Faisal A. Alzahrani
Islam M. Saadeldin *Editors*

Role of Exosomes in Biological Communication Systems

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Preface

Exosomes as membrane-bound extracellular vesicles (EVs) are an active area of research and, known as universal minute nanosized vehicles, can be released from all cells in all prokaryotes and eukaryotes to transfer genetic instructions between cells. EVs deliver proteins, mRNAs, miRNAs, lipids, metabolites, and enzymes to alter cell functions on the physiological or pathological levels. There are tremendous increase and thousands of publications related to the isolation, characterization, and functional analysis of EVs. The physiological purpose of generating EVs remains largely unknown and needs investigation. One speculated role is that EVs likely remove excess and/or unnecessary constituents from cells to maintain cellular homeostasis. Recent studies reviewed here also indicate a functional, targeted, mechanism-driven accumulation of specific cellular components in EVs, suggesting that they have a role in regulating intercellular communication. More attention has been given to the regenerative capabilities of stem cells-derived EVs for overcoming the setbacks of cellular therapy and towards a cell-free therapy. EVs are associated with reproductive functions, basic immune responses, parasitic pathogenicity, urinary system diseases, liver diseases, cardiovascular diseases, central nervous system-related diseases, and cancer progression. Proteins, metabolites, and nucleic acids delivered by EVs into recipient cells effectively alter their biological response. These EVs-mediated responses can be disease promoting or restraining. EVs can be artificially engineered to deliver diverse therapeutic cargoes, including siRNAs, antisense oligonucleotides, chemotherapeutic agents, and immune modulators. Additionally, EVs also have the potential to aid in disease diagnosis as they have been reported in all biological fluids and considered as a reliable biomarker for different diseases. In this book we highlight the work from different laboratories and interested researchers regarding the vital aspects of EVs and exosomes including their role in physiological and pathological communications as well as their therapeutic uses in different physiological and pathological levels. Two chapters illustrate the isolation and characterization of EVs. Three chapters discuss the roles of EVs in male and female reproduction as well as the early embryonic life. Two chapters highlight the beneficial roles of EVs derived from stem cells and the regenerative and therapeutic potentials. Moreover, five chapters discuss the EVs in diseases of the urogenital system, nervous system, liver, and stem cells-derived EVs. Four more chapters uncover the critical roles of EVs in different cancers and metastasis. Finally,

the trend for generating therapeutic EVs and exosomes is also covered by two chapters. The potentials of EVs to be employed in translational medicine, especially as biomarkers, and therapeutic delivery system are promising for developing novel therapeutic and diagnostic tools for clinical practice.

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Editors

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1 Isolation and Characterization of Extracellular Vesicles: Classical and Modern Approaches

Ahmed E. Noreldin, Asmaa F. Khafaga, and Rasha A. Barakat

Abstract

Extracellular vesicles (EVs) are tiny membrane vesicles containing detailed cellular information. Recently, researchers have been focusing on EVs due to their role in intercellular communication, and prognostic, diagnostic, and therapeutic usage in medical purposes. In this chapter, we summarize the available technologies for EV characterization and describe their limitations and potential. Moreover, we highlight the emerging technologies with their development.

Keywords

Extracellular vesicles · Exosomes · Isolation · Electron microscopy · Ultracentrifugation

Abbreviations

AF4	Asymmetric flow field-flow fractionation
AFM	Atomic force microscopy
AKI	Kidney injury

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CCD	Coupling system
cryo-EM	Cryogenic electron microscopy
CSF	Cerebrospinal fluid
DLS	Dynamic light scattering
DUC	Differential ultracentrifugation
EM	Electron microscopy
EVs	Extracellular vesicles
FC	Flow cytometry
FCS	Fluorescence correlation spectroscopy
FIC	Fluorescence imaging system
Fl-NTA	Emitted fluorescence
IFC	Image flow cytometer
LSPRi	Localized SPR imaging
LTRS	Laser tweezers Raman spectroscopy
MISEV	Minimal information for studies of extracellular vesicles
MSC	Mesenchymal stem cell
MVBs	Multivesicular bodies
NTA	Nanoparticle tracking analysis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCS	Photon-correlation spectroscopy
PEG	Polyethylene glycol
PMT	Photomultiplier tube
Sc-NTA	Scattered light
SEA	Fluorescent microscopic analysis
SEC	Size-exclusion chromatography
SEM	Scanning electron microscopy
SERS	Surface enhanced Raman spectroscopy
Sp-IRIS	Single-particle IRIS
SPR	Surface plasmon resonance
SPT	Single-particle tracking
TEM	Transmission electron microscopy
TRPS	Tunable pulse resistive sensing

1.1 Introduction

Extracellular vesicles (EVs) are phospholipid bilayer vesicles secreted by most cells. EVs have attracted great interest in the field of biomedical research in recent years due to their pivotal biological role in disease and normal physiology (Bank et al. 2015; Colombo et al. 2014; Quek and Hill 2017). Recently, EV-mediated cell-to-cell communication has been highly investigated in cancer, where spreading of EVs to the tumor microenvironment enhances modulating immune, matrix remodeling and angiogenesis (Al-Nedawi et al. 2008; Andreola et al. 2002; Huber et al. 2005; Luga et al. 2012; Skog et al. 2008). On the other hand, tumorigenesis is enhanced by the

transfer of EVs to tumor cells through elevating tumor cell migration, propagation, resistance to chemotherapy, and epithelial-to-mesenchymal transition (Au Yeung et al. 2016; Leca et al. 2016; Luga et al. 2012; Richards et al. 2017). Moreover, EVs can also move farther from the tumor site where they create a pre-metastatic niche (Alderton 2012; Costa-Silva et al. 2015; Peinado et al. 2012; Somasundaram and Herlyn 2012).

Chargaff and West (1946) detected EVs in blood. Then, Wolf (1967) called EVs as "platelet dust." Later, EVs had been created by rectal adenoma microvillus cells and called "plasma membrane fragments" (De Broe et al. 1977). Moreover, in 1983, detailed investigations showed that vesicles are formed by the union of plasma membrane with multivesicular bodies (MVBs) (Harding et al. 1983). After that, Raposo and colleagues reported that these vesicles, separated from virus-transformed B lymphocytes, had the ability to stimulate T cell responses (Raposo et al. 1996). In 2007, EVs attracted more attention as mediators of communication between one cell to another cell due to detection of RNA in EVs (Valadi et al. 2007).

