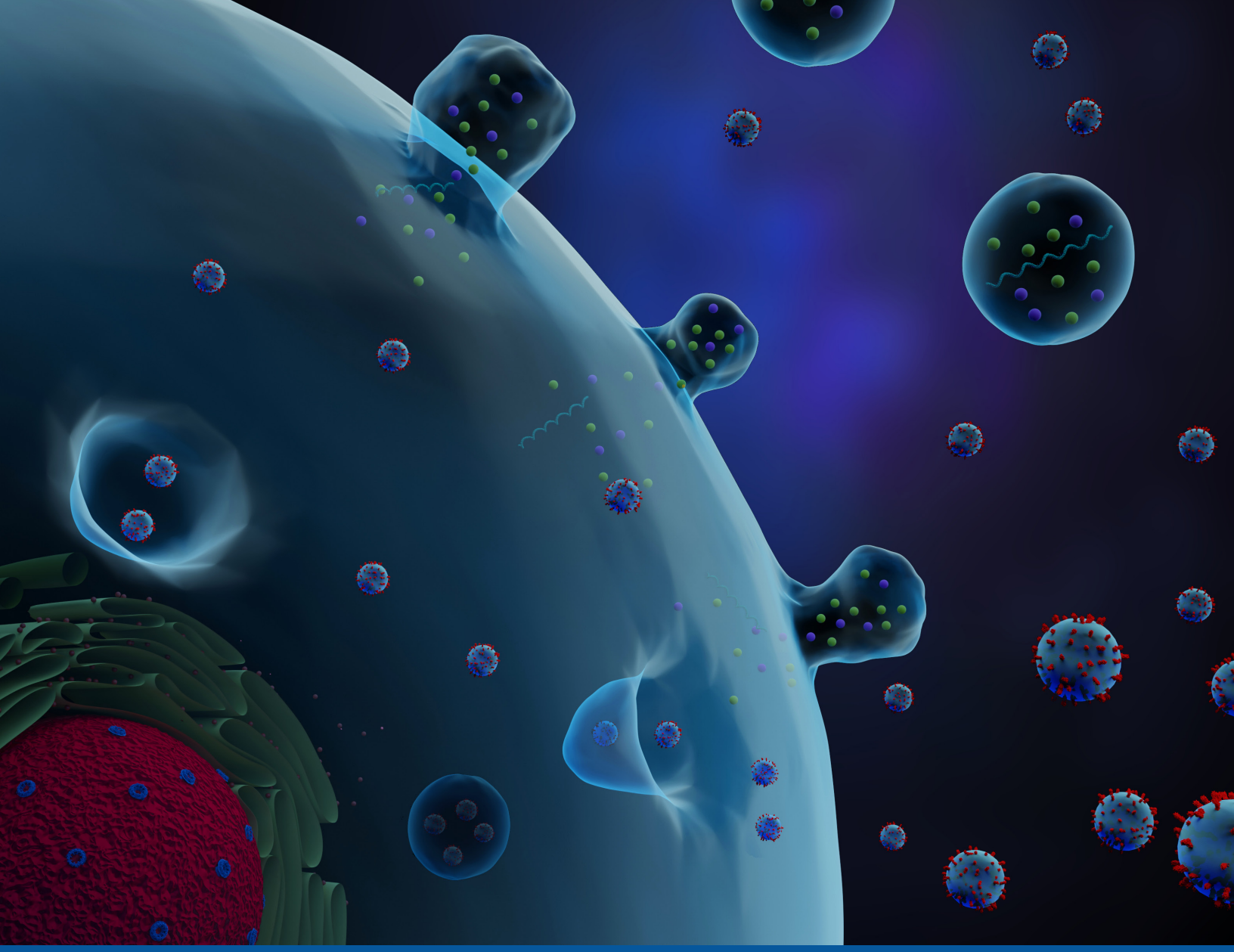

Flow Cytometry in Extracellular Vesicle Analysis

ARTICLE COLLECTION

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FURTHER READING AND RESOURCES

Introduction

Extracellular vesicles (EVs) are small, membrane-bound particles that are secreted by cells and play important roles in intercellular communication. EVs carry unique surface markers, proteins, and nucleic acids that reflect the origin and state of the cells they were secreted from. Flow cytometry is a powerful tool for analyzing EVs due to its ability to measure the size, concentration, and surface markers of individual particles in a heterogeneous sample. Flow cytometry can help identify and quantify EV subpopulations that are associated with disease, which can aid in early detection, diagnosis, and monitoring of disease progression. In this article collection, we have gathered papers that highlight the role of flow cytometry in the evaluation of extracellular vesicles in fundamental research, biomarkers, and cellular therapy products.

First, Couch et al. (2021) provide an overview of the role of flow cytometry in the evaluation of EVs and their importance as diagnostic and therapeutic tools. The authors highlight the use of flow cytometry as a powerful tool for analyzing EVs and emphasize the importance of standardization in both EV isolation and EV experimental approaches. They also underscore the importance of accurate and consistent evaluation of EVs for the effectiveness of cellular therapies. Overall, the importance of EV flow cytometry as a valuable technique for studying the biology of EVs is covered. For additional reading on flow cytometry in the analysis of EVs, including a selection of appropriate markers and standardization of protocols for EV isolation, labeling, and detection, we suggest the thorough overview by Welsh et al. (2023).

Next, Rikkert et al. (2018) highlight the use of flow cytometry in EV based biomarker research and demonstrate EV isolation methods. They consider various protocols to isolate EVs with different characteristics and apply a model to estimate the effect of centrifugation on sample purity. The authors point out the importance of understanding the technical limitations and challenges associated with EV analysis, such as the need for standardized protocols and the lack of consensus regarding EV isolation and characterization. They show that flow cytometry enhances EV research and aids in the development of innovative diagnostic and therapeutic approaches.

Lastly, Welsh et al. (2021) discuss the use of flow cytometry in the analysis of EVs and describe a reporting framework to facilitate consistency between EV measurement, data reproducibility, and experimental interpretation. The authors highlight the importance of standardization in EV analysis, including the development of reference materials and the establishment of guidelines for EV characterization. They also provide an overview of the current challenges associated with EV analysis, such as the small size of EVs, signal calibration, and swarm detection. The framework presented by the authors provides a roadmap for transparent and reproducible EV data generated by flow cytometry, which ultimately expedites the advancement of EV-based diagnostic and therapeutic applications.

Accurate and consistent evaluation of EVs is crucial for the effectiveness of cellular therapies. Our aim is to enable users to explore the potential of using flow cytometry for their specific EV research objectives by highlighting its benefits. Instruments such as the newly released BD FACSymphony ™ A1 Cell Analyzer with BD® Small Particle Detector are ideal flow cytometry tools for EV research. To access further information, we suggest visiting the product page of the BD FACSymphony™ A1 Cell Analyzer (bdbiosciences.com) and exploring the resources available there.

By Christene Smith, PhD
Editor

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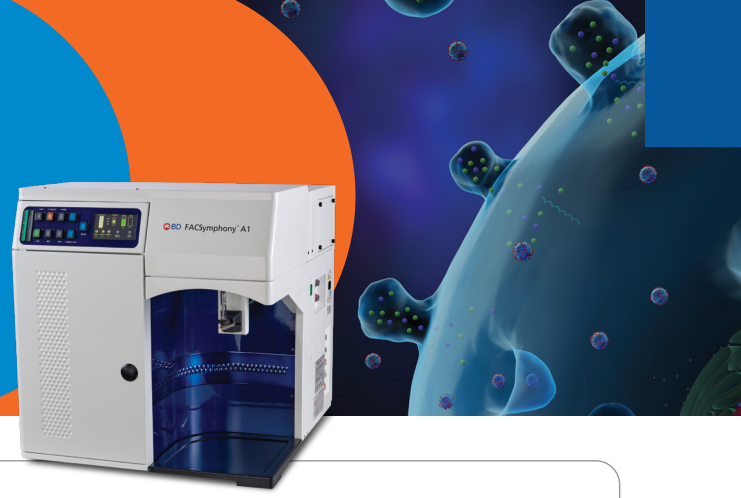
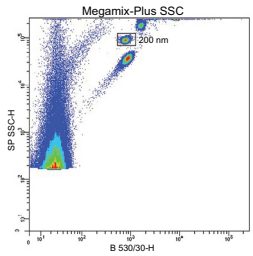
Flow cytometric analysis of extracellular vesicles

with BD FACSymphony™ A1 Cell Analyzer, BD FACSDiva™ Software, FlowJo™ Software and Rosetta Calibration Plugin

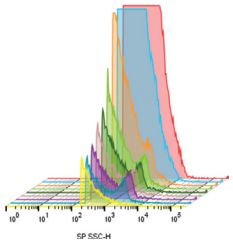




Fine tune your small particle detector (SPD)

STEP 1: Prepare your BD FACSymphony™ A1 Cell Analyzer using standard small particle beads

- Prepare SPD bead mixture
- Align SPD using Picomotor Application (if needed)
- Adjust SP SSC voltage to place bead peaks on target



Ensure reproducible low background noise

STEP 2: Perform start-up procedure to reduce background noise

- Run BD® Detergent Solution Concentrate
- Run sample diluent and check background noise

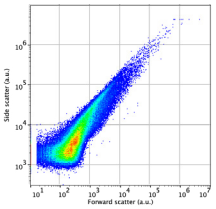
3.1	Buffer only*	3.5	Single-stained controls*
3.2	Buffer with reagents*	3.6	Procedural controls*
3.3	Unstained controls*	3.7	Serial dilution*
3.4	Isotype controls	3.8	Detergent-treated EV samples

Welsh JA, et al. *J of Extracell Vesicles.* 2020;9(1):1713526.

