of this step depends on tissue type and dissociation method. A study investigating the benefits or caveats of filtered versus non-filtered dissociated tissue has not been described, as far as the authors are aware. Pre-clearing centrifugation steps are recommended to remove cells, cellular debris, or large apoptotic

bodies (if these vesicles are not required for analysis). Typically, centrifugation times and speeds are 300–500 × *g* for 5–10 min, then 2000–3000 × *g* for 10–20 min, followed by 10,000–16,500 × *g* for 20–30 min at 4°C. If larger vesicles are of interest, the 10,000–16,500 × *g* pellets can be considered for further analysis. All these parameters need to be optimized since they depend on factors like tubes, volumes and whether fixed-angle or swing-out rotors are used. Repeated pre-clearing centrifugation steps for ST-EV enrichment have been published by several groups (Deng et al. 2009; Huleihel et al. 2016; Loyer et al. 2018); however, it is unclear if this is necessary or beneficial.

Following pre-clearing, ST-EV separation is recommended either by UC at 100,000–150,000 × *g* for 90 min to 3 h (Perez-Gonzalez et al. 2012; Cvjetkovic et al. 2014), or by directly processing the 10,000 × *g* supernatant without a crude UC step (Vella et al. 2017; Cheng et al. 2020; Huang et al. 2020; Vassileff et al. 2020). Either way, additional separation of ST-EV from non-EV material is recommended given the complexity of the starting material. Discontinuous density gradients (DG) are most commonly performed with sucrose or iodixanol as medium; various specific protocols have been reported for ST-EV isolation (Pérez-González et al. 2017; Vella et al. 2017; Huang et al. 2020; Crescitelli et al. 2021; Ruan et al. 2021; Mangolini et al. 2024). Bottom-loaded flotation gradients have been reported to improve purity over top-loaded gradients for brain-derived ST-EVs (Hurwitz et al. 2018).

Several studies have provided a direct comparison of DG to non-ultracentrifugation-based methods for ST-EV enrichment (Huang et al. 2020; Zhang, Yu, et al. 2023). Although time consuming, DG-UC yielded the highest purity of ST-EVs and enrichment of EV associated proteins from brain tissue, over SEC and affinity selection (Zhang, Yu, et al. 2023). However, SEC coupled with subsequent UC may improve particle/protein ratio over DG-UC (Huang et al. 2020). A protocol demonstrating efficient absorption of soluble proteins after SEC-enrichment using mixed mode chromatography resin has also been reported (Ter-Ovanesyan et al. 2023; Bonner et al. 2024; Zimmerman et al. 2024). This method could be a valuable approach to adopt in the future, as it has the potential to significantly reduce the lengthy process currently required for separating ST-EVs from tissue. In addition to chromatography-based enrichment, immunoaffinity-based isolation approaches targeting tissue-specific ligands could be used to capture defined EV subtypes. At the same time, fluid-flow-based separation techniques (such as Tangential Flow Filtration and Asymmetric Flow Field-Flow Fractionation), along with microfluidic and acoustic enrichment strategies, could represent alternatives that reduce sample handling and improve the scalability of separation methods (Welsh et al. 2024). A key point to consider is that different separation methods may enrich different EV subpopulations. This can be useful depending on the research question being addressed, but it is also a limitation (Huang et al. 2020).

Each method offers advantages and limitations regarding processing time, cost, ST-EV yield, and ST-EV subpopulation selection, as well as soluble protein contamination. Since no single method is universally suitable for all tissue types, protocols must be tailored to fit the specific requirements of each tissue. However, key factors need to be considered, and recommendations can be made to improve consistency and reliability, as well as to achieve optimal outcomes within and across experiments (Table 3).

2.4 | Characterization of ST-EVs and Efficacy Assessment of Separation Procedure

Multiple characterization methods are essential not only to confirm the presence of ST-EVs but also to demonstrate that ST-EV preparations contain limited processing-related co-isolates (Paolini et al. 2022). These include non-EV vesicles such as synaptic vesicles (brain tissue), microsomes or released intracellular vesicles, exogenous proteins and cellular debris (Paolini et al. 2022; Radeghieri and Bergese 2023).