Therefore, EVs are a potential origin of biomarkers for various diseases because they indicate the secretion of the cells such as lipids, nucleic acids, and proteins. EV-containing "liquid biopsies" such as urine (Duijvesz et al. 2011), blood (Caby et al. 2005), cerebrospinal fluid (CSF) (Chen et al. 2013), and saliva (Yang et al. 2014) are easily obtained and are considered as a good alternative to common biopsies (Wu et al. 2017). Currently, the biomarkers for EVs are being investigated in many diseases, including cancer (Choi 2015; Merchant et al. 2017; Moon et al. 2016).

In addition to prognostic and diagnostic potential, the therapeutic usage of EVs or liposomes as targeted therapy delivery vehicles is being investigated (Crivelli et al. 2017; Usman et al. 2018; van der Meel et al. 2014). In preclinical models, human mesenchymal stem cell (MSC)-derived EVs have proved their therapeutic ability. For instance, the treatment of mice suffering from acute kidney injury (AKI) with MSC-derived EVs supporting the functional recovery of AKI, compared with the administration of MSCs only (Bruno et al. 2009). Furthermore, cardiac function was enhanced by treatment with MSC-derived EVs after myocardial ischemia/reperfusion injury (Arslan et al. 2013). To utilize the biomedical ability of EVs, methods are essential to estimate their concentration in samples and to determine their molecular composition.

The complex nature of clinical and biological EV samples and EV heterogeneity hamper EV analysis. The family of EVs can be classified according to their biogenesis into three major categories: apoptotic bodies, microvesicles, and exosomes (Raposo and Stoorvogel 2013). Exosomes have a small diameter (40–100 nm) and are synthesized in endosomal compartments and excreted by the coalition of the plasma membrane with multivesicular bodies. Microvesicles have a large diameter ranging from 100 nm to 1000 nm and are created directly by blebbing of the plasma membrane. The diameter of apoptotic bodies, secreted by membrane budding during programmed cell death, ranges from 50 nm up to 5 μm. The major pathways for EV biogenesis and release are illustrated in Figs. 1.1 and 1.2.

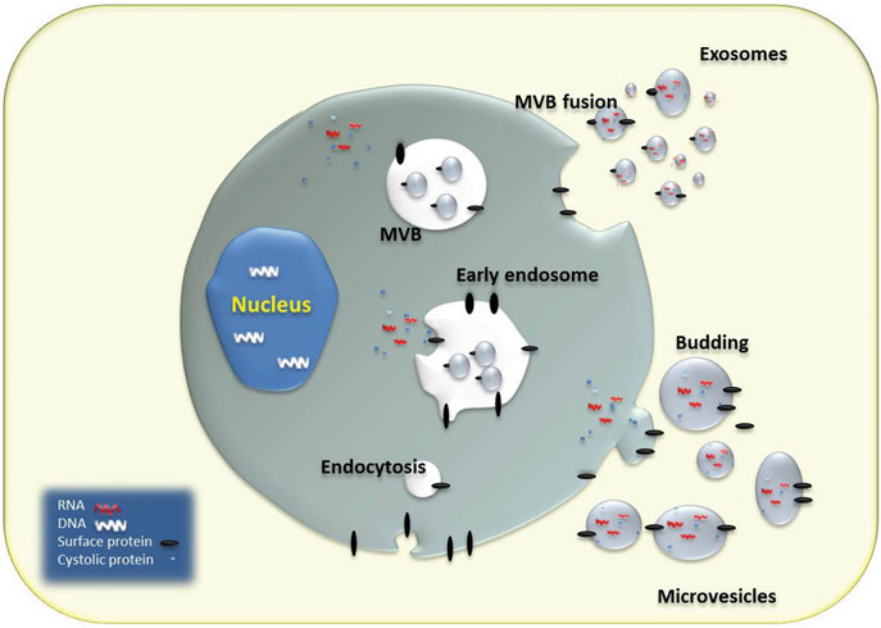

Fig. 1.1 Major pathways for EV secretion and biogenesis. The exosomes are formed via the inward budding of endosomes and secreted through a fusion of plasma membrane to the MVBs

Recently, the heterogeneity of EVs has been revealed. By utilizing cryogenic electron microscopy (cryo-EM), various shapes of EVs in body fluid samples have been detected (Hoog and Lotvall 2015). About 41% of EVs in human semen are found to be double vesicles, oval vesicles, triple vesicles, double special vesicles, lamellar bodies, and tubules, while the rest are single vesicles (Hoog and Lotvall 2015). The different forms of EVs indicate the presence of various subpopulations, which may have various biochemical characters. Due to the elevated heterogeneity of EVs, it is imperative to arrange them into particular groups to understand their composition and functions in pathological and physiological operations. On the other hand, the means of the analysis of EVs of various intracellular origins are still under development. Recently, a multidimensional EV refining plan has been used to obtain extremely refined EV subgroups for the following analysis of EV cargo. Therefore, more properties in the function and composition of particular EVs and EV-based biomarkers are obtained.

This chapter aims to show the novel developments in technologies for EV characterization and quantification and to reveal the recent technologies with a high possibility for more progress. In this chapter, we make our best endeavor to supply the reader with wider aspects of the current status of the field. First, we reveal a general summary of the most utilized methods for EV isolation. Then, we classify the methods of EV characterization.