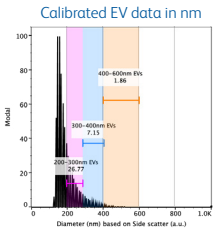
Acquire your EV samples and explore the power of "OR" thresholding

STEP 3: Apply proper controls, calibration and thresholding strategy

- Use "OR" thresholding for SP SSC and relevant fluorescent parameters for maximum information
- Consider running serial dilution series to check for absence of swarming
- Use *MiFlowCyt-EV* framework for publication to guide you on assay controls and SP SSC and fluorescence calibration



Uncalibrated EV data in A.U.



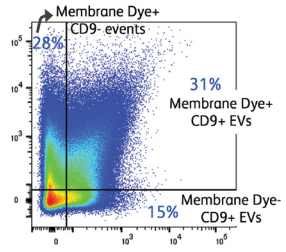
Measure Rosetta Calibration beads and your EV samples

STEP 4: Optional for EV size calibration

- Run Rosetta Calibration beads and your EV samples at the same scatter detector voltage and gain
- Configure the threshold and voltage of the scatter channel so that you detect the smallest beads in the mixture while retaining maximum dynamic range

Flow cytometric analysis of extracellular vesicles

with BD FACSymphony™ A1 Cell Analyzer, BD FACSDiva™ Software, FlowJo™ Software and Rosetta Calibration Plugin



Analyze your EV data

STEP 5: Take advantage of the analysis power of BD FACSDiva™ Software, FlowJo™ Software and the Rosetta Calibration Plugin

- Output files from recommended calibration software compatible with BD FACSDiva™ and FlowJo™ Software for further analysis
- Use the Rosetta Calibration Plugin to derive calibrated size measurements of your EV samples

Still need help?

- Follow troubleshooting guidelines in the BD FACSymphony™ A1 Cell Analyzer User's Guide
- Contact your BD support team for assistance

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Rosetta Calibration webinar: **https://flowjo.wistia.com/medias/92eyr9axmf**

More at: **https://www.exometry.com/products/rosettacalibration**

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REVIEW ARTICLE

A brief history of nearly EV-erything – The rise and rise of extracellular vesicles

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Abstract

Extracellular vesicles (EVs) are small cargo-bearing vesicles released by cells into the extracellular space. The field of EVs has grown exponentially over the past two decades; this growth follows the realisation that EVs are not simply a waste disposal system as had originally been suggested by some, but also a complex cell-to-cell communication mechanism. Indeed, EVs have been shown to transfer functional cargo between cells and can influence several biological processes. These small biological particles are also deregulated in disease. As we approach the 75th anniversary of the first experiments in which EVs were unknowingly isolated, it seems right to take stock and look back on how the field started, and has since exploded into its current state. Here we review the early experiments, summarise key findings that have propelled the field, describe the growth of an organised EV community, discuss the current state of the field, and identify key challenges that need to be addressed.

KEYWORDS
ectosome, exosome, extracellular vesicle, microparticle, microvesicle

1 | THE EARLY EXPERIMENTS

The experiments in which EVs were specifically identified as biological entities, with enzymatic and functional potential, began during the 1980s and 1990s. Prior to this period there are numerous studies that hint at potential structures that *would subsequently* be described as EVs, or that describe experiments in which we can retrospectively speculate *may have* involved the activity of EVs. In this sense the story of the origins of EV research arguably begins with the studies of coagulation.

As a topic this dates back to the mid-1600s and is covered in excellent reviews elsewhere (Hargett & Bauer, 2013; Quick, 1966). For the purposes of this article we will start with Chargaff and West and their studies on blood clotting, performed in New York in the 1940s. West was a clinician, with an on-going interest in anaemia and haemophilia, and Chargaff was a biochemist. Chargaff had begun a series of papers in 1936 in the Journal of Biological Chemistry entitled *Studies on the Chemistry of Blood Coagulation* and made an observation in paper XIX of the series – *Cell Structure and the Problem of Blood Coagulation* – which can be interpreted as the beginning of the field of EV biology. When spinning down blood to establish a centrifugation protocol to separate clotting factors from cells, Chargaff observed that "*the addition of the high speed sediment to the supernatant plasma brought about a very considerable shortening of the clotting time*" (Chargaff, 1945). Enigmatically he went on to say "*this will be discussed in detail on a later occasion*"; that later occasion turned out to be his paper published with Randolph West in 1946 on *The Biological Significance of the Thromboplastic Protein of Blood*. Here they discovered a 'particulate fraction' which sedimented at 31,000 *g* and had high clotting potential, as well as a 'thromboplastic protein'. The authors suggested that this fraction "*probably includes, in addition to the thromboplastic agent, a variety of minute breakdown products of blood corpuscles*" (Chargaff & West, 1946). However, it would be some years before these were specifically identified as EVs.

In fact, 17 years would pass until Peter Wolf described a "*material in minute particulate form, sedimentable by high-speed centrifugation and originating from platelets, but distinguishable from intact platelets*" which we now know as the EV fraction. Wolf published electron microscopy images of these particles, which he described as 'platelet dust' (Wolf, 1967). Following this, in 1971, Neville Crawford published further images of these vesicles—which were now being described as 'microparticles'—obtained from platelet-free plasma. Crawford also showed they contained lipid and carried cargo including ATP and contractile proteins (Crawford, 1971). These pioneering experiments with platelets were the first to describe the presence and coarse structure of such cell-free components and hinted at their potential biological importance.

Between the mid-1960s and early 1980s other early electron microscopy studies described structures consistent with the sub-micron size of EVs. In the summer of 1966, Sun described vesicle-like structures released from alveolar cells into the alveolar space (Sun, 1966). In the late 1960s, H. Clarke Anderson and Ermanno Bonucci described 'matrix vesicles'. These small membrane-bound vesicles of different sizes are embedded in the matrix of hypertrophic cartilage and could potentially play a role in bone mineralisation (Anderson, 1969; Bonucci, 1967). Nunez et al., and Gershon (1974) described the presence of small (1-10 nm) extracellular vesicles in the bat thyroid gland during arousal from hibernation (Nunez et al., 1974). In fact, this paper was one of the first to describe the presence of multivesicular bodies (MVBs) close to the apical membrane. The authors proposed that "*fusion of the outer or limiting membrane of the multivesicular body with the apical plasma membrane might lead to the release of the vesicles contained within the structure into the luminal space*" (Nunez et al., 1974). Indeed, we now define a subtype of EV, commonly called the exosome or small EV, as being formed when the endosomal MVB structure fuses with the plasma membrane, leading to the release of the intraluminal vesicles (for an illustration of the different types of EVs *see* Figure 1).

In addition to these experiments where vesicles were found in a happenstance manner, others were specifically looking for vesicles. Between 1950 and 1970 there were several researchers who were hoping to prove that viruses caused diseases beyond infection, specifically that they caused cancer. In looking for 'virus-like particles' in biofluids they often came across particulate matter (Levine et al., 1967; Seman et al., 1971) but could not identify anything they thought might actually be viral in nature (Dmochowski et al., 1968; Haguenau, 1959; Levine et al., 1967). Moreover, the particles seemed to be present in control fluids as well as those from cancer patients (Fawcett, 1956; Lunger et al., 1964; Prince & Adams, 1966). By the mid-1960s the consensus was that it was unlikely that particles found in biofluids were attributable to viruses but were rather an artefact of separation (Prince & Adams, 1966). Finally, in 1975 Dalton published a paper studying fractions of filtered and unfiltered foetal bovine serum and demonstrated that the sera held similar particles to an epithelial cell line. He put an end to the reign of the virus-like particle by saying that '*to call structures with the morphology of normally occurring vesicles of multivesicular bodies and of microvesicles associated with epithelial cells "virus-like" is unwarranted*' (Dalton, 1975).

Studies in other organisms suggested that vesicular structures extruded from cells were not unique to mammals. A study of *Ochromonas danica*, a flagellated alga, revealed the presence of a range of vesicles that could be visualised budding from cells and isolated by centrifugation (Aaronson et al., 1971). Preparations including EVs released by the yeast *Candida tropicalis* were shown to decrease growth of other cultures of yeast (Chigaleichik et al., 1977). Different kinds of vesicles were shown to be released by Corynebacterium, some of which were shown to induce cell agglutination (Vysotskii et al., 1977); Acinetobacter, which were seen to release phospholipid-rich EVs (Käppeli & Finnerty, 1979); and the gram negative bacteria, *Escherichia coli*, which was shown to produce EVs containing lipopolysaccharide complexes (Käppeli & Finnerty, 1979). Whilst these studies began to unravel the ultrastructure of cells and the potential existence of EVs the research had yet to gain the momentum to unite as a cohesive field.

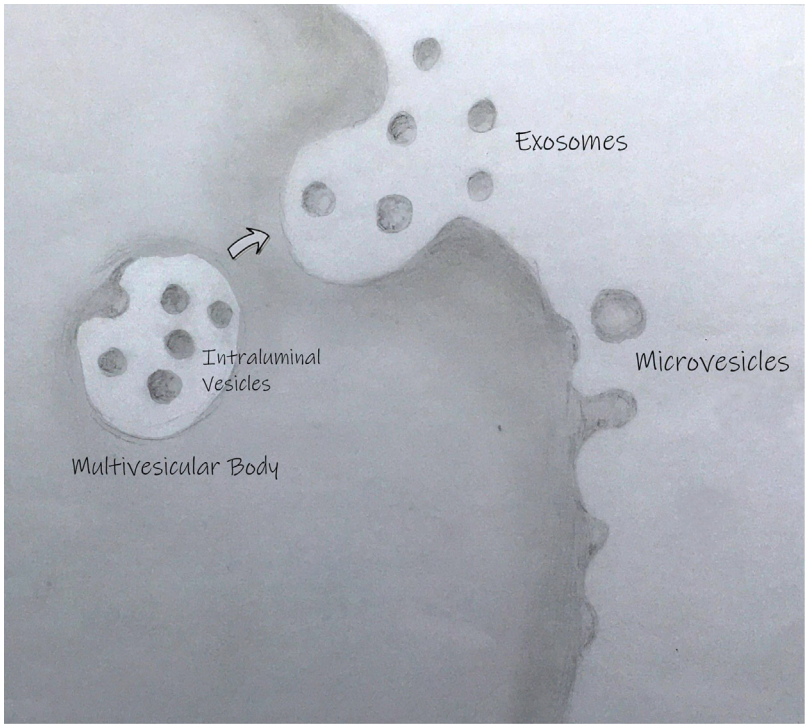

FIGURE 1 The primary routes of extracellular vesicle biogenesis. Exosomes are released from cells when a multivesicular body (which is formed when an early endosome matures and inwardly buds to form intraluminal vesicles) fuses with the plasma membrane. Ectosomes (more commonly called microvesicles and microparticles) are formed when the plasma membrane buds outwardly and pinches off. Cargo can be loaded into both intraluminal vesicles (which are released as exosomes) and ectosomes. Other types of vesicles such as apoptotic bodies (not shown) can be released by dying cells.

