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Microfluidic systems in extracellular vesicles single analysis. A systematic review

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ABSTRACT

Extracellular vesicles (EVs) are key elements in cell-to-cell communication and important circulating carriers of molecular biomarkers. There is an increasing interest in the analysis of EVs, as they can facilitate the identification and assessment of new biomarkers in liquid biopsy, allowing an earlier and more precise diagnosis of several pathologies like cancer or degenerative diseases. However, current analytical approaches are based on bulk EV analyses, being unable to provide precise information about the contents and the cells of origin of EVs. Thus, comprehensive EV research requires the analysis at a single particle level. Multiple studies have been conducted to achieve this goal, employing more sophisticated techniques such as microfluidic systems to separate single particles. In this systematic review we have identified 23 studies describing single EV analysis using different microfluidics approaches. Most of the reviewed works in this article have been performed during the last 3 years and have described several strategies for EV isolation, sorting, labeling, signal amplification, and sensing. These works provide new opportunities for further studies in the field and unravel the potential of microfluidic systems as the key to develop a single EV analysis with high sensitivity, robustness, and specificity.

1. Introduction

Extracellular vesicles (EVs) are small particles composed of a double phospholipid membrane. EVs are produced by all kinds of eukaryotic cells by cell membrane invagination and released to the extracellular compartments by different mechanisms [1,2]. In general, EVs can be divided into three main groups according to their biogenesis: EVs produced by multivesicular bodies (MVBs), or exosomes, EVs originating from the plasma membrane, or ectosomes, and EVs produced by cell fragmentation during apoptosis called apoptotic bodies (ABs). It is important to mention that exosomes are the most abundant population of EVs and play a prominent role in intercellular signaling [3]. Exosome size ranges from 20 to 150 nm in diameter and these vesicles can be sub-classified into three groups according to their size and cargo. The smallest particles, exomeres, are defined as non-membranous structures of ~35 nm with reduced protein cargo. Then, both small exosomes, with a diameter ranging from 60 to 80 nm, and large exosomes, with a diameter from 90 to 120 nm, are highly enriched in proteins, RNA, and miRNA, and are the principal source of information in EV analysis for liquid biopsy applications [4].

EVs were first reported by Chargaf and West in 1946, who observed small particles in human blood [5]. After that, Wolf and collaborators described EVs as “platelet dust” in 1967 [6]. Finally, it

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Abbreviations

AAO	Anodic aluminum oxide
ABs	Apoptotic bodies
BAL	Bronchoalveolar lavage
BF	Biological fluids
CDPA	Carboxydecylphosphonic acid
CM	Conditioned media
ddELISA	Droplet digital enzyme-linked immune sorbent assay
DEVA	Droplet-based extracellular vesicles analysis
EpCAM	Epithelial cell adhesion molecule
EVs	Extracellular vesicles
HNCIB	High-throughput nano-bio chip integrated system for liquid biopsy
HRP	Horseradish peroxidase
MITEV	Microfluidic isolation of tumor-derived extracellular vesicles
MVBs	Multivesicular bodies
PDMS	Polydimethylsiloxane
Pdots	Polymer dots
PEEK	Polyetheretherketone
PMMA	Polymethyl methacrylate
STED	Stimulated emission depletion
TIRFM	Total internal reflection fluorescence microscopy
TBE	Tris-borate-EDTA
TRPS	Tunable resistive pulse sensing

was in the 70′s when EVs were first described as double membrane particles generated in multivesicular bodies and possibly produced by plasmatic membrane release [7]. In the 80's, scientists discovered,for the first time, intercellular transport of proteins through EVs [8,9], thus challenging the idea that EVs were inert cell junk. Later on, Raposso et al. demonstrated that these EVs, usually called exosomes at that time, presented biologically active major histocompatibility complexes that were able to present antigens and activate T cell response [10]. Based on this evidence, Zitvogel et al. proposed dendritic cell derived EVs as potential cancer vaccines in 1998 [11]. Nevertheless, advances in the field were moderate and controversial and it was not until 2007 when EV cargo was better characterized by Valadi et al. and EV interaction was better understood [12]. As a consequence, there was an exponential increase in the interest of the study of EVs and their role in cell-to-cell communication in all biomedical areas [12–14]. Finally, in 2015, Melo et al. discovered that EVs from tumor cells were produced at a higher rate than those from non-malignant cells and could be detected in blood earlier than any other diagnostic markers [15], increasing the potential of EVs as liquid biopsy biomarkers in solid tumors.