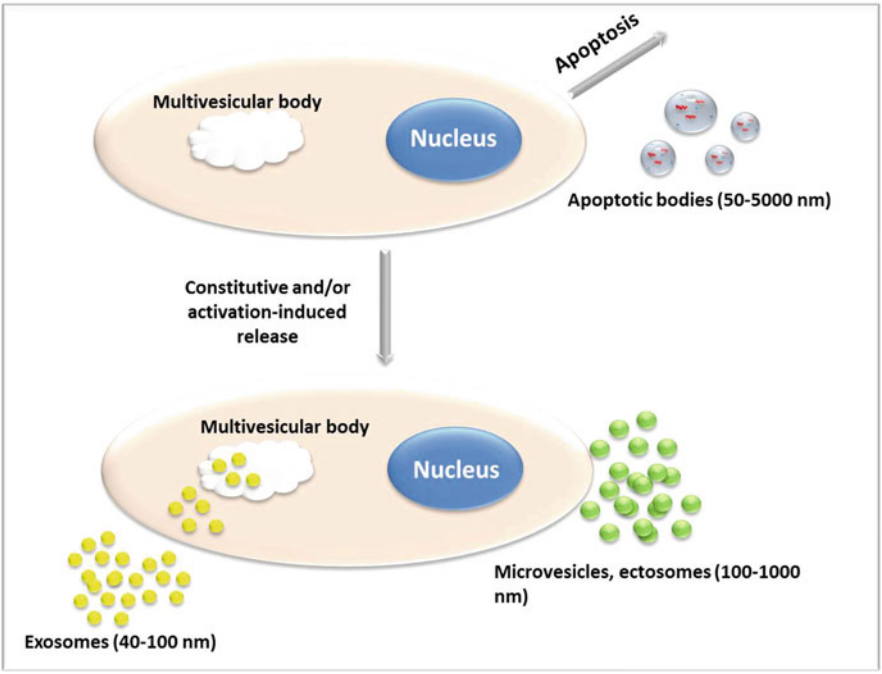

Fig. 1.2 Release of EVs. Exosomes and microvesicles are released from live cells constitutively or by activation. The microvesicles are released via direct budding of the plasma membrane, while exosomes are formed from multivesicular bodies. Cells undergoing programmed cell death form apoptotic bodies by membrane budding

1.2 Isolation Techniques

Five main subpopulations of EV isolation techniques have been promoted, namely size-based techniques, differential ultracentrifugation (DUC)-based techniques, polymer-based precipitation, immunoaffinity capture-based techniques, and microfluidic techniques. Different subpopulations of EVs are illustrated in Fig. 1.3.

1.2.1 Ultracentrifugation-Based Techniques

DUC procedures are the most common techniques used for EV isolation. In DUC, particulates are precipitated according to their shape, size, and density. The supernatant is undergone to next centrifugation with high centrifugal force, then the pellet is transferred to a suitable medium. Then, groups of EVs are isolated at various times of centrifugation (Yamashita et al. 2016). Thus, the pelleting time relies on the solvent viscosity and the physical characteristics of the particles. On the other hand, EV pellets procured are mainly contaminated with lipoproteins, protein collections, and

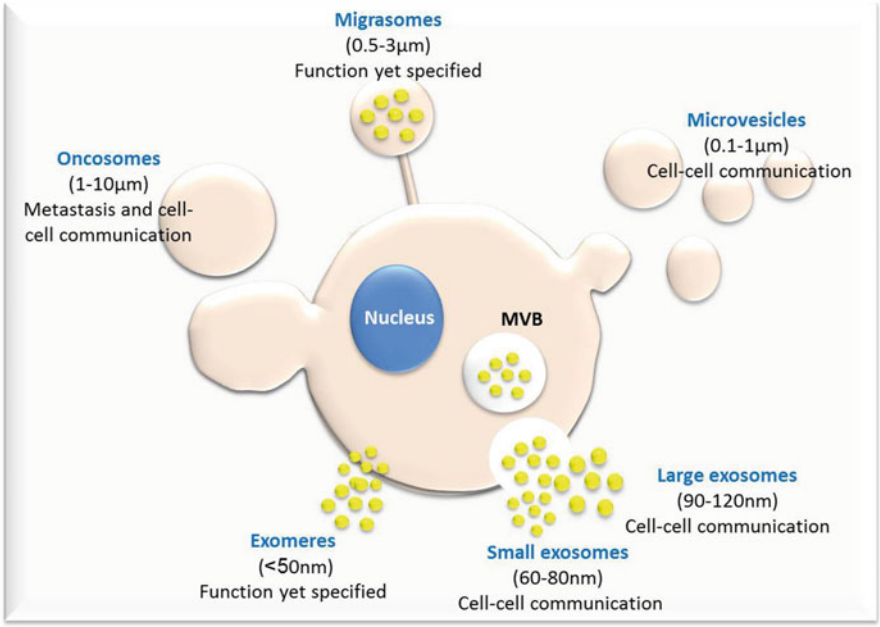

Fig. 1.3 Different subpopulations of EVs. Various subtypes of EVs showed various sizes and release pathways. Exosomes are released from MVBs to carry protein and mRNA cargo for cell-cell communication; they can be characterized as small and large exosomes. Exomeres are small-size nanoparticles (<50 nm) that carry proteins responsible for metabolism. Microvesicles are larger than exosomes and can support cell-cell communication. Oncosomes are large EVs that are released from cancer cells by budding to support the invasion of tumor cell. Migrasomes are formed after the migration of cells with unknown function

other particulates when body fluids are analyzed. After ultracentrifugation, contaminant ejection can be carried out by density gradient ultracentrifugation. This tactic could be treated as the "gold standard" for EV sorting (Mateescu et al. 2017). On the other hand, density gradient ultracentrifugation entails high-cost equipment (around US$50,000–100,000) (Aalberts et al. 2012; Palma et al. 2012) and consumes a lot of time ranging from 62 to 90 h in analysis of convoluted biological samples (Taylor and Shah 2015). Therefore, treatment of a high number of samples is not suitable for resource-poor settings and normal hospital laboratories (Liga et al. 2015). Moreover, lipoprotein contaminant, deterioration of EV integrity by centrifugation, and low EV yield (5–25% recovery) (Lamparski et al. 2002) make this method very difficult to apply in a clinical approach. Also, DUC protocols enhance accumulation of EVs in extremely condensed suspensions. Furthermore, EVs are damaged by repetitive freeze and thaw cycles (Bosch et al. 2016) and alternate their biological activity (Kim et al. 2005). It is indicated that including 25 mM trehalose may protect the membranes of EVs during thawing and freezing cycles and lower the accumulation of EVs during ultracentrifugation (Bosch et al. 2016). The classic ultracentrifugation protocol is illustrated in Fig. 1.4.