2 | THE START OF SOMETHING ~~BIG~~ SMALL

The early 1980s mark the start of the era of expansion and more specific understanding in EV research. Whilst the significant explosion of papers, theories, arguments about nomenclature and EV-related societies wouldn't begin for another 20 years or so, the cohesion began here. Two seminal and complementary papers published by the Johnstone and Stahl laboratories made a watertight case for the release of intraluminal vesicles from the cell, and defined them as exosomes (Harding et al., 1983; Pan & Johnstone, 1983). Whilst these papers are now considered seminal and the origins of our field, Rose herself felt the discovery to be happenstance, saying they had an '*Alice in Blunderland approach which led to the discovery of exosomes*' (Johnstone, 2005). Both laboratories were using reticulocyte maturation as a model; Stahl's group to investigate membrane trafficking, and Johnstone's lab to study the biochemistry of the plasma membrane. Their work showed that during reticulocyte maturation the transferrin receptor was lost via the release of vesicles. Cliff Harding, then an MD/PhD in the Stahl laboratory, produced some stunning EM images demonstrating that these vesicles were released from the lumen of MVBs upon fusion with the plasma membrane. Conceptually, the Harding et al. (1983) paper revealed the existence of a novel intracellular sorting and trafficking pathway, now referred to as the exosome secretion pathway. Although Trams et al., and Heine (1981) originally coined the term 'exosome' to describe EVs shed from the surface of the cell (Trams et al., 1981), Rose Johnstone applied the name to those vesicles specifically released following fusion of MVBs with the plasma membrane and in this context the name caught on (Johnstone et al., 1987; Witwer & Théry, 2019).

As well as defining one of the hallmarks of EV vernacular, an early lecture by Rose Johnstone may have been responsible for the global opinion of EVs as just 'waste disposal mechanisms' for the ensuing decade. In 1991 she gave the Jeanne Manery-Fisher Memorial Lecture which she titled *'Maturation of reticulocytes: formation of exosomes as a mechanism for shedding membrane proteins'* which was primarily based on her paper from the same year where she suggested that exosomes were a '*major route for externalization of obsolete membrane proteins*' (Johnstone et al., 1991). This paper demonstrated the presence of the transferrin receptor on exosomes, and the presence of the nucleoside transporter. The authors demonstrated that different cellular stresses resulted in the internalization and shedding of these membrane components at different times. Whilst they did not speculate on the mechanisms of this, the message that this was a way for the cells to shed 'obsolete' proteins stuck in the minds of researchers for some years to come.

Despite this, these early studies laid the foundation for the explosion of interest that followed over the next 35 years. In terms of the period between these seminal papers and the start of the massive expansion in EV research at the millennium, form seemed to come before function. Articles on platelet derived microparticles, microvesicles and exosomes dominated, with some important early advances in the understanding of the fundamental nature of EVs. These early studies demonstrated lateral diffusion of lipids and proteins in vesicle membranes (Gawrisch et al., 1986) and the presence and function of flippases (Vidal et al., 1989). Studies revealed glimpses of the iconic components of EVs we know today such as Rab, ARF (Vidal & Stahl, 1993) and the tetraspanins

(Escola et al., 1998). As early as 1986 there were concerns about storage of blood and its effects on the EV population (George et al., 1986). In addition to work on mammalian EVs, a wealth of knowledge was developed about bacterial EVs in studies from Liverpool on *Porphyromonas gingivalis* (Kay et al., 1990; Smalley & Birss, 1987; Smalley et al., 1988, 1989). These last papers demonstrated not only the presence of bacterial EVs but the interaction of these EVs with mammalian cells in the body (Kay et al., 1990).

During the 1980s and 1990s several articles reported the quantification of EVs, demonstrating altered EV numbers in disease. The phenomenon started around 1993 with a paper on elevated microparticles in transient brain ischemia and other infarctions (Lee et al., 1993), but goes on to be explored in diseases such as angina (Singh et al., 1995) and Crohn's (Powell et al., 1996). Papers describing the physical and biochemical characteristics of EVs also began to emerge. Rose Johnstone's 1989 paper demonstrated exosomes released from reticulocytes are enzymatically active (Johnstone et al., 1989). Membrane vesiculation was shown to be a potentially protective mechanism to prevent cell lysis (Iida et al., 1991), and a way of specifically exposing phosphatidyl serine to enhance clotting (Chang et al., 1993). It was also revealed that other active enzymes could exist in EVs (Fourcade et al., 1995). Outside the field of platelet biology, it was discovered that EVs from immune cells are capable of presenting antigen (Raposo et al., 1996). This last paper, in particular, was a watershed moment that caught the imagination of many and helped to catalyse increased interest in the field of EVs. It showed that EVs had the potential to be harnessed as anti-tumoral vaccines; indeed, this study led the Amigorena lab to investigate whether dendritic cells secrete EVs that, when loaded with tumor peptides, can eradicate tumours (Zitvogel et al., 1998), and led to clinical trials over the next decade (Escudier et al., 2005). Importantly, it showed that EVs could play functional roles in biological processes. Taken together, these ideas that EVs could have physiological roles, that they could be used as biomarkers, and that they could have therapeutic applications, led to the explosion of interest in EVs in the early 21st century.

3 | A ROSE BY ANY OTHER NAME

In 2018 Roy and colleagues performed a systematic survey of all the papers published in the field since 2000, demonstrating the exponential growth of the field since the millennium (Roy et al., 2018). This included not only thousands of papers but also patent applications and grant funding. The specific search criteria to isolate key papers for this current review identified 1017 articles published in the 15 years between 1985 and 2000, and more than four times that number in the 10 years to 2010. The issue, still plaguing the field today, although vastly improving (Witwer & Théry, 2019), was the issue of nomenclature (Box 1) (Gould & Raposo, 2013; Witwer & Théry, 2019). Of the > 4000 papers from 2000 to 2010 the most popular search term was 'microparticles'. This proves challenging as a search criterium because not only can it refer to platelet microparticles, but also microparticles of iron oxide (frequently used as an imaging agent) and synthetic microparticles for drug delivery. Sifting out the relevant papers remained challenging. During this period 'exosomes' remained more popular than 'microvesicles' or 'ectosomes' (respectively 945, 664 and 261 papers; though it should also be noted that the term 'exosome' also describes RNA-processing machinery). The term 'extracellular vesicles' was barely seen at all with a mere 31 papers.

In the decade following the year 2000 the first reviews began to be published in the field of EV biology (Denzer et al., 2000; Schartz et al., 2002). The growing community of researchers started to explore the nature of EVs in more depth, investigating the proteome of EVs from various cell types (Bard et al., 2004; Théry et al., 2001; Wubbolts et al., 2003) as well as the lipidome (Subra et al., 2007). Cytokines were shown to be shed via EVs (Mackenzie et al., 2001) and EVs derived from immune cells were found to play a key role in the function of the immune system (Skokos et al., 2003; Van Niel, 2003). The increased interest in tumor-derived EVs (Wolfers et al., 2001), combined with new knowledge of the role of EVs in the immune system, led to their potential as anti-tumor therapy (Chaput et al., 2003). As the decade winds down, the real expansion in EV research began. Papers began to demonstrate the functional effects of EVs in vivo, protecting animal models from disease (Colino & Snapper, 2007). The functional transfer of nucleic acids was demonstrated (Ratajczak et al., 2006; Skog et al., 2008; Valadi et al., 2007), and a report that plant cells can use EVs as a means of communication was also published (An et al., 2007). The increased interest in EV-based therapy was merged with burgeoning interest in stem cells as therapy, and 2009 saw the emergence of a plethora of papers on mesenchymal stem cell (MSC)-derived vesicles (Bruno et al., 2009), further increasing the therapeutic opportunities afforded by EVs.

Some key EV milestones from 1940 to 2010 are summarised in Figure 2. From 2010 to today the expansion of the field has been enormous. EVs have been shown to be involved in numerous biological processes across many species, and they contribute to a plethora of diseases when deregulated. It would be unfair to pick out individual contributions to this latest decade of work as it has become so diverse and specialised, and the reader is directed to more recent reviews (Mathieu et al., 2019; Raposo & Stahl, 2019; Welsh et al., 2020).

The early 2000s also saw the first organized EV meetings take place, and the regular meetings of the International Society for Extracellular Vesicles (ISEV) now have thousands of participants working in a multitude of disciplines from all over the world. Now came the time to organize these disparate researchers and bring them together with a common purpose.