Previous studies on ST-EVs have addressed this point by examining multiple biological and biophysical properties with orthogonal techniques such as Western blot (WB), ELISA, proteomics, lipidomics, metabolomics, transcriptomics (Vella et al. 2017; Huang et al. 2024; Vassileff et al. 2024), nanoparticle tracking analysis, resistive pulse sensing, transmission electron microscopy (TEM) and flow cytometry (Brenna et al. 2020; Su et al. 2021; Li et al. 2024). Proteomics (with cellular component ontology) or immunoblotting of the tissue and EV preparations can be useful in demonstrating that the preparation is enriched in EVs while depleted in process-related co-isolates. For example, protein markers of intercellular organelles as the endoplasmic reticulum or the Golgi network can help rule out cellular debris co-isolation, while synaptic vesicle markers can help report on their co-isolation (Brenna et al. 2020; Li et al. 2024), although synaptic vesicle molecules can also be present in EVs (Wang et al. 2011). Authors should assess the presence of exogenous soluble proteins in the preparations using assays such as the total particle number/total protein ratio (Crescitelli et al. 2020, 2021) or the colorimetric nanoplasmonic assay (Théry et al. 2018; Zendrini et al. 2019; Welsh et al. 2024). This will help confirm that the assays used to measure protein concentration in the samples specifically measure EV proteins, rather than soluble proteins.

The presence of blood-derived contaminants (if perfusion of the tissue was not possible) such as haemoglobin, lipoproteins, platelets should also be determined and reported as suggested in the MISEV guidelines (Théry et al. 2018, Welsh et al. 2024). This is particularly relevant when using non-tumour tissues as starting material for ST-EV separation, as tumour tissues may exhibit heterogeneous vascularization and infiltration of immune cells within the microenvironment (Lugano et al. 2020). Nonetheless, authors should provide detailed reporting on these factors.

To further ensure the specificity and accuracy of ST-EV characterization, it is recommended to determine cellular origin by analysing cell-specific markers (e.g., protein markers) associated with the cell types in the tissue. This approach serves two purposes: it validates the tissue-specific origin of the ST-EVs and provides an assessment of the cell types contributing to the ST-EVs enriched in the preparation. Different strategies can be applied to identify the tissue-specific markers such as proteomics, immunohistochemistry, WB, in situ hybridization analysis of the starting-tissue (Casadei et al. 2022; Watanabe et al. 2022) or referring to known markers in the literature (Bub et al. 2022; Matamoros-Angles et al. 2024). If confirmation of the cellular

TABLE 3 | Recommendations and reporting guidelines for tissue EV separation.

Recommendations
· Ultracentrifugation is not recommended as a standard method without further enrichment steps including DG-UC, SEC and/-or affinity selection (Welsh et al. 2024)
· Avoid using filters with small cutoff size (0.20–8 μm for example) and high speed/pressure filtration. These may result in exclusion of EV subpopulations, reduced yield, and creation of EV mimetics (Lässer et al. 2017).
· All steps should be performed at 4°C (when possible) and buffers should contain protease inhibitors or be diluted to minimize protein degradation (Hurwitz et al. 2019; Matamoros-Angles et al. 2024).
Reporting guidelines
· Report methodological details according to the MISEV2024 checklist (Welsh et al. 2024, 2023 guidelines), and reposit methods in the EV-TRACK platform (Van Deun et al. 2017).
· Report ST-EV yield relative to starting material.
· Report pre-clearing steps and include filter sizes, membrane, and company.
· If filtration is used, report if it is gravity driven or vacuum assisted.
· Report details of all separation steps including pre-clearing and enrichment.
Outstanding questions
· Do separation methods vary in effectiveness across different tissue types?
· Is there a need for tissue-specific optimization of standard EV separation protocols, or can universal methods be developed?

origin is not feasible, for instance, if tissue- or cell type-specific phenotypes are lost due to abnormal cell growth (tumours, e.g.) (Moreno-Sánchez et al. 2016), this limitation should be reported. It is acknowledged that systematic investigations into intra-organ heterogeneity of EV characteristics are currently lacking, which is important to note given that most clinical samples are obtained from biopsies rather than entire organs. This gap adds an additional challenge to the characterization of ST-EVs and represents an area for future study.