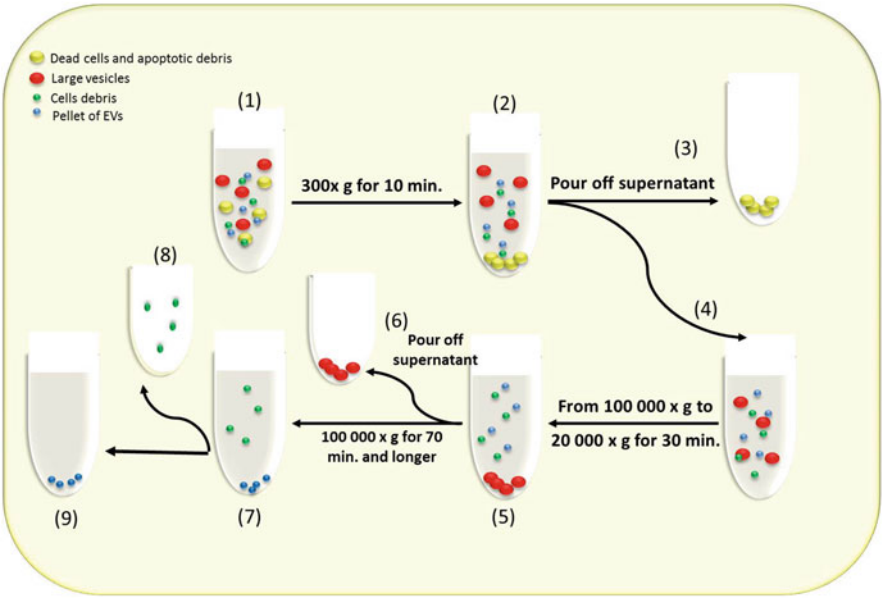

Fig. 1.4 Classic ultracentrifugation protocol. To produce small size pellet, the RCF (g) and the centrifugation period are increased. (1, 2) Body fluid or conditioned medium is centrifuged at 300 × *g* for 10 min to discard apoptotic debris and dead cells. (3, 4) The precipitate is discarded and supernatant is kept for the next step. (5, 6) Centrifugation is done for 30 min at 100,000 × *g* to 20,000 × *g* to eliminate large vesicles. (7, 8) On the contrary, pellets containing EVs are kept after the 100,000 × *g* centrifugations for 70 min and longer, while the supernatants are discarded. (9) Pellets are then resuspended in PBS

1.2.2 Size-Based Techniques

These techniques, for example, size-exclusion chromatography (SEC) and ultrafiltration, subdivide EVs according to their size. In ultrafiltration, a membrane of known sized pores is used to permit the crossing of tiny particulates only. Ultracentrifugation is slower than ultrafiltration and does not need additional reagents and special equipment. On the other hand, poor biological actions and protein contamination are expected because of the dissolution of large vesicles and shear-force-induced distortion. Moreover, EVs damage because of connection to the membrane may distort the consequences of downstream analysis (Batrakova and Kim 2015). SEC is another size-based isolation technique that is applied in EV isolation. In SEC, a pored fixed phase is used to group macromolecules according to their size.

1.2.3 Immunoaffinity Capture-Based Techniques

These techniques use magnetic beads or a molecule-coupled substrate to catch EVs holding objective molecules on their surface. After that, EVs will be restored using a

certain washing solution. EVs have been known to contain different membrane biomarkers. The requirement of an excellent biomarker for immunoselection is to be highly concentrated on the surface of EVs and lacking soluble counterparts. The immunoaffinity technique with small volumes has generated results similar to those obtained by ultracentrifugation. It has more efficiency than ultracentrifugation due to specificity, availability, and affinity between the EV surface marker and captured molecule (Tauro et al. 2012).

1.2.4 Polymer-Based Precipitation

EVs may be filtered from biological fluids by changing their dispersibility or solubility by supplying polymers, like polyethylene glycol (PEG). PEG is mainly utilized to separate viruses. Low-speed centrifugation can pellet EV precipitation. Thus, polymer precipitation does not need any special equipment and is easy to utilize. This makes it suitable for clinical utilization and for large sample sizes (Batrakova and Kim 2015). On the other hand, precipitating polymers and proteins mainly contaminate the isolates (Taylor and Shah 2015). Pre- and post-sorting steps must be done to reduce the contamination. The pre-isolation step is to discard subcellular particles like lipoproteins. The post-isolation step is for deletion of the polymer by using a desalting column like Sephadex G-25 (Taylor and Shah 2015).

1.2.5 Microfluidic Techniques

The rapid development in microfabrication technology has provided a possibility for the isolation of EVs efficiently by manufacture of microfluidic-based devices, according to biochemical and physical characteristics of EVs on a small scale. For clinical purposes, a novel method for EV characterization has been developed by fabrication of microfluidic techniques for EV detection. These techniques need lower quantities of samples and are more sensitive and faster than classical methods. Microfluidic immune-affinity techniques for EV sorting have been reported (He et al. 2014; Kanwar et al. 2014). The quantity and quality of RNA extracted from trapped EVs are enough for microarray analysis or polymerase chain reaction (PCR). On the other hand, the immune-affinity technique only isolates EVs having a specific surface protein (Liga et al. 2015). Porous polymer sieves with microfluidic devices can collect EVs without immunoselectivity.

Making the filtration by electrophoresis could lessen contamination, EV trapping, and pore clogging. A much lower voltage could be utilized by microfluidic devices because of their tiny size (Davies et al. 2012). Wang et al. have revealed size-based targeting of liposomes utilizing ciliated nanowire-on-micropillar hierarchical structures (Wang et al. 2013). Solving of silicon nanowires in phosphate-buffered saline (PBS) overnight can release targeted particles. Recently, characterizing and sorting of polymers, nanoparticles, EVs, and proteins by asymmetric flow field-flow fractionation (AF4) technology has been performed (Gigault et al. 2014). In AF4,

samples introduced into a flat channel by laminar tangential flow, and after that, a transverse flow is introduced to group samples according to their diffusion coefficients. However, most sorting technologies still need more off-chip steps like nucleic acid extraction and sample preparation.

1.3 Characterization of Extracellular Vesicles

A major requirement is to standardize the size of EVs with their concentration. To understand the kinds of EVs (microvesicles, apoptotic bodies, and exosomes), EV size is utilized; however, the correlation between EV type and EV size is lower realized than indicated. It was reported that EVs vary in concentration and size at various phases of many kinds of cancer, indicating that these measurements are helpful for clinical diagnostics (Baran et al. 2010; Kim et al. 2003; Szajnik et al. 2013).