BOX 1 – EV NOMENCLATURE

In the early years of the field, a variety of terms were used to describe the structures that were observed, including 'extracellular microvesicles', 'microparticles, 'pequenas particulas' (small particles), and 'virus-like particles'. The term 'exosome' was first used in the context of EVs by Trams *et al* (Trams et al., 1981) to describe vesicles that are produced directly by outward budding at the plasma membrane. Later, Rose Johnstone used the term 'exosome' to describe vesicles released following the fusion of MVBs with the plasma membrane (Johnstone et al., 1987), and this has become ISEV's recommended term for this type of vesicle (Théry et al., 2018). As the field grew, and understanding of the variety of biogenesis pathways increased, it became clear that distinct and precise nomenclature was required (Gould & Raposo, 2013). It was suggested that the catch-all term 'extracellular vesicles' should be used to describe non-replicating structures that are delimited by a lipid bilayer (György et al., 2011), and this was formalised into the current recommendations within the MISEV guidelines (Théry et al., 2018). Confusion in nomenclature can arise due to the assignment of arbitrary size ranges for different types of vesicle; in fact, the proposed names for different types of EVs are based on biogenesis pathways (Théry et al., 2018) (see also figure 1). The range of terms used to describe the different types of EV continues to grow, and authors should clearly define what type of EV they are referring to (Théry et al., 2018; Witwer & Théry, 2019). The issue of EV nomenclature has caused controversy over the years, and not all researchers agree with current recommendations (Witwer & Théry, 2019). The use of the term 'exosome' as a general term for EVs continues to pervade the literature (Roy et al., 2018), despite the fact that most (if not all) EV samples contain a heterogeneous mixture of vesicle types (Van Deun et al., 2017). This prevalence for the term 'exosomes' to describe EVs may be due to the anecdotally reported perception of exosomes as a more 'desirable' term, particularly in the context of industrial applications of EVs (Witwer & Théry, 2019). Similarly, the terms ectocytosis (Stein & Luzio, 1991), proposed to design specifically release of EVs from the plasma membrane, and ectosomes for such EVs (Cocucci & Meldolesi, 2015; Hess et al., 1999), are still less commonly used than the term 'microvesicles' for plasma membrane-derived vesicles. It is therefore important that the field continues to discuss the best way to describe these exciting extracellular voyagers, and clear reporting is crucial to reduce confusion in nomenclature.

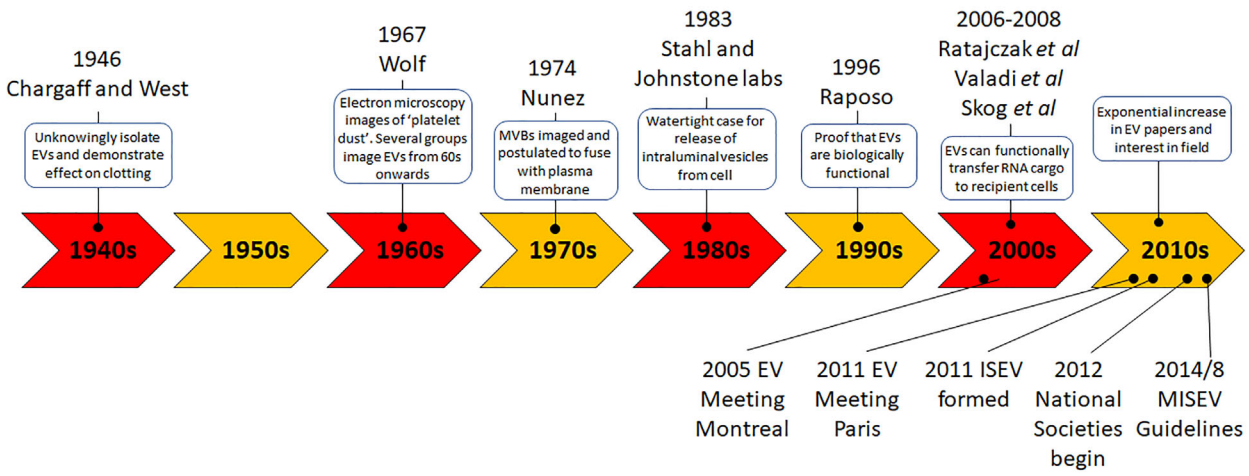

FIGURE 2 Timeline of selected milestones in the EV field

4 | BRINGING ORDER TO THE CHAOS

The first international meeting for EVs (called exosomes at the time) was organised by Rose Johnstone and held in Montreal in 2005 (Couzin, 2005). An international meeting in 2010 in Oxford focused on advances in methodologies for measuring EVs (including new biophysical approaches such as Nanoparticle Tracking Analysis (Dragovic et al., 2011), which led to the publication of the first book on EVs (Harrison et al., 2014). A seminal moment came at a vibrant (and oversubscribed) international meeting organised by Clotilde Théry and Graça Raposo, held in Paris in 2011. At this meeting of over 200 attendees, Jan Lötvall (who fortunately was allowed to attend despite a late registration!) proposed the formation of an International Society to represent the interests of the field. Following extensive consultations with members of the community, the International Society for Extracellular Vesicles (ISEV) was formed in 2011. The first ISEV meeting was held in 2012, in Gothenburg, Sweden,

and attracted more than 400 participants and was also oversubscribed. Subsequent ISEV annual meetings in Boston (2013), Rotterdam (2014, 2016), Washington (2015), Toronto (2017), Barcelona (2018) and Kyoto (2019) saw rapid growth in attendee numbers with over 1000 attendees recorded for the last 2 years. With the eruption of a global pandemic in 2020, the ISEV meeting went virtual, holding its first international online conference to great success. The society also organises and supports a variety of other focused workshops and surveys that lead to 'position papers' (Hill et al., 2013; Lener et al., 2015; Mateescu et al., 2017; Russell et al., 2019; Witwer et al., 2013), survey outputs (Gardiner et al., 2016; Soekmadji et al., 2018), and meeting reports (Araldi et al., 2012; Clayton et al., 2018; Hu et al., 2017; Soares et al., 2017), many of which are published in the society's 'Journal of Extracellular Vesicles' (JEV) (Lötvall et al., 2012). These have played an important role in helping to collate and focus the efforts of the field. This is perhaps best exemplified by the publication of 'Minimal Information for Studies of EVs' (MISEV) guidelines in 2014 (Lötvall et al., 2014), which has been more recently reviewed, in 2018 (Théry et al., 2018). ISEV has therefore provided an effective platform for researchers around the world to come together and share their work on EVs.

As the EV field expands, so too does the number of researchers in each country. This has led to the formation of numerous 'National Societies' or local networks who conduct their own local meetings and support EV research within their own countries. For a field where for many years there was considerable scepticism about whether EVs were just cellular debris, local support networks capable of validating findings and sharing new ideas, reagents, models and techniques, are crucial. These networks began in the US in 2012 with the American Society for Exosomes and Microvesicles and expanded from there; the Grupo Español de Innovación e Invesigación en Vesículas Extracelulares (in 2012), the UK, French and German Societies for EVs (in 2018), are but a few of the many national groups working together with the common goal of forwarding EV research. These National Societies help to coordinate national meetings and support regional networks of EV researchers, providing opportunities for newcomers to the field to network with established labs. Together with ISEV they provide an important support mechanism in the rich research ecosystem for the field.

ISEV has also strived to produce educational material for those new to the EV field. This includes the production of two popular and free Massive Open Online Courses (Lässer et al., 2016), the production of a 3D animated video on EV function, and posters on the basics of EVs (Nieuwland et al., 2018). This not only helps give new researchers perspective on the field, but also helps with some of the challenges and disputes the field has had, and continues to have, regarding standardization and nomenclature.

5 | CHALLENGES

The proliferation of EV research around the world has propelled the field forwards at an ever-increasing pace, but this brings with it a different set of problems. The EV field, as with science more generally, may suffer from a lack of reproducibility (Begley & Ellis, 2012; Neuhaus et al., 2017). This is exacerbated by the relatively young state of the field and the 'hype', which drives accelerated publication of 'exciting' new findings. The technical challenges and position papers from ISEV and other international groups have been outlined comprehensively elsewhere (Ramirez et al., 2018). Below are some of the major issues the field continues to contend with.

6 | STANDARDISATION AND REPORTING

There is no universal agreement on many aspects of methodology in EV research, including the best methodology for enrichment, and protocols vary between laboratories (Gardiner et al., 2016). In the 1990s the International Society on Thrombosis and Haemostasis (ISTH) vascular biology subcommittee (SSC) initiated the discussion and early standardization efforts of microparticle measurements. The SSC has continued to publish important articles on pre-analytical variables, inter-laboratory studies and standardization of flow cytometry. On-going standardization and collaboration between the ISTH SSC, ISAC (International society for advancement of cytometry) and ISEV continue. A recent consortium-effort to catalogue EV research revealed a total of 1,742 experiments with 190 different isolation methods and 1,038 unique protocols to isolate EVs (Van Deun et al., 2017). While it is too early to pronounce which methodology is 'right or wrong', the heterogeneity in approach and frequent lack of complete reporting make comparing and interpreting the results of different studies more difficult and reaching general conclusions more challenging. This is further compounded by a lack of experimental reference materials and controls that can be reliably used to standardise experiments between labs. Initiatives such as EV-TRACK (Van Deun et al., 2017), the MISEV guidelines (Théry et al., 2018), EV databases (Kalra et al., 2012; Simpson et al., 2012), attempts to generate reference materials (Welsh et al., 2020). ISEV taskforces and ISEV workshops on 'Rigour and Reproducibility' aim to address these issues, but transparency in reporting and standardisation of methodology remain two of the greatest challenges for this nascent field.