During the last decade, EVs have been gaining special relevance in biomedical research due to their role in cell-to-cell communication, both with local or distal tissues [16]. As the EV cargo represents, in part, the composition of the cells or origin, EVs have become an important source of biomarkers in an uncountable number of diseases [17]. Since all biological fluids, such as blood, urine, or cerebrospinal fluid, collect disease-related EVs form different organs and tissues, researchers have focused on characterizing EVs isolated from these fluids with the aim to find more accurate clinical tools for early diagnosis, monitoring, and prognosis [18]. For examples, EVs circulating in blood or urine have been suggested as promising carriers of information and biomarkers in epithelial cancers [19]. In particular, pancreatic cancersare commonly diagnosed in advanced stages what explains the associated high mortality rate of 93% and the high difficulty for an early diagnosis of this disease. However, the detection of blood EVs carrying biomarkers like EphA2, EGFR, or CA19-9 could allow for an earlier diagnosis and hence a possible curative intervention [20]. In breast cancer, several EV biomarkers like CD47, GPC-1, HER2, and EpCAM(Epithelial cell adhesion molecule)have been described, potentially allowing an earlier diagnosis of the disease [20,21]. Single EV markers like miRNA-21 or the ratio of PD-1/PD-L1 mRNAs have been proposed as suitable markers for early diagnosis and the prediction of treatment outcomes in patients with non-small cell lung cancer [20,22. Similarly, other cancer types and other diseases with difficult access could benefit from the advantages of EV analysis in blood. One example are neurodegenerative diseases, where brain-derived NCAM-positive EVs observed in plasma have been identified as disease-related biomarkers [23].

The analysis of circulating EVs in body fluids is technically limited by their small size (majority around 50–120 nm), their limited cargo (few molecules per each marker), and the heterogeneity of the pathological cells producing them [24]. Therefore, most studieshave performed bulk analysis of circulating EVs, in which a variation in the molecular signature is presented as a clinical tool [25]. While bulk analysis provides relevant information for cancer status, single EV analysis is positioned as the most robust strategy to determine specific molecular and phenotypic features of the disease, including physical, genetic, lipidic, proteomic, and metastatic variations. Thus, recent scientific advances in the area of single EV analysis show promising strategies to develop analytical systems for protein and nucleic acid determination. Interestingly, most of these new approaches apply microfluidic systems as a key tool in the analytical process, allowing label-free or affinity-based isolation of EVs according to their physical or biological properties [26,27]. After EV isolation and sorting, biologically meaningful information has to be retrieved from these particles using sophisticated labeling, signal amplification, and sensing techniques [28]. This systematic review recapitulates microfluidic applications for single EV analysis and summarizes important challenges in EV manipulation, labeling, signal amplification, and sensing.

2. Objectives

The main aim of this systematic review was to summarize all scientific works where microfluidics was employed for single EV analysis, as well as those studies which performed relevant approaches for single EV characterization. This review focused not only on the methods, materials,and design of the microfluidic systems employed for EV isolation, but also on the labeling, signal amplification, and methods of signal detection, which are the most important challenges for further development in this field.

3. Methods

PRISMA-Preferred Reporting Items for Systematic Reviews and Meta-Analysis Methodguidelines were followed to perform this systematic review [29]. According to the evolution of published works in the field, we established our literature search from January 1st, 2007 and July 30th, 2022, using the Scopus, Pubmed, Science direct, and Web of Science databases. Articles were searched for combinations of the following key expression in the title, abstract or keywords: (“microfluidic”) AND (“single EV” OR

"single EVs" OR "single Exosome" OR "single Exosomes") NOT ("review").

Inclusion Criteria: Original research publications with an abstract, which presented experimental results that applied microfluidic systems during the process of separation or potential identification, quantification, or characterization of single EVs. Studies reporting qualitative and quantitative data and referring to the source of where EVs were obtained from.

Exclusion Criteria: Original research in a language different from English. Articles without microfluidic systems in any step in the single EV analysis process. Articles without single EV analysis or without the potential to reach it by improving signal amplification or sensitivity of the detection method.