In cases where enzymatic digestion is employed (i.e., collagenase, DNAse), it is recommended to ensure that the enzyme does not alter or digest the surface proteins (Vella et al. 2017; Brenna et al. 2020; Crescitelli et al. 2021; Matamoros-Angles et al. 2024). This can be evaluated by comparing the protein content and profile of ST-EVs isolated without enzymatic digestion or by comparing undigested tissue, digested tissue, and EVs using single protein detection methods such as WB, Flow Cytometry or ELISA or protein profiling with mass spectrometry. These techniques can help identify any changes in the presence or abundance of specific surface proteins. This point is crucial if studying the EV 'biomolecular corona', a spontaneous self-assembly layer of biomolecules (proteins, lipids, metabolites and nucleic acids) (Radeghieri and Bergese 2023) that covers the EV surface nanoparticles when interfacing with a biological matrix (Toth et al. 2021; Radeghieri et al. 2022). The surface characteristics can influence the biological identity and function of the EVs, impacting ST-EV interaction with cells and tissues, ST-EV biodistribution and clearance from the body (Hallal et al. 2022; Radeghieri and Bergese 2023).

If a particle count is performed, normalizing the results to the mass of the starting material to enable comparability across samples can be performed (Crescitelli et al. 2021; Estrada et al. 2021; Huang et al. 2022). However, it is important to acknowledge key caveats: differences in the nature of the starting tissue (e.g., atrophied vs. non-atrophied tissue) can introduce variability, impacting EV yield and composition (Matamoros-Angles et al. 2024). Additionally, the multiple processing steps required to separate EVs from tissue invariably lead to some degree of EV loss, which may differ between samples. Therefore, normalization strategies should consider not only tissue weight, but also potential disparities in tissue composition and processing efficiency to ensure accurate interpretation of results.

In addition, morphological integrity of preparations enriched in ST-EVs should be demonstrated through imaging techniques such as transmission/cryo electron microscopy (Crescitelli et al. 2021) or atomic force microscopy (Mangolini et al. 2024), to provide visual confirmation of intact vesicle structures and a qualitative indication of the presence/absence of cellular debris. Table 4 outlines the recommendations and reporting guidelines for the characterization of EVs after separation from tissue.

3 | Conclusion and Perspectives

The study of ST-EVs represents a promising and rapidly evolving field within EV research. Two approaches have been used to investigate tissue-derived EVs. One is to use ST-EVs, which are directly harvested from a tissue sample (Perez-Gonzalez et al. 2012; Polanco et al. 2016; Jeurissen et al. 2017; Vella et al. 2017; Crescitelli et al. 2020; Huang et al. 2020), while another is to culture tissue samples and separate EVs released by the specimens (Jeurissen et al. 2017; Jingushi et al. 2018). This article focuses on ST-EVs, which capture in vivo EV signatures within the native tissue.

There are many pre-analytical variables when separating ST-EVs. These include storage and tissue processing considerations and details of such parameters should be reported. Dissociation needs careful attention and must be optimized as there is a risk of co-isolation with other particles, the formation of microsomes, and contamination with other cellular components due to cell

TABLE 4 | Recommendations and reporting guidelines for ST-EV characterization.