The most popular methods for determination of EV diameter are either indirectly by utilizing electrical readouts or directly by high-resolution imaging. Direct high-resolution imaging of immobilized EVs utilizing atomic force microscopy (AFM) or electron microscopy (EM) obtains the precise size of individual EVs with nanometer resolution. On the other hand, direct imaging does not give accurate EV number because of the complicated procedures involved in the preparation of samples. Using indirect estimation of EVs, the concentration of EVs and/or size from other detectable properties like their interaction with light, their diffusion trajectories, or their influence on the electrical current within a detector can be determined. For indirect technologies, the number of EVs is higher than direct imaging technologies enhancing them markedly more precise in measuring EV concentration. However, the estimated size distribution is less accurate. Moreover, indirect methods are limited by the sensitivity of detectors or the interference with other biological particles exist in samples full by EVs. In this part of the chapter, the concentration is on epitomizing the most common techniques for the physical measurement of EVs by grouping them according to the principle utilized by these methods for EV size estimation.

1.3.1 Electron Microscopy

The best method for estimation of the morphology and size of individual EVs is electron microscopy (EM). EM uses a condensed electron beam as an alternative to the light beam, which acquires high-resolution images of nanoscale objects. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are the most prevalently used kinds of EM. The image of SEM is acquired by scanning the topography of the EV surface with a concentrated electron beam and revealing the reflection of the electron beam on the analyzed area. TEM utilizes electrons that penetrate the sample, instead of utilizing secondary electrons, to make a two-dimensional image of the EVs. Therefore, TEM images provide information

about the inner structure of the studied objects. Utilizing of TEM needs 50 nm thin slices of analyzed material, making it difficult to visualize tissues and cells; however, it does not restrict the EV analysis (Bozzola and Russell 1999).

The major restriction of utilizing EM on biological samples like EVs or cells is the requirement of visualizing under the vacuum circumstances that needs drying and fixation of the biological objects. Complicated sample preparation is a major obstacle in the translation of examined samples into the familiar morphology of EVs. Despite these restrictions, the morphology and size of EVs can be easily estimated utilizing both TEM (Linares et al. 2017) and SEM (Casado et al. 2017; Nanou et al. 2018; Sokolova et al. 2011).

Cryogenic transmission EM (cryo-TEM) techniques have been developed to prevent sample dehydration, which is most suitable for investigating EVs. In cryo-TEM, the image resulting from the visualization of ultra-thin vitrified film is made by flash-freezing at a very low temperature (<-100 °C) of thin liquid film of EV suspension (Cizmar and Yuana 2017). This modified technique is commonly utilized to measure the ultra-structure of EVs and gives high-resolution imaging of biological samples (Buzhynskyy et al. 2009; Gustafsson et al. 1995; Issman et al. 2013). Moreover, the utilizing of immunogold labeling facilitates the diagnosis of specific groups of EVs in the clinical samples (Linares et al. 2017; Thompson et al. 2016). For instance, Brisson et al. combined immunogold labeling with cryo-TEM to investigate EVs extracted from platelets under many kinds of induction and contrasted their morphology, size, and levels of expression of CD63 and CD41 proteins (Brisson et al. 2017).

Nowadays, cryo-TEM is considered the most reliable technology for estimation of EVs. On the other hand, it is less accurate for measuring EV concentration because of the powerful effect of TEM grid interactions with EV and influences of sample blotting. The analysis of the low number of EVs is not representative of the population of heterogeneous EVs exist in clinical and biological samples. Interestingly, to estimate the heterogeneity in size of EVs, the minimal information for studies of extracellular vesicles (MISEV) initiative indicates analyzing an adequate number of overview images holding multiple EVs joined by close-up images of single EVs (Thery et al. 2018). On the other hand, this limits routine usage of EM for EV analysis in clinical applications.

1.3.2 Atomic Force Microscopy

Recently, a new kind of scanning probe microscopy has been making images of the surfaces with nanometer resolution called atomic force microscopy (AFM). In this technology, the imaging of the surfaces is by scanning the area with a sharp tip and translating its deviation into the surface features. There is no need for sample labeling in AFM (Allison et al. 2010). Utilization of AFM is to estimate the size and structure of dry immobilized EV samples. During the drying step, samples may be damaged, which may be blocked by binding EVs to complementary antibodies or by immobilizing EVs on a surface through electrostatic interactions (Biggs et al.

2016; Hardij et al. 2013). Casado et al. utilized AFM to investigate the forces that stimulate EV secretion from certain cells and detected a high correlation between the size of EVs and the size of revealed protrusions of the cell membrane (Casado et al. 2017). Moreover, AFM provides information about mechanical properties of vesicles such as elasticity and stiffness (Vorselen et al. 2018a). For instance, Wuite et al. detected clear variations in membrane rigidity of platelet-derived EVs between a patient with hereditary spherocytosis and a healthy donor (Vorselen et al. 2018b). On the other hand, AFM is limited to be widely applied in EV research due to the need for specific equipment and skills.

1.3.3 Dynamic Light Scattering

Dynamic light scattering (DLS) measures the size distribution of vesicles. This is based on analyzing temporal intensity fluctuations of laser light, scattered when freely movable EVs are dispersed. On the contrary, EM and AFM measure the individual EV size, and DLS assesses the cumulative mobility (diffusion coefficient) of the diffused vesicles. The distribution of the final size is defined by its medium size and polydispersion (Berne and Pecora 2000).

Simplicity and speed of accurate measurements (several minutes) distinguish DLS from other methods, making it an important technique for routine EV analysis. DLS is appropriate for quantitative analysis of relatively monodisperse samples. However, the distribution of the particle size (e.g., mono- or multimodal) is essential to the measurement of isolated EVs scale and synthetic variants (e.g., liposomes) is considered to be sufficient in DLS samples (Baddela et al. 2016; Pearson et al. 2017; Zhang et al. 2016). In a biological and biomedical context, DLS is used, to some extent, in the analysis of minimally processed biofluids, although it detects all diffused objects in solution.

1.3.4 Nanoparticle Tracking Analysis

Speed of particle diffusion is determined by its size in a static solution according to Brownian motion. The diffusion coefficient is determined by this relationship, and by analyzing the motion trajectories and the size of vesicles. This methodology is called single-particle tracking (SPT) and is considered as the base for a commonly utilized EV study technique known as nanoparticle tracking analysis (NTA). Many business networks have been developed for NTA activity. Tracing a time-lapse of particulates with Brownian motion by imaging them utilizing either emitted fluorescence nanoparticle tracking analysis (Fl-NTA) or scattered light nanoparticle tracking analysis (Sc-NTA) is the basis of this method (Dragovic et al. 2011). It is likely to estimate the consent of particle and scale distribution even in poly-distributor samples by analyzing many individual trajectories.