7 | TECHNICAL CHALLENGES

There are many technical challenges associated with working on EVs which are detailed well elsewhere (Ramirez et al., 2018). Briefly, there are several techniques available for isolating EVs; they all have pros and cons, and the best choice depends on the intended downstream applications, the type of EV of interest, and level of homogeneity required (Gardiner et al., 2016). However, there is still a need to develop improved methodology to enrich higher yields, with greater homogeneity, faster time, and lower cost. The challenge is because the fluids that EVs are enriched from are typically complex matrices containing multiple contaminants, often of similar size and/or density (Ramirez et al., 2018). Improved tools are also required for characterising and quantifying EVs. A key problem here is the relatively small size of most EVs, which makes specifically counting and characterising EVs a challenge, and there is currently no perfect instrument for quantifying and characterising EVs. Another issue is their relative paucity of material when isolating EVs. To obtain sufficient material for testing using most 'bulk methods' (in which material from multiple vesicles is aggregated for testing), such as Western blotting, a lot of EVs are required. More sensitive methods are therefore required to make EV characterisation less onerous on laboratories. 'Single-EV' methodology must be developed and improved to allow a greater range of experiments to be performed and new insights generated into EV biology. Finally, improved in vivo methods are required for studying the biology of EVs. These challenges, amongst many others, are being addressed by multiple labs around the world, and as these technical issues are addressed our ability to test hypotheses about EV function will improve.

8 | UNANSWERED BIOLOGICAL QUESTIONS

There are some areas of EV biology where more is known, and some where almost nothing is known (Soekmadji et al., 2018). One area that needs addressing is the lack of suitable markers for specifically identifying different types of EVs. Some excellent work has been done to address this (Jeppesen et al., 2019; Kowal et al., 2016; Zhang et al., 2018). However, due in most part to the overlap in EV biogenesis mechanisms and the overlap in size and density of different EV types, it has proven difficult to generate reliable markers for different EV subtypes. Despite this, several laboratories have shown that different subtypes of EVs may exist, with different cargo, release mechanisms, and different functions (Goberdhan et al., 2019; Willms et al., 2016; Yeung et al., 2018). Better understanding of these subpopulations is a key goal for EV research over the coming decade. Another area in need of further work is EV uptake, and in particular, how EVs functionally deliver cargo to recipient cells (Mulcahy et al., 2014; Russell et al., 2019). This is thought to be a fairly low-efficiency process, and it is understood that a significant number of EVs go to the lysosome, where they presumably are destroyed (Russell et al., 2019). The development of novel in vitro and in vivo systems for modelling EV transfer and cargo release is therefore another priority for the field. An increased understanding of cargo delivery would not only help us to understand EV biology, but it would help us to engineer vesicles specifically to avoid lysosomal destruction, resulting in the rapid emergence of strong EV therapeutic platforms.

9 | CONCLUSIONS AND FUTURE PERSPECTIVES

Since the early electron microscopy and biochemistry studies from the 1940s through to the 1980s, the EV field has rapidly progressed. The range of functions that have been assigned to EV grows by the week. The reasons for this increased interest are manifold. The idea that these small messengers can carry cargo from one cell and deliver it for functional use by another cell is a highly attractive one that has captured the imagination. The results of many studies confirm the work of early pioneers in the field, indicating an important functional role for EVs in cell-to-cell communication. Their roles in many biological processes, and their deregulation in disease have fuelled further interest. EVs have been found in every biological fluid tested thus far (Carollo et al., 2019; Garcia-Contreras et al., 2017; Jansen & Li, 2017; Lee et al., 2019; Li et al., 2019; Meng et al., 2019; O'farrell & Yang, 2019) so perhaps the greatest translational prospect for them lies in their diagnostic, prognostic and therapeutic abilities (Box 2). They have the potential to be modified for the delivery of therapeutic cargo in the treatment of different disorders (Clemmens & Lambert, 2018; Melling et al., 2019; Wiklander et al., 2019). Both their therapeutic and diagnostic potential stems from their ability to protect cargo in circulation, and their functionality as natural cell-to-cell transporters of multiple complex biological cargo.

In the coming years we expect the increase in EV research observed over the past two decades to continue. This will carry on yielding incremental improvements in our knowledge of EV biology, and the translational benefits will follow.

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BOX 2 EVS IN DIAGNOSTICS AND THERAPEUTICS

- The growth of the EV field has been accompanied by a growth in patents to use EVs as diagnostic markers, and therapeutic delivery vehicles. Between 2000 and 2020 there were > 500 patents filed in the US which included any of the various terms for EVs (Roy et al., 2018). As a more specific example of their use, between 2000–2020 > 30 clinical trials specified using EVs, either as diagnostic tools or as therapeutics, mainly in the field of cancer biology.
- There are two major ways that EVs might be useful as biomarkers for disease. Firstly in the acute setting as *diagnostic* markers, to determine whether someone has had an ischemic or a haemorrhagic stroke for example. And secondly in the *prognostic* setting, to help determine the course of a disease such as cancer, or the responsiveness of a patient to, for example, anti-depressant therapy. The first CLIA/FDA approved diagnostic test using EVs is the EPI ExoDx platform, a rule-out test for prostate cancer which uses gene expression to determine whether patients are positive for cancer-specific markers (Mckiernan et al., 2018).
- The potential for EVs as therapeutics is vast. EVs can potentially be engineered to deliver specific therapeutics, including proteins and RNA. Non-engineered EVs, for example those produced from MSCs, also have the potential to be used in a therapeutic context. The first clinical trials using EVs as therapeutics used autologous EVs derived from patient dendritic cells and demonstrated that EVs are capable of boosting the immune response to lung cancer in both phase I and phase II/III studies (Besse et al., 2016; Escudier et al., 2005; Morse et al., 2005). Several more trials have since been established studying the potential of several types of EVs, from autologous EVs to plant-derived EVs in diseases from cancer to stroke (Nassar et al., 2016; Wiklander et al., 2019). While challenges remain, the potential of EVs in diagnostic and therapeutic is beginning to be unlocked and there is much excitement for the translational applications of EVs in the coming decades.

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CONFLICT OF INTEREST

Edit Buzas: Shere Gene Tharapeutics Inc. Boston, MA, US. Advisory Board Member. David Carter: Evox Therapeutics Ltd, Employee. Yong Song Gho: Founder and CEO of Rosetta Exosome, INC. Philip Stahl, Graca Raposo, Kenneth Witwer and Yvonne Couch: no conflicts of interest.

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Cytometry PART A
ISAC Journal of Quantitative Cell Science

Centrifugation Affects the Purity of Liquid Biopsy-Based Tumor Biomarkers

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Abstract

Biomarkers in the blood of cancer patients include circulating tumor cells (CTCs), tumor-educated platelets (TEPs), tumor-derived extracellular vesicles (tdEVs), EV-associated miRNA (EV-miRNA), and circulating cell-free DNA (ccfDNA). Because the size and density of biomarkers differ, blood is centrifuged to isolate or concentrate the biomarker of interest. Here, we applied a model to estimate the effect of centrifugation on the purity of a biomarker according to published protocols. The model is based on the Stokes equation and was validated using polystyrene beads in buffer and plasma. Next, the model was applied to predict the biomarker behavior during centrifugation. The result was expressed as the recovery of CTCs, TEPs, tdEVs in three size ranges (1–8, 0.2–1, and 0.05–0.2 μm), EV-miRNA, and ccfDNA. Bead recovery was predicted with errors <18%. Most notable cofounders are the 22% contamination of 1–8 μm tdEVs for TEPs and the 8–82% contamination of <1 μm tdEVs for ccfDNA. A Stokes model can predict biomarker behavior in blood. None of the evaluated protocols produces a pure biomarker. Thus, care should be taken in the interpretation of obtained results, as, for example, results from TEPs may originate from co-isolated large tdEVs and ccfDNA may originate from DNA enclosed in <1 μm tdEVs. © 2018 The Authors. Cytometry Part A published by Wiley Periodicals, Inc. on behalf of International Society for Advancement of Cytometry.

Key terms

biomarker; centrifugation; circulating cell-free DNA; circulating tumor cells; exosomes; extracellular vesicles; flow cytometry; microparticles; miRNA; tumor-educated platelets

POTENTIAL biomarkers in the blood of cancer patients include circulating tumor cells (CTCs) (1–3), tumor-educated platelets (TEPs) (4), tumor-derived extracellular vesicles (tdEVs) (5), EV-miRNAs (6), and circulating cell-free DNA (ccfDNA) (7,8). Typically, the first step to isolate a biomarker involves (differential) centrifugation, which isolates particles based on size and density. After centrifugation and optionally further processing, the biomarker is measured based on antigen exposure (CTCs and tdEVs) or on the composition of RNA (TEPs and EV-miRNAs) or DNA (ccfDNA). Fractions containing the biomarker of interest may be impure and contain substantial quantities of "contaminants", for example, other biomarkers. These contaminants may affect the obtained signal and potentially lead to the misinterpretation of results. Therefore, we applied a model based on the Stokes equation to assess the purity of biomarkers after centrifugation according to protocols used by other groups to study their biomarkers of interest (1–8).