Schematic representations of the devices included in the figures have been created using BioRender.com.

4. Results

4.1. Results of PRISMA statement evidence search and selection

PUBMED search identified 100 publications, another 115 were found in Web of Science, 16 in Science Direct, and 54 using SCOPUS databases. The total number of identified records was 285, of which 107 were duplicates and were removed before screening. The remaining 178 records were manually evaluated based on title and abstract, having 11 records excluded as they were conference abstracts (n = 2), reviews (n = 8), or book chapters (n = 1). Finally, 167 were assessed for eligibility, of which 144 did not pass the inclusion criteria as no single EV analysis/or microfluidics approaches were reported (n = 83), were not relevant (n = 59), or were not written in English (n = 2). A detailed diagram of the selection process can be seen below in Fig. 1.

From the final 23 studies included in this review, the first one was published in 2012 and the number of publications increased from 2018 to 2020 and slightly decreased in 2021 (Fig. 2.). The studies were classified in two categories according to the type of analysis: static measurement or flow cytometry analysis. Static measurement methods comprise immobilization of EVs by an array, antibody capturing, or adsorption on a slide. These were divided into 3 subtypes; single EV analyses identifying physical and chemical properties of EVs (n = 3); single EV analyses of target membrane proteins (n = 9), and single EV analyses of miRNA, RNA, or DNA (n = 4). Flow cytometry methods included single EV analyses of target membrane proteins (n = 4) and methods identifying physical properties (n = 1).

4.2. Single EV analysis by physical or chemical characteristics

For single EVs analysis, researchers have developed microfluidic systems that allow single particle characterization by label-free methods based on the physical and chemical features of these membranous particles. Riazanski and collaborators developed a microfluidic device that performs tangential flow filtration using a 100 nm thick nanoporous silicon nitride (NPN) membrane that traps EVs (Fig. 3A.) [30]. These NPN-captured EVs were measured using biodipy™ for lipid staining and acridine orange as cytosolic pH indicator. Then, EVs were analyzed by confocal imaging and by Stimulated Emission Depletion (STED) super resolution imaging, defining EVs as particles positive for both dyes. Rodriguez-Quijada and Dall investigated the mechanical properties of single apoptotic bodies with the help of a microfluidic device on top of an inverted light microscope [31]. This device was composed of two microfluidic flow channels converging in a center, where vesicle visualization and imaging was performed (Fig. 3B.). By applying a flow in the opposite direction of the sample, the membranes of the apoptotic bodies deformed. The magnitude of the deformation was determined in the obtained images in order to assess the stiffness of the apoptotic body membranes [31]. Another way to measure and quantify EVs larger than 50 nm is using tunable resistive pulse sensing (TRPS), a classical technique that has recently been applied to the quantification and sizing of EVs. With this analytical technique, particles are suspended in an aqueous electrolyte which crossed through a single pore membrane into the microfluidic system. The membrane is subjected to an electric potential that drives an ionic current that is interrupted when an EV passes through the pore, thus generating a resistive pulse (Fig. 3C.) [32]. To date, the QNano device fabricated by IZON is the most widely used tool for EV analysis by TRPS methodology [33]. Table 1 summarizes the principal EV analysis methods based on their physical and chemical characteristics.

4.3. Single EV analysis by protein labeling

Protein characterization of single EVs is particularly challenging and several technical problems remain unsolved, such as internal protein labeling [24,34]. One of the principal problems for single EV protein analysis is the small size and the low concentration of proteins in the EV membrane. Protein detection and analysis must be sensitive enough to catch a very low signal and also specific enough to separate this signal from the unbound background [24]. To date, researchers have tackled these problems by applying several strategies, such as signal amplification, to increase the probabilities for detection or the addition of washing steps, to remove signal background without loss of EVs [28]. In this context, Liu and collaborators developed a microfluidic co-flow system able to sort EVs in three size ranges (Fig. 4A.) [35]. For protein detection, they first labeled EVs with fluorescent Cy5-conjugated HER2 (human epidermal growth factor receptor 2) and FAM-conjugated EpCAMaptamers. Labeled EVs were injected with a dilution of viscoelastic λ-DNA and Tris-borate-EDTA (TBE) buffer into the central channel of the microfluidic device and TBE buffer is injected into a secondary inlet as sheath fluid. Due to the equilibrium between flow forces and viscoelastic characteristics of these dilutions, small EVs flowed through the center of the channel along with the λ-DNA, while other larger EVs moved to the periphery. Finally, several central and peripheral EVs were sorted in divergent channels patterned at the final section of the chip, where labeled EVs were imaged by fluorescence microscopy [35].