Recommendations
· If enzymatic digestion has been used for tissue dissociation, demonstrate that the enzyme has not digested surface proteins by examination of EV surface markers (Crescitelli et al. 2021; Matamoros-Angles et al. 2024)
· If possible, demonstrate depletion/absence of EV mimetics, exogenous proteins and cellular debris (Vella et al. 2017).
· Demonstrate the morphological integrity of ST-EVs (Perez-Gonzalez et al. 2012).
· If possible, demonstrate the ST-EV cellular origin (Abdelmohsen et al. 2023; You et al. 2023).
Reporting guidelines
· Report on purity from non-EV proteins, non-EV markers, contamination of released particles from dissociated tissues and blood.
· Report enzymatic digestion influence on EV profile.
· Report EV morphological integrity.
· Report presence of marker of cellular origin
Outstanding questions
· What is the impact of tissue dissociation on ST-EV composition and characterization?

lysis (Shimizu et al. 2003; Vilcaes et al. 2021; Solana-Balaguer et al. 2023). Once the dissociation step is optimized, the implementation of subsequent separation methods is similar for EVs from biofluids. However, specific and multiple characterization steps are required to assess the efficacy of the separation procedure and determine an enrichment of intact ST-EVs in the preparations.

The potential of applications of ST-EV analyses is multiple and ranges from cellular biology to clinical outputs, both in diagnostics and as novel therapeutics. Unlike EVs derived from body fluids, ST-EVs offer a direct representation of the tissue microenvironment. This will provide a more comprehensive understanding of the biological and functional significance of EVs, including their role in cell-to-cell communication, their ability to interact with surrounding or remote cells and organs, both in physiological and pathological conditions (Zhang, Yang, et al. 2023). ST-EVs allow for the investigation of tissue-specific pathophysiology by enabling the analysis of EV populations originating from tissues containing heterogeneous or multiple cell types.

Profiling of ST-EVs has been used to identify organ specific protein markers in serum (Abdelmohsen et al. 2023; You et al. 2023). In the case of brain tissue-derived EVs, whereby ST-EV-associated ATP1A3 was shown to have a high level of specificity for neurons, enabling the subsequent isolation of neuron-derived EVs from blood. ATP1A3-associated EV provides better diagnostic prediction of Alzheimer's disease over other plasma biomarkers (You et al. 2023). This and other examples highlight the potential of ST-EVs for novel biomarker discovery in pathological conditions such as cancer, infectious diseases, central nervous system diseases, and metabolic disorders (Cheng et al. 2020; Qin et al. 2021; Zhang, Yang, et al. 2023; Lee et al. 2024).

ST-EV could be used to support organ transplantation and regenerative medicine, by exploiting their ability to maintain homeostasis and structural integrity of different tissues (Lee et al. 2021, 2024), as well as a source of tumour antigens to support the development of cancer vaccines (Park et al. 2021).

There are many pre-analytical variables, tissue and species differences, that can affect ST-EV quality, composition, and function. To address this, reproducibility studies will be crucial in validating findings and ensuring consistency across different settings. Collaborative efforts will be essential to expand the full potential of ST-EVs in both research and clinical applications.

Author Contributions

Rossella Crescitelli: conceptualization, investigation, writing–original draft, writing–review and editing, funding acquisition, visualization. **Yiyao Huang**: writing–review and editing, writing–original draft, investigation. **An Hendrix**: writing–review and editing, writing–original draft, investigation. **Andrew F. Hill**: writing–original draft, writing–review and editing, investigation. **Stephanie N. Hurwitz**: investigation, writing–original draft, writing–review and editing. **Tsuneya Ikezu**: investigation, writing–original draft, writing–review and editing, funding acquisition. **Efrat Levy**: investigation, writing–original draft, writing–review and editing. **Berta Puig**: investigation, writing–original draft, writing–review and editing. **Lucia Paolini**: conceptualization, investigation, writing–original draft, funding acquisition, writing–review and editing, supervision. **Laura J. Vella**: conceptualization, investigation, writing–original draft, writing–review and editing, supervision, validation.

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Conflicts of Interest

Rossella Crescitelli has developed multiple EV-associated patents for putative clinical utilization. Rossella Crescitelli owns equity in Exocure Bioscience Inc. All the other authors have no conflict of interests.

Data Availability Statement

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary Table: jev270185-sup-0001-tableS1.xlsx