Materials and Methods

Stokes Model

Figure 1 shows the principle of centrifugation. Before centrifugation, particles are uniformly distributed in a medium. Upon centrifugation, particles denser than the medium will travel toward the bottom of the tube. After centrifugation, the top

fraction is collected, defined here as the "supernatant." We define the "pellet" as the fraction remaining in the tube. The dashed line in Figure 1 is the interface between the supernatant and the pellet. The recovery of particles in the pellet is the fraction of particles in the pellet after centrifugation. This recovery depends on the starting point of the particle in the medium, as well as the densities and diameters of the particles. Consequently, the pellet will contain a high concentration of large and high density particles compared to the supernatant. For a swing-out rotor, the distance traveled by a particle can be modeled by the Stokes equation. In the Stokes equation, the speed of the particle results from the balance between the buoyant force and the drag force on a particle in a medium. In a centrifuge, the gravitational acceleration g depends on the distance from the axis of rotation.

$$g = R\omega^2 \tag{1}$$

where R is the distance to the axis of rotation and ω is the angular velocity of the centrifuge. After time T, the distance R of a particle starting at R_0 is given by the following equation:

$$R_T = R_0 e^{\frac{d^2(\rho_P - \rho_M)}{18 S \eta}\omega^2 T} \tag{2}$$

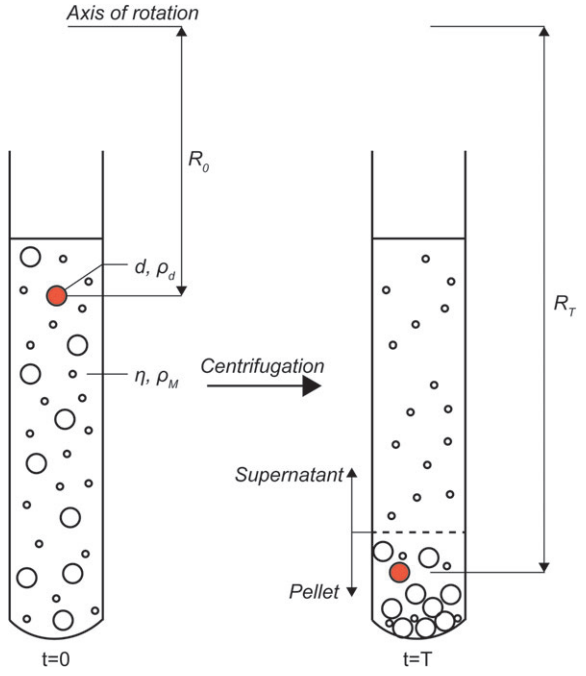

Figure 1. Centrifugation to isolate biomarker of interest from whole blood. The position of a particle after centrifugation can be described with the stokes equation (Eq. 2). Centrifugation of particles that are uniformly distributed throughout the sample causes large, high-density particles to move down to the pellet (below the dashed line), while small, low-density particles stay in the supernatant (above the dashed line). [Color figure can be viewed at wileyonlinelibrary.com]

where d and ρ_P are the diameter and the volumetric mass density of the particle and ρ_M and η are the volumetric mass density and viscosity of the medium. To allow nonspherical shapes such as platelets, the shape factor S is introduced (S = 1 for spheres). S accounts for the additional drag these nonspherical particles experience (9). Note that for nonspheres, d is the diameter of a sphere with equivalent volume. Model assumptions are (1) negligible wall effects, (2) instantaneous formation of the packed cell fraction during centrifugation of whole blood, and (3) aspiration sufficiently gentle not to disturb the pellet (see (10,11) for a complete description of the assumptions in the Stokes model). The model was programmed in Matlab (v2017a; MathWorks, Natick, MA) and applied to the evaluated centrifugation protocols.

Stokes Model Sample Properties

Table 1 shows the assumed sample properties, which were derived from the literature for a temperature of 20°C (see Supporting Information Table S1 for additional background). If available, volumetric mass densities were taken from references that applied density media with neutral osmotic effects. Platelets have a log-normal volume distribution (12) and a shape factor S of 2.0 (9,13). Platelets can have a volumetric mass density of 1.05–1.09 g/ml, where the lower density is associated with platelets that have secreted their α-granule content but with an unaffected platelet volume distribution (14,15). Most EVs have a diameter of <200 nm, which means that the volumetric mass density is substantially affected by the membrane density. A model describing EVs as cytoplasm enclosed by a cell-like membrane (16) leads to a size-dependent volumetric mass density. For EVs 100, 200, and 1,000 nm, the density is 1.099, 1.081, and 1.064 g/ml, respectively, which is in fair agreement with literature estimates between 1.08 and 1.11 g/ml (17–21). Finally, the mass density of ccfDNA is only available in cesium chloride media and the length of ccfDNA is unknown. Assuming a length of <1,000 base pairs, the ccfDNA would be smaller than 50 nm.

Model Validation

To validate the model, polystyrene beads were diluted in phosphate-buffered saline (PBS) or blood plasma. The concentration of beads was measured by flow cytometry before and after centrifugation at 300*g* for 20 min or 2,700*g* for 22 min using a Rotina 380R centrifuge (Hettich, Tuttlingen, Germany) or 15,800*g* for 60 min using a SW 41 Ti rotor and Optima L-80 XP ultracentrifuge (Beckman Coulter, Fullerton, CA), all at 20°C and with deceleration set to the minimum possible value. Polystyrene beads were 400, 799, 994, and 3,005 nm in diameter (Thermo Fisher Scientific, Waltham, MA) with a density of 1.05 g/ml. PBS (154 mM NaCl, 1.24 mM $Na_2HPO_4{\cdot}2H_2O$, 0.2 mM $NaH_2PO_4{\cdot}2H_2O$; pH 7.4) was 0.22 µm filtered (Merck, Darmstadt, Germany). Blood from anonymous healthy donors was obtained after written informed consent in accordance with the Dutch regulations and approved by the medical–ethical assessment committee of the Academic Medical Center, University of Amsterdam. Whole blood was drawn using a 21G needle in EDTA

Table 1. Stokes model sample properties

TYPE AND PROPERTY	VALUE USED IN MODEL	REFERENCES
CTCs		
Density (g/ml)	1.053	(24,25)
Diameter (μm)	Uniform. 8–20	(26)
Platelets		
Density (g/ml)	Norm., mean 1.069, SD 0.0053	(14,15,27)
Diameter (μm)	Lognorm., median 2.4, SD 0.6	(12,28)
Shape factor	2.0	(9,13)
EVs		
Density (g/ml)	Core 1.060, 8 nm shell 1.155	(17–21)
Diameter (μm)	Uniform. 0.05–1	
ccfDNA		
Density (g/ml)	1.7	(16,29)
Diameter (μm)	≤0.05	(16)
Polystyrene beads		
Density (g/ml)	1.05	MFG
Diameter (μm)		MFG
PBS		
Density (g/ml)	1.004	(30)
Viscosity (mPa s)	1.193	(30)
Plasma		
Density (g/ml)	1.0253	(31,32)
Viscosity (mPa s)	1.75	(33–35)

Values at 20°C (see Supporting Information Table S1 for more details). Lognorm., lognormal distribution; MFG, manufacturer specifications; Norm., normal distribution; SD, standard deviation; Uniform., uniform distribution.

vacutainers (BD Biosciences, San Jose, CA) and processed within 15 min after collection. Plasma was obtained from whole blood by double centrifugation at 2,500*g* for 15 min at 20°C using a Rotina 380R centrifuge. The supernatant was pooled between centrifugation steps and pooled before mixing with the polystyrene beads. The concentration of beads before and after centrifugation was measured on side scatter of a flow cytometer (FACSCalibur; BD, Franklin Lakes, NJ, 15 mW 488 nm laser, flow rate ~60 μl/min calibrated by weight (22), SSC 400 V, gain 0, threshold 0), and data were analyzed using FlowJo (v10; FlowJo LLC, Ashland, OR).

Predicted Performance of Centrifugation Protocols

We modeled the protocols as described in the literature and shown in Table 2. The dimensions of the low-speed centrifugation tube were derived from the 57.462 Sarstedt centrifugation tube and for ultracentrifugation from the 344059 Beckman Coulter centrifugation tube. For the model, we assume that centrifugation <10,000*g* is performed using a Rotina 380R centrifuge and centrifugation >10,000*g* using a SW41 Ti rotor and Optima L-80 XP ultracentrifuge. For most centrifuges, the set *g*-force is the force at the bottom of the tube. The force elsewhere is given by Eq. 1. This means that a

Table 2. Modeled centrifugation protocols

PROTOCOL	BLOOD VOLUME AND VACUTAINER	PROTOCOL SUMMARY	REFERENCES
CTCs/tdEVs	10 ml CellSearch	WB 7.5 ml:6.5 ml PBS 800*g*, 10 min → (10) Pel	(1–3,5)
TEPs	6 ml EDTA	WB 120*g*, 20 min → (3) Sup 360*g*, 20 min → (10) Pel, wash 2×	(4)
EV-miRNA	6 ml EDTA	WB 900*g*, 7 min → (5) Sup 2,500*g*, 10 min → (5) Sup 500*g*, 10 min → (5) Sup	(6)
ccfDNA (Speicher)	10 ml PAXgene ccfDNA	WB 200*g*, 10 min, 1,600*g*, 10 min → (3) Sup 1,600*g*, 10 min → (3) Sup	(8)
ccfDNA (Dawson)	10 ml EDTA	WB 820*g*, 10 min → (5) Sup 20,000*g*, 10 min → (10) Sup	(7)

ccfDNA, circulating cell-free DNA; CTCs, circulating tumor cells; EV-miRNAs, extracellular vesicle-associated miRNAs; TEPs, tumor-educated platelets; tdEVs, tumor-derived extracellular vesicles; WB, whole blood; → (*xx*) Pel, collect pellet *xx* mm above bottom of tube or buffy coat (for WB); → (*xx*) Sup, collect supernatant *xx* mm above bottom of tube or buffy coat (for WB).

10 cm high sample in a Rotina centrifuge set to 2,500*g*, has a *g*-force of 900*g* at the top of the sample. The same centrifugation protocol on a rotor with a larger diameter will increase the *g*-force at the top of the sample and thus result in an increased recovery in the pellet. However, we did not model multiple rotor diameters, because most rotors suitable for large tubes have diameters comparable to the Rotina 380R. Results are expressed as the % recovery of CTCs, TEPs, tdEVs in three size ranges from large (1–8 μm, "apoptotic bodies", "oncosomes", or "tumor microparticles"), intermediate (0.2–1 μm, "microparticles"), to small (0.05–0.2 μm, "exosomes") tdEVs, EV-miRNAs, and ccfDNA.