The research is virtually restricted by relatively short measured pathways because of the constant dispersion of the vesicles within and out of focus, though NTA is able to measure the vesicle's size distribution. The predicted statistic error is ~35%, leading to statistically significant hesitation, for example, also in the 20-step trajectory, and this results in an expansion of the size distribution achieved (Qian et al. 1991). Study of much longer paths of particles will minimize virtually nonpractical statistical uncertainties or mathematical models that test and correct the magnitude and the doubt of measurement (Saveyn et al. 2010). Moreover, because of the existence of many scatter origins (e.g., protein aggregates), scattered light could well distort the EV scale distribution and concentration of combined biofluids (McNicholas et al. 2017). Fl-NTA is utilized more efficiently to differentiate EVs from other particles (Carnell-Morris et al. 2017), makes utilizing of high shining and photo-stable fluorescent labels (e.g., quantum dots) owing to identification and to prevent excessive bleaching. EV-NTA can only be properly identified by monitoring fluorescent artifacts (Carnell-Morris et al. 2017). Eventually, it is now in a productive role as NTA in a relatively new technique (Vestad et al. 2017).

1.3.5 Tunable Resistive Pulse Sensing

Tunable resistive pulse sensing (TRPS) is a technology that reveals individual nanoparticles by estimating alternates in electric currents at an adjustable nanopore. This volume can be accurately correlated with the magnitude of the electric current drop reported (blocking event). Lately after calibration by common standards, like the polystyrene of known size nanoparticles, TRPS was utilized to measure the unlabeled EVs' volume and density. The universal application of TRPS for EV analyses on minimally processed biological samples is still a challenge, though it is a valid technique, as demonstrated by its use in nanoparticle study (Weatherall and Willmott 2015), mainly because of the heterogeneous nature of EV populations, which obstruct pores from larger EVs and due to the need of a buffer equivalent calibration of the system (Maas et al. 2017). The availability and sensitivity of commercial TRPS equipment, nevertheless, make studying of pure EV-containing biofluids possible (Akers et al. 2016; Mork et al. 2016). Besides estimating the size and concentration of EVs, TRPS can accurately quantify EV surface load by estimating the spent time for each EV inside the nanopore as a function of the applied voltage and pressure (Vogel et al. 2017). Because of the important role of EV surfaces in their pharmacokinetic characteristics, they are used for the design of EVs for therapeutic use (Charoenviriyakul et al. 2018). Moreover, TRPS is useful to test pure EV suspension due to its flexibility and precision, and several testing groups are currently working on standardizing the TRPS protocols for better effective use of methods in EV work (Vogel et al. 2016).

1.3.6 Flow Cytometry

Flow cytometry is mostly used for cell analysis and also implemented for EV analysis (Gardiner et al. 2016). In flow cytometry (FC), a hydrodynamic solution allows one-cell illumination of multiple lasers in a flow chamber. The scattered light is detected with several detectors as a consequence of the variation in the refractive indices between the fluid and cells. Size of individual cells is provided by forward scatter, while their granularity and composition are provided by side scatter. EV application is an important challenge given the fact that the EV sensitivity is low, it is a structured and reliable process for cell analyses at a speed of 1000 cells per minute (Chandler et al. 2011). As a result, EV scatters ten-fold less light relative to polystyrene beads usually utilized for calibration because they are of small scale, and have a low refractive index difference with solution (Chandler et al. 2011). Consequently, even a single EV over ~500 nm in size can only be observed by most traditional flow cytometry (Chandler et al. 2011). Because of the swarm effect—several EVs that have simultaneous laser illumination—a smaller EV is collectively detected (van der Pol et al. 2012). The combined dispersion of these products thus reaches the defined detection limit, which means they are counted as a single much larger particle. Thus, the counts observed consist of single detections and swarm detections, which lead to incorrect measurements in complex samples. Samples can be calculated in serial dilutions so that the swarm effect can be tested and resolved by a linear correlation between the dilution and calculated concentrations (van der Pol et al. 2012).

FC is increasingly used in research groups for the study of larger EVs (e.g., microvesicles) (Chandler 2016; Nolan and Jones 2017). A study of the expression and detection of particular EV subpopulations of different surface antigens is enabled by the recognition of dispersed and fluorescent light from EVs marked by fluorescent antibodies or general fluorescent membrane labels in most of these tests (Arraud et al. 2016; Ayers et al. 2011); however, the inability to individual detection of small specific vesicles. This, together with the low volume of bonded fluorophores, is a significant limitation to the study of biological samples for sensitive sensors for identification of less abundant (disease) common EV biomarkers. This strong requirement has recently led to the creation of dedicated flow cytometers (Pospichalova et al. 2015; Stoner et al. 2016; van der Vlist et al. 2012).

The addition of special changes and a high-power 488 nm laser to the optical detection system increased fluorescent signals and scatter intensity from the EVs to decrease the forward scattering detection angle. Besides identification and measuring of different EV subpopulations as low as 100 nm, the utilizing of immunofluorescent antibodies to target EV-related membrane proteins is preferred (Stoner et al. 2016). Additionally, in order to allow a post-analytic detection inspection to be utilized to separate true EVs from protein aggregates or noise, a commercial image flow cytometer (IFC) was innovated, which includes both traditional flow cytometry and the fluorescence imaging system (FIC) (Erdbrugger et al. 2014). Instead of a photomultiplier tube (PMT), which has greater dynamical range and less noise, IFC

uses a charged coupled device (CCD) sensor, so it is more appropriate to estimate low/weak EV fluorescent signals in the range 100–200 nm (Erdbrugger et al. 2014).