In a different approach, Beekman et al. reported a multi-modal analysis platform for the specific capture of tumor-derived EVs on antibody-functionalized stainless-steel substrates [36]. In this study, three polydimethylsiloxane (PDMS)microfluidic devices were placed on top of acarboxydecylphosphonic acid (CDPA) functionalized stainless-steel substrate (Fig. 4B.). The first PDMS device was employed as a reservoir to immobilize antibodies on the substrate. Next, after removal of the first PDMS reservoir, the second PDMS microfluidic device, composed of an inlet, a central channel, and an outlet, was placed on top of the substrate. Besides introducing the sample and facilitating EV capture on a functionalized substrate, this device allowed sample washing and fixation. The third PDMS device allowed EV navigation. This device was comprised 3 microchannels, two featuring navigation markers that flanked a third sample microchannel. Cyanoacrylate superglue (Tesa SE, Norderstedt, Germany) was injected in the side channels to create the navigation markers and finally, after removing the PDMS piece, EVs were ready for multimodal analysis by Raman microscopy, atomic force microscopy (AFM), and scanning electron microscopy (SEM). This methodology captured EVs with the antibody-functionalized central channel (against EpCAM for epithelial EV capture). The navigation channels identified each

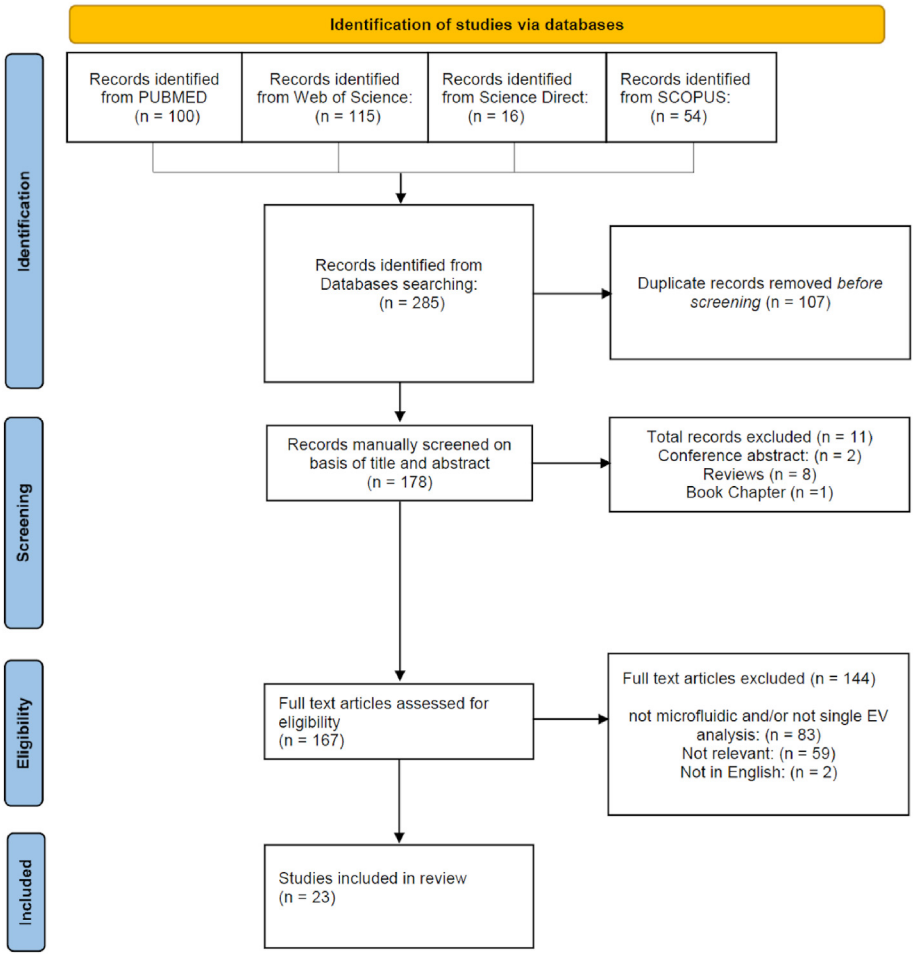

Fig. 1. Flow diagram summary of item selection for this systematic review and meta-analysis (PRISMA).