Results

Model Validation

Figure 2 shows the modeled and measured bead recovery in PBS (top) and plasma (bottom). As expected, the recovery of beads in the pellet increases with increased particle diameter, *g*-force, and centrifugation time. In addition, because plasma is more viscous than PBS, the recovery of beads in the pellet is lower in plasma compared to PBS. Modeled and measured recovery are in fair agreement (all errors <8%) for both PBS and plasma at 300*g* and 2,700*g*, as well as for PBS at 15,800*g*. The model overestimates the particle speed at 15,800*g* for plasma, with a maximum error of 20%. Thus, the model is a reasonable approximation to predict the behavior of spherical particles when applied to the centrifugation protocols as shown in Table 2.

Predicted Performance of Centrifugation Protocols

Table 3 shows the predicted recovery of number of particles based on the centrifugation protocols as shown in Table 2. The volume of the supernatant plus the volume of the pellet equals the starting volume. The volume reduction can be calculated by dividing the starting volume by the volume of supernatant or pellet. To obtain the particle concentration after centrifugation, the predicted recovery in the number of particles needs to be multiplied with the volume reduction, which is shown in the last column of Table 3.

The CTC protocol recovers 100% of CTCs but co-isolates substantial fractions of TEPs, large tdEVs, and ccfDNA. In subsequent epithelial cell adhesion molecule (EpCAM)-based magnetic enrichment of CTCs, the TEPs and ccfDNA will be removed, but tdEVs exposing EpCAM will be co-isolated. The latter is confirmed by a previous study, in which the CTC fraction was shown to contain large $EpCAM^+$ EVs (5).

The protocol to isolate TEPs predicts a yield of 71% together with 22% large tdEVs and <3% CTCs, smaller tdEVs, and ccfDNA. Because the subsequent processing includes detection of all mRNA present in the sample, our model indicates that additional evidence is needed to prove that the obtained RNA profiles indeed originate from platelets.

EV-miRNA samples contain intermediate tdEVs (40%), small tdEVs (57%), ccfDNA (57%), and TEPs and large tdEVs (<1%). By subsequent size exclusion chromatography (23), ccfDNA is removed, resulting in a relatively pure tdEV sample with high yield. The model predicts that the concentration of tdEVs is unaffected by the final centrifugation step

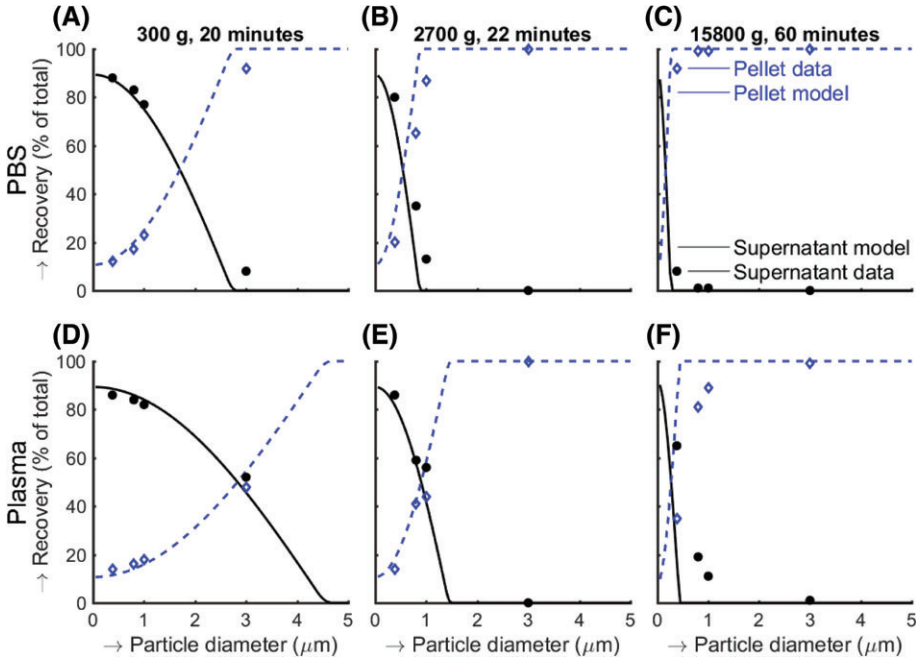

Figure 2. Centrifugation of beads diluted in PBS or plasma. The modeled bead recovery in pellet (blue dashed line) and supernatant (black, solid line) compared to measured bead recovery in pellet (open blue diamonds) and supernatant (closed black circles) for three centrifugation conditions. [Color figure can be viewed at wileyonlinelibrary.com]

Table 3. Predicted recovery (%) of centrifugation protocols

	CHANGE IN NUMBER (% OF ORIGINAL)						VOL. RED.
PROTOCOL	CTCS	TEPS	LARGE TDEVS	INTERMEDIATE TDEVS	SMALL TDEVS	CCFDNA	(FOLD)
CTCs/tdEVs	**100.0**	39.4	**81.1**	16.7	13.3	13.3	2.9
TEPs	0.0	**71.1**	21.7	3.2	2.2	2.2	3.0
EV-miRNA	0.0	0.01	**0.7**	**39.7**	**57.0**	56.9	1.7
ccfDNA (Speicher)	0.0	0.05	2.4	63.4	82.0	**82.0**	1.2
ccfDNA (Dawson)	0.0	6.10^{-7}	0.0	6.0	49.0	**48.4**	1.7

ccfDNA, circulating cell-free DNA; CTCs, circulating tumor cells; EV-miRNAs, extracellular vesicle-associated miRNAs; TEPs, tumor-educated platelets; tdEVs, tumor-derived extracellular vesicles; Vol. red., volume reduction. Bold font highlights the target component.

of the protocol, which is confirmed by the authors upon inquiry (6).

For ccfDNA, two centrifugation protocols were evaluated. The Speicher protocol (8) predicts the 82% recovery of ccfDNA, together with large tdEVs (2%), intermediate tdEVs (63%), small tdEVs (82%), and negligible CTCs and TEPs. The Dawson protocol (7) yields 48% of ccfDNA, but with lower contamination of intermediate tdEVs (6%), small tdEVs (49%), and negligible CTCs, TEPs, and large tdEVs. Thus, both protocols do not yield pure ccfDNA. Because the volumetric mass density difference between DNA and plasma is at least ninefold higher than the density difference for EVs, it is difficult to separate DNA from small EVs. Consequently, a 35 nm DNA "particle" travels at the same speed as a 100 nm EV.

Discussion

Centrifugation can be described with a model based on the Stokes equation. The model illustrates that centrifugation is effective at recovering particles that can be pelleted but that additional methods are needed to obtain a pure fraction. Particles with similar sedimentation rates are always co-isolated during centrifugation. This is especially relevant for TEP and ccfDNA protocols, because the co-isolated tdEVs may have substantial impact on the outcome. This model offers a tool to (re)design centrifugation protocols and at a minimum to establish whether available candidate protocols differ sufficiently from each other to warrant comparison. The model includes a shape factor to account for the nonspherical shape of platelets. This shape factor was set to 2.0 based on literature but was not experimentally validated.

For technologies evaluating the presence of CTCs, such as the CellSearch system, whole blood is centrifuged at 800*g* for 10 min. For this application, the model predicts a 100% effective isolation of CTCs, in addition to a recovery of 81% of the large tdEVs and 39% of TEPs. However, because of the use of imaging to identify each particle, tdEVs and TEPs can be distinguished from CTCs, and thus, the co-isolation does not affect the determined CTC concentration. Nevertheless, a purer CTC sample may be obtained by adjusting the centrifugation protocol. To reduce the recovery of TEPs or intermediate size tdEVs, the *g*-force or centrifugation time should be reduced. Because of their size, ccfDNA or small tdEVs' recovery in the pellet is mainly reduced by a reduction of the collection height.

The model predicts that a single biomarker cannot be purified by centrifugation alone. Substantial fractions of co-isolated other biomarkers remain present in all evaluated protocols, which may affect the obtained results and interpretation thereof.

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Conflict of Interests

The authors declare that the research was carried out in the absence of any personal, professional, or financial relationship that could potentially be construed as a conflict of interest.

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MIFlowCyt-EV: The Next Chapter in the Reporting and Reliability of Single Extracellular Vesicle Flow Cytometry Experiments

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What Is MIFlowCyt-EV?

Following in the footsteps of the minimum information to report about a flow cytometry experiment (MIFlowCyt), an extracellular vesicle (EV) flow cytometry working group composed of members from the international societies of extracellular vesicles (ISEV), advancement of cytometry (ISAC), and thrombosis and haemostasis (ISTH) developed the recent MIFlowCyt-EV framework position statement, with the aim to improve reporting and reliability of single EV flow cytometry experiments (1,2).

Why Is MIFlowCyt-EV Important?

MIFlowCyt-EV was developed in response to the growing body of single EV flow cytometry literature that lacks rigor and standardization, thereby leading to irreproducible results. MIFlowCyt-EV builds upon pre-existing frameworks for reporting and best practices in both flow cytometry and EV research; MIFlowCyt and MISEV, respectively (2–4). A need for MIFlowCyt-EV exists because (1) single EV flow cytometry experiments face specific technical challenges, which may lead to artifacts that are not covered by MIFlowCyt, and (2) in contrast to *Cytometry Part A*, which requires manuscripts to be supplemented with a MIFlowCyt template, journals in the field of EVs lack a formal requirement tailored to EV flow cytometry. To emphasize the need of MIFlowCyt-EV, Table 1 shows differences between the technical challenges and opportunities involved in EV and cell detection. This is mainly because EVs have signal intensities just below and above the detection limit, signal intensities overlap with buffer contaminants and unstained reagents, swarm detection may occur, and sensitivity differences strongly affect measured EV concentrations. On the other hand, the morphological properties of EVs offer new flow cytometric opportunities, such as particle sizing and refractive index estimation (5–8).