Aside from the need for more sensitive cytometers, the growing requirement for comparison of data with various equipment and calibration of sample processing has also been demonstrated in increasing numbers of EV studies using the flow cytometry (FC). In addition, EV FC performance should be enhanced through the advancement of calibration methods and data processing. Van der Pol et al. designed a model that could be applied to the observed dispersal strength of beads with a known refractive index and size. Vesicles are coupled to aldehyde-sulfate latex beads with 4 μm diameter to enable EV detection in traditional cytometers (Lozano-Ramos et al. 2015; Thery et al. 2006). Bead-based cytometry was previously used to couple an E marker antibody with the beads and EV characterization has been accomplished (Cvjetkovic et al. 2016; Torregrosa Paredes et al. 2014). The method for semi-quantitative analyses of the EV samples has been updated by Suárez et al. (2017). The aldehyde -sulfate beads were used directly by Suárez et al. (2017) and they do not limit binding to include the entire population of vesicles in the study (Wahlgren et al. 2012).

The mean refractive index of EVs could well be utilized for estimating EV's size on the basis of the dispersion rate, whereas the refractive index of EVs is unknown in individual samples (van der Pol et al. 2012). It is noteworthy that the level of scattering of EVs is affected by the heterogeneity of the membrane and cargo composition and possibly causing errors in the calculated EV sizes (Gardiner et al. 2014). Standardization of microparticle quantification is continuously improved by MISEV and several multi-center initiatives that use flow cytometry to refine test protocols, using similar calibration and control samples, and by enhancing detailed documentation of experimental information (van der Pol et al. 2018). Recent papers have also discussed important issues in high-resolution flow cytometry and provide strong recommendations for using the technology. This results in better comparable data generated by different groups of research while minimizing the impact of the coincidences/swarm effects described previously (Libregts et al. 2018; Nolan and Duggan 2018).

Taken together, recent advances in the area of FC, particularly the ability to analyze biomarkers for single vesicles up to 100 nm, will allow the identification, application, and understanding of novel unique subpopulations of EV diagnostic markers. The need for highly advanced and frequently personalized instruments is therefore a major downside and makes this approach less available and more clinically adaptable. The potential production of nano flow cytometers, which are able to analyze tiny EVs at a relatively low cost and may definitely be the cue for overcoming these obstacles, would allow microfluidics to be applied in current techniques of EV analysis (Friedrich et al. 2017).

1.3.7 Laser Tweezers Raman Spectroscopy

Raman spectroscopy is a molecular dispersion–based technique that detects dynamics and chemical properties at the organelle level or single cell level (Puppels et al. 1990), which corresponds to the frequency of much of the scattered irradiation, as monochromatic events impact a sample and interfere with the sample molecule, similar to the frequencies of Rayleigh scattering. Owing to an inelastic collision between the monochromatic occurrence of radiation and sample molecules, a tiny part of the dispersed radiation is of a different frequency that constitute the stampede of the Raman (Settle 1997). The consistency and quantity of the samples are demonstrated by their frequency and intensity (Bumbrah and Sharma 2016). There were several positive tests on biomedical subjects such as cancer detection (Haka et al. 2005) and orthopedic control (Tchanque-Fossuo et al. 2013), and drug-of-abuse evaluations were successful applications (Day et al. 2004). Laser tweezers Raman spectroscopy (LTRS) is a type of Raman spectroscopy that uses a closely focused laser beam as the incident light for the pickup of the particle (Carney et al. 2017). Raman signals collected from the focal volume are allowed by LTRS supported by a setup of confocal identification, making the detection of lipid droplets (Argov et al. 2008) and EVs possible (Smith et al. 2015). Vibrational or fluorescence spectroscopy can be integrated with LTRS to provide a comprehensive characterization of single EVs (Carney et al. 2017). LTRS is capable of time-course analysis through its unlabeled and contact-free feature (De Oliveira et al. 2014). Nevertheless, the response time and the throughput still do not fulfill the profiling requirements. The weak signals of Raman are typically one million times less than for fluorescence labeling. LTRS will, for example, take about 5 minutes to obtain a single EV spectrum (Smith et al. 2015).

1.3.8 Dark-Field Microscopy

Dark-field microscopy absorbs only light emitted from the sample, so the image typically has a good signal-to-noise ratio (S/N). On-chip microcapillary electrophoresis has been integrated with dark-field microscopy to evaluate the single EV's zeta potential (Akagi et al. 2014). By uncovering the dispersed laser light, the movement of individual EVs can be visualized in a dark area, and to profile the biochemical compositions of EVs the mobility changes of the EVs after immunolabeling could well be implemented (Akagi et al. 2015). The distribution of the zeta potential of non-treated EVs was found to be symmetric with a mean of −10.2 mV and biased toward −3.4 mV if antibodies are labeled (Akagi et al. 2015).

1.3.9 Fluorescence-Based Techniques

In various fields of life sciences, many analytical methods depend on fluorescence. In the sense of EV research, fluorescent labeling by means of common lipophilic

stains or precise labeling of antibodies makes them to be viewed and monitored, which enables an evaluation of their size and their concentration. Greater sensitivity and quality of EV analysis resulted from endless enhancement of configurations of lighting profiles and/or optical systems. For example, light sheets were recently used for measurement of the size of cell-derived EVs by fluorescence-based SPT in a microfluidic system. The dramatic decrease in the history of the unbound stain by provoking the fluorophores only within the light sheet, compared to epifluorescence microscopy, greatly improves the contrast and allows detection of vesicles with higher accuracy (Deschout et al. 2014). Moreover, using fluorescence correlation spectroscopy (FCS), the size of EVs has been studied. EV researchers became fascinated with FCS only very recently, while FCS has been already used to analyze protein dynamics and even to calculate the synthetic lipid vesicles.

Recently, Wyss et al. reported an accurate analysis of highly purified EVs from cell culture using FCS with an algorithm for single event analysis. This has provided a possibility for the estimation of CD63 on EVs besides its size and concentration (Wyss et al. 2014). Although Wyss et al. analysis offers a fluorophore sensitiveness that makes it a promising tool for EV study, recent multiplexed biomarker analysis of individual EVs was made possible through the creation of a single EV analysis (SEA) technique. FCS needs more validation and calibration for the analysis of EV-containing samples. The EVs are immobilized using a microfluidic system and immunostaining (up to three markers) and fluorescence images are obtained in the chip. More than ten different markers can be detected on the same EVs by breaking the fluorophores existing on EVs, pursued by three other detection antibodies and then reiterating this protocol (Lee et al. 2018).