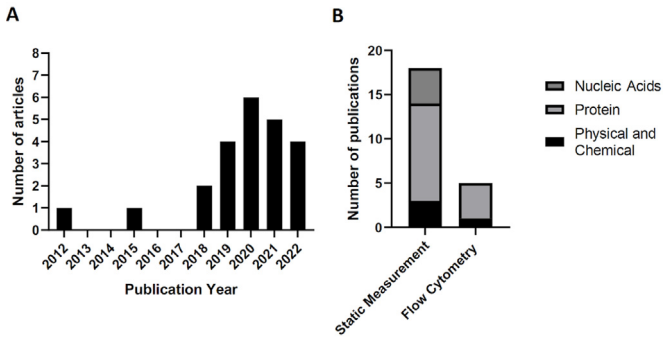

Fig. 2. Results of literature search. A) Number of publications per year since the first publication in 2012. B) Number of publications performed according to the detection method. Static Measurement means Extracellular vesicles immobilized by an array, antibody captured, or adsorbed on a slide.

captured EV for successive analyses [36]. Another interesting device to isolate and characterize EVs is the ExoChip developed by Kanwar and collaborators [37]. This device fabricated in PDMS and glass had 6 circular chambers interconnected through straight and narrow channels, in which the flow was highly reduced to facilitate the recognition and capture of EVs by the antibody-functionalized surface. Then, after washing, bound EVs were labeled with a fluorescent dye that allowed their quantification using a plate reader or fluorescent microscopy for single EV analysis [37].

Yang et al. developed a microfluidic system composed of a nanoporous membrane (100 nm pore size) and a polymethyl methacrylate (PMMA) base (Fig. 4C.) [38]. This anodic aluminum oxide membrane was covered by CD9-antibody functionalized gold nanoclusters that allowed the capture of EVsthat were subsequently marked with a secondary antibody conjugated to gold nanorods. These nanorods amplified the signal making EVs detectable by SEM and dark field microscopy. As a result, researchers identified and analyzed single EVs expressing a biomarker of interest [38]. In another work, Lu and collaborators developed a microfluidic system for the isolation and characterization of PD-L1 in single EVs from cell lines and patients with lung cancer with the aim to monitor immunotherapy response [39]. This microfluidic device was included a serpentine-shaped channel and an incubator chamber for exosome isolation and labeling and then, micropillar arrays for the analysis of PD-L1 protein markers (Fig. 4D.) [39]. EV samples were injected into the chip together with CD9 antibody functionalized beads that flowed through the serpentine channel up to the incubator chamber. There, microbeads and CD9 positive EVs were retained in a magnetic field. Next, EVs were washed and labeled with PD-L1 protein immunofluorescent marker. Finally, the magnetic beads were released and captured in the micropillar array that was scanned by fluorescent microscopy to identify PD-L1 positive EVs-decorated beads. Despite direct single EV analysis was not described in this work, dilutions combined with signal amplification made it possible.