Table 1. Differences between the technical challenges and opportunities involved in EV and cell detection

EXTRACELLULAR VESICLES (EVS)	CELLS
EVs <500 nm are spherical core-shell particles, allowing diameter and refractive index estimation	Cells have organelles and have varying shapes, complicating diameter, and refractive index estimation
Signals below and above detection limit	Signals exceed detection limit
Signal calibration is essential: sensitivity differences strongly affect measured EV concentrations	Signal calibration is optional: sensitivity differences weakly affect measured cell concentrations
Signal intensities overlap with buffer contaminants or unstained reagents	Signal intensities exceed buffer contaminants or unstained reagents
Swarm detection	Coincidence detection

Coincidence detection: Simultaneous illumination and detection of two or a few particles. Swarm detection: Special case of coincidence detection, where instead of two or a few particles, multiple (tenths to hundreds) particles at or below the limit of detection are simultaneously and continuously illuminated and erroneously measured as single event.

As the field has progressed, essential controls have been identified to ensure (1) the detection of single EVs, and (2) providing reassurance that the detected events are EVs and not artifacts, such as free fluorescent labels. In line with the controls, calibration methods have been identified that allow for data to be reported in comparable, standardized units with a quantifiable limit of detection, which allows validation and comparison of data on flow cytometers that differ in sensitivity. Despite the identification and acknowledged importance of essential controls and calibrations, these currently lack widespread adoption by the scientific community, as evidenced by the absence of utilization in the published literature. Hence, now it is time to disseminate the solutions to these EV specific technical challenges in the form of a field standard: MIFlowCyt-EV.

What Are the Contents and Objectives of MIFlowCyt-EV?

The MIFlowCyt-EV reporting framework is composed of seven main components which include: (1) preanalytical variables and experimental design, (2) sample preparation, (3) assay controls, (4) instrument calibration and data acquisition, (5) EV characterization, (6) flow cytometry (FC) data reporting, and (7) FC data sharing. Together, these components ensure measurement of single EVs, data reproducibility, and the facilitation of data interpretation. Each component of the reporting framework aids in different areas of increasing reported data integrity. Components 1 and 2 provide the necessary details to repeat a reported assay. Component 3 provides evidence that single EVs of interest are being detected, and not labeling/purification artifacts or coincidence events. Components 4 and 5 allow for validation of assays irrespective of differences in instrument settings and/or limits of detection. Finally, Components 6 and 7 provide evidence of how the data was analyzed proof of raw data quality. Whereas the framework has a focus on EVs, it is also applicable to and useful for flow cytometry experiments on submicrometer particles other than EVs, such as viruses.

What Is the Impact of MIFlowCyt-EV Being Used in the Literature?

First, the completion of the MIFlowCyt-EV components will aid the peer-review process by allowing reviewers and readers to have access to essential information about the conducted experiment to assess the quality of data and validity of findings. Second, the field will get insight into the actual concentration of EVs in biofluids within reported detection ranges. Because hitherto detection ranges are unreported, the concentration of EVs in biofluids is virtually unknown. Third, the framework raises awareness about the essential criteria to consider when designing and performing a single EV flow cytometry experiment to researchers who may be new to single EV flow cytometry.

Steps toward Adoption of MIFlowCyt-EV Framework

The MIFlowCyt-EV framework was established to address key issues identified in the field of EV research, specifically pertaining to the quality of flow cytometry data. For this framework to have the intended impact on improving transparency and data quality in literature, ubiquitous adoption and integration into the research workflow is required. Figure 1 shows a diagram containing eight steps toward adoption of the MIFlowCyt-EV framework. *Step 1 and 2*, which involve the identification of key issues and the establishment and publication of the framework, have been carried out. Although this framework has been endorsed and acknowledged by international societies and key opinion leaders alike in flow cytometry and EV research fields (Fig. 1, Step 3), a clear understanding of the rationale and methods behind each component of the framework is required (Fig. 1, Step 4) to achieve widespread adoption. To educate the field, several recent initiatives led by the ISEV-ISAC-ISTH EV FC Working Group have contributed to a growing body of online educational resources (www.evflowcytometry.org). Software tools to facilitate data calibration are available both commercially and free-of-charge online with resources and tutorials on how to use these tools (5,9,10). A concerted effort has also been made by the ISEV, ISAC, ISTH communities to dedicate time for disseminating and discussing developments within the EV flow cytometry community at international meetings and workshops for several years, which will continue to aid understanding and uptake of best practices (11).

We envision that the next step toward adoption of the MIFlowCyt-EV framework is the involvement of shared resource labs (SRLs). SRLs can substantially contribute to the

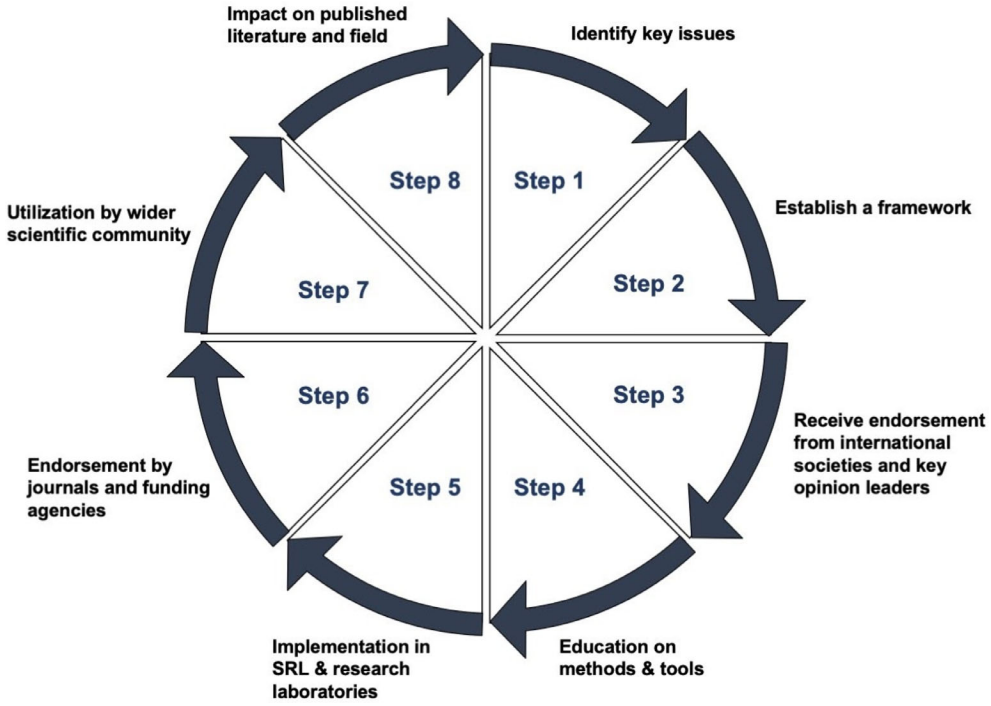

Fig 1. Strategy consisting of eight steps to realize adoption of the MIFlowCyt-EV framework. SRL, shared resource laboratory. [Color figure can be viewed at wileyonlinelibrary.com]

effort to standardize EV measurements, as SRLs are a source of both education and instrument expertise in the research setting (Fig. 1, Step 5). SRLs play a crucial role in instrument maintenance, calibration, and educating users on instrument best practices, such as instrument settings, calibration methods, experiment controls, FC data sharing, and reporting. For example, calibrating light-scatter, which is not required to be performed on an individual experiment basis, can potentially be a service performed by the SRL whenever instrument alignments have been adjusted. Components of the MIFlowCyt-EV framework, such as sample preparation and assay controls, instrument calibration, and data reporting can also be incorporated into user training for EV flow cytometry. As more EV researchers turn to flow cytometry for the analysis of EVs, the onus will be on flow cytometry core facilities to adequately support this application. The MIFlowCyt-EV framework provides an ideal reference for core facilities to provide these services and on the other hand, SRLs have the role to support and promote best practices; making them ideal candidates to disseminate the MIFlowCyt-EV framework for adoption in the wider scientific community.

The implementation of MIFlowCyt-EV will enable clearer interpretation and increased confidence in the reported single EV flow cytometry literature. However, to reach the goal of increasing transparency and confidence in the published literature (Fig. 1, Step 7–8), widespread adoption and implementation is required by academia, industry, and commercial vendors. Similar to the requisite by *Cytometry Part A* with compliance to MIFlowCyt guidelines for publication, endorsement for MIFlowCyt-EV by major journals and funding agencies for manuscript and grant submissions containing single-EV flow cytometry data would be crucial for the success of this initiative (Fig. 1, Step 6). We further believe that industry should take responsibility and participate in this effort, for example, by incorporating calibration procedures that lead to data in comparable units into instrument data acquisition and analysis software. Moreover, additional investment in research and development of reference materials resembling EV properties as well as calibration materials would significantly enhance standardization efforts.

In summary, to improve reporting and reliability of flow cytometry experiments on EVs, we have developed the MIFlowCyt-EV framework and a strategy to disseminate and adopt the framework. While no gold standard exists for single EV flow cytometry assays, the field is at a stage where methods have been identified that enable confirmation of single EV detection and calibration of results across instruments. Improvements to existing procedures and expansion of current knowledge about EVs in biofluids can only be achieved with proper reporting. We believe the endorsement of MIFlowCyt-EV by *Cytometry Part A*, like MIFlowCyt for cellular analysis, is a big step forward for the small particle field and will aid in the development of optimal instrumentation, settings, staining assays, and future standards. This progress can only be made if it is built upon transparent, reproducible data, for which the MIFlowCyt-EV framework will aid researchers to identify.

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Conflict of Interest

EvdP is shareholder of the company Exometry B.V., Amsterdam, The Netherlands.

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