1.3.10 Surface Plasmon Resonance

Surface plasmon resonance (SPR) is the basis for several emerging technologies for EV analysis. SPR enables highly sensitive label-free sensing on an SPR-active surface, such as gold or silvery nanoparticles, through its immunological capture. Quantifying tumor-derived EVs is an application of these techniques based on selected protein markers. For example, it has been shown that gold-stabilized nanoparticles with DNA aptamers demonstrate a simple change of color because of the unique binding of EVs to these aptamers (Jiang et al. 2017). Protein content analysis of EVs allows for a multiplexing approach, both visually and spectrophotometrically. The surface format used is highly variable and can be modified in accordance with the test format. A microfluidic SPR platform uses changes in transparency of a thin gold layer, with nanoholes created by the immunosuppression of EVs, to determine the levels of EVs with many ordinary protein markers (Im et al. 2017). Alternatively, single capture events of EVs originated from the distilled cell line of brain cancer have been observed using localized SPR imaging (LSPRi) of a nano-manufactured gold nanopillar array coated with anti-CD63 antibodies (Raghu et al. 2018). Finally, self-assembled gold nanoparticles have been immunomodified on glass to measure the density of EVs derived from a series of cultured cell types

(Thakur et al. 2017). Surface enhanced Raman spectroscopy (SERS) has become a gateway to biochemical testing of low-abundance biomarkers over the past decade. Detection of single molecules deposited on antibody-modified metal nanoparticles is enabled by signal enhancement through the SERS effect. Nevertheless, SERS-based tests and estimation devices for concentration and the profiling of EV proteins (Ertsgaard et al. 2018; Kwizera et al. 2018; Park et al. 2017; Tian et al. 2018; Wang et al. 2018) have been described in several studies. Differences in dried EVs derived from cells in the lung cancer system compared to normal alveolar EVs are indicated by the use of SERS as a clinical relevance. (Park et al. 2017). A number of sandwich-style SERS tests have been published. EVs are concentrated first by immunocapture and then by immunolabeled SERS nanoprobes. This strategy allowed multiplexed tests with EV concentration sensitivity to ~4 to 104 mL^{-1} (Tian et al. 2018; Wang et al. 2018). In a glass-slide-based test, gold nanorods were utilized as SERS nanotags to count plasma EVs and assess their level by a specific protein biomarker (Kwizera et al. 2018). Through integrating dielectrophoretic trapping of model vesicles with SERS imaging, Ertsgaard et al. calculated the level of EV material in the Raman system with a new high-speed analysis technique for EVs (Ertsgaard et al. 2018). A similar cheap substitution to the existing gold nanoparticles as the SERS surface is the utilizing of compact disc recortable (CD-R) discs as a base for a silver nanolayer. This method is used for the Raman hemoglobin and plasma EV spectrum analysis (Yan et al. 2019).

1.3.11 Interferometric Imaging

Daaboul and his colleagues have recently reported a very popular count for individual EVs and digital optical detection according to interferometric imagery of EVs captured on a layered silicone substrate, enabling the EV size to be linked to the contrast between the detected bound vesicles (Daaboul et al. 2016). Single-particle IRIS (Sp-IRIS) is a tool for multiplexing analysis using an abundance of CD9, CD63, and CD81 markers, seen in size and protein profiling of isolated EVs from cerebrospinal fluid. Specifically, it is a tool for the development of an array of immobilized antibodies. The approach is sufficiently sensitive to even detect EVs of 40 nm according to the authors. The technology under the name "ExoView" is currently being built into a platform for cartridges.

1.3.12 Detection of Exosomes by Western Blot

Cells secrete exosomes in pathological and normal circumstances and include different cytosolic and membrane proteins. Therefore, exosomal proteins can be utilized in clinical diagnostics. Pivotal ten proteins detected in the exosomes contain CD63 antigen (CD63), heat shock protein 8 (HSPA8), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta actin (ACTB), enolase 1 alpha (ENO1), cytosolic heat shock protein 90 alpha (HSP90AA1), CD9, CD81, tyrosine 3-monooxygenase/

tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), and muscle pyruvate kinase (PKM2) (Mathivanan and Simpson 2009). Specific functional classes include exosome proteins including tetraspanins (CD9, CD63, CD81) and heat shock proteins (HSC70 and HSC90), membrane conveyors (GTPases), and proteins that are lipid bonding.

Tetraspanins are common markers unique to the exosome. The proteins are membrane proteins such as CD9, CD63, and CD81. In exosome development, tetraspanin is involved. In antigenic cells, MHC-II functions are regulated by their incorporation into the tetraspanin CD9 enriching cytoplasmic membrane regions (Buschow et al. 2009). In order to treat many tumors and infectious diseases, tetraspanins can be used. In patients with melanoma and other cancer forms, exosomes of CD63+ were shown in particular to be significantly increased (Logozzi et al. 2009). CD63 has therefore been proposed as a cancer protein marker. In addition, CD81, another member of tetraspanin family, played a key role in the cell input of hepatitis C virus. Exosomal CD81 in patients with chronic hepatitis (Welker et al. 2012) has been shown to be significantly increased, suggesting that CD81 will mark the diagnosis of the viral hepatitis C infection.

1.4 Conclusion and Future Perspective

The rapid development of a wide variety of innovative tests for EV analysis has drove although some of the problems in EV study have still not been solved. There is high attention in completing techniques which can define parameters such as, molecular contents and size at the single vesicle stage. In addition, the recognizing of clinical subpopulations related to EVs and the analysis of individual EVs are important and can provide new potential biomedical possibilities for the correct explanation of EV-related investigations, as they can offer new possibilities for diagnostic testing and biomarker research. The growing attention toward EV in biomedical research and ongoing collaboration with application of biophysics and chemistry, mostly based on detection concepts that have previously been applied in these fields of research, are evidenced by an increasing number of advanced approaches being developed for EV characterization. In addition, the rapid growth of microfluidic and lab-on-a-chip technology has led to a minimization in the usage of many technologies, combining EV purification, isolation, and analysis in a single assay. Therefore, the sample volume required for analyses in biomedical and clinical applications has substantially decreased. Future progress will be made on these issues. Further changes in the reproducibility and accuracy of the mentioned data will benefit from the active and ever-increasing EV research society and our growing awareness of pitfalls and obstacles. Triple, accurate EV measurement and characterization guarantees at a low cost will be a future step.

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