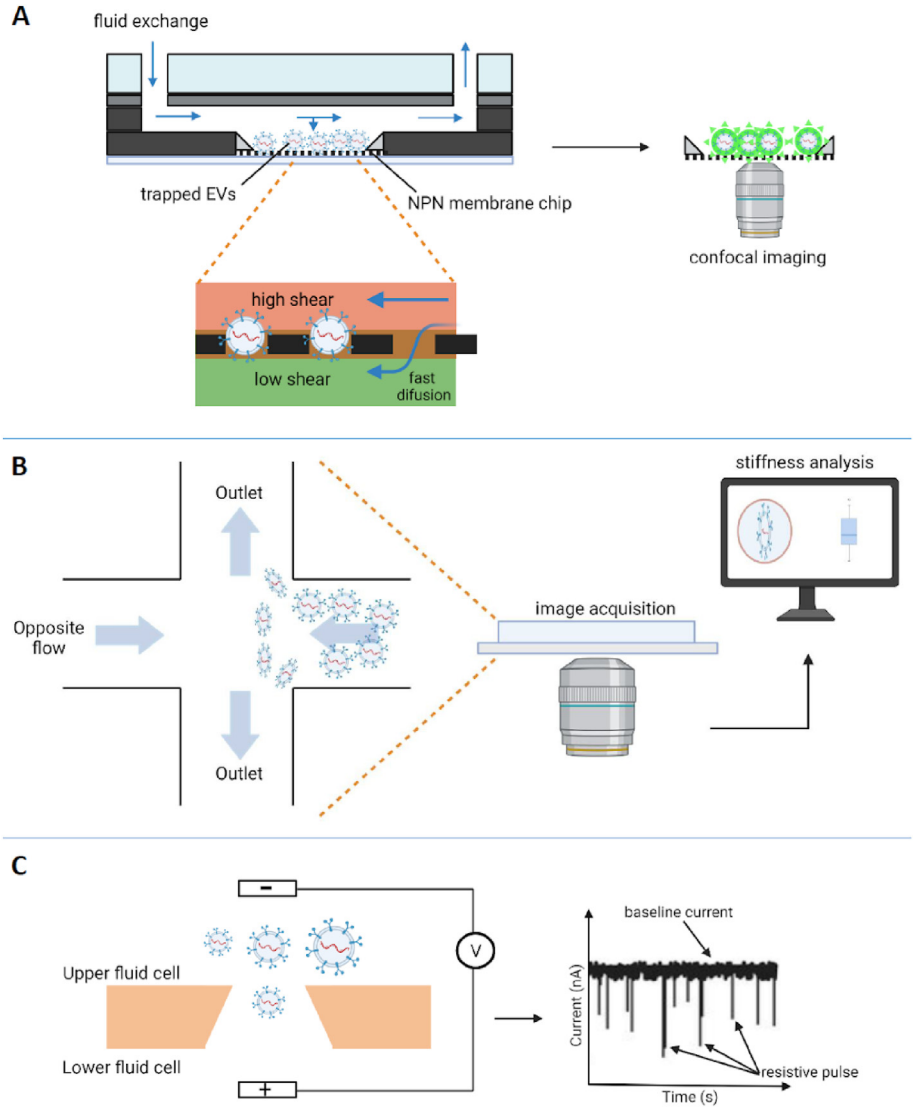

Fig. 3. Single EV analysis performed by their physical or chemical properties. A) Schematic representation of the work performed by Riazansky et al. B) Device described by Rodriguez-Quijada and Dall. C) Design of the TRP sensing, where EVs produced a determined resistive pulse by E. Weatherall and Willmott. EVs: Extracellular vesicles, NPN: nanoporous silicon nitride, nA: nanoamperes.

Table 1
EV analysis methods based on their physical and chemical characteristics.

	Methods based on physical and chemical characteristic						
	Title	Target	Single or multiplex	Sample	Biological application	Year	Ref.
1	Real time imaging of single extracellular vesicle pH regulation in a microfluidic cross-flow filtration platform	pH	single	BAL and CM	Luminal pH stability	2022	[30]
2	Non-contact microfluidic mechanical property measurements of single apoptotic bodies	stiffness	single	Blood and plasma	Mechanical behavior in biological flow	2021	[31]
3	Applications of tunable resistive pulse sensing	electric resistance	single	BF and CM	Size and number analysis	2015	[32]

BAL: Bronchoalveolar lavage; CM: Conditioned media; BF: Biological fluids.

In 2020, Kim and collaborators developed a microfluidic chip for size fractionation and single analysis of EVs called EV-Ident [40]. This device used several anodic aluminum oxide membrane filters with pores of 200, 100, and 20 nm in a disc chamber for the centrifugation of cell culture supernatant or plasma. These filters allowed the isolation and capture EVs of specific sizes in each compartment (Fig. 4E.). Then, using the same chip, EVs were immunolabelled with fluorescent dyes and washed to be finally analyzed by fluorescent confocal microscopy. In addition, Zhang and collaborators fabricated a nano-engineered chip for multiplexed EV immunophenotyping [41]. This chip was composed of 3D porous serpentine nanostructures with patterned colloidal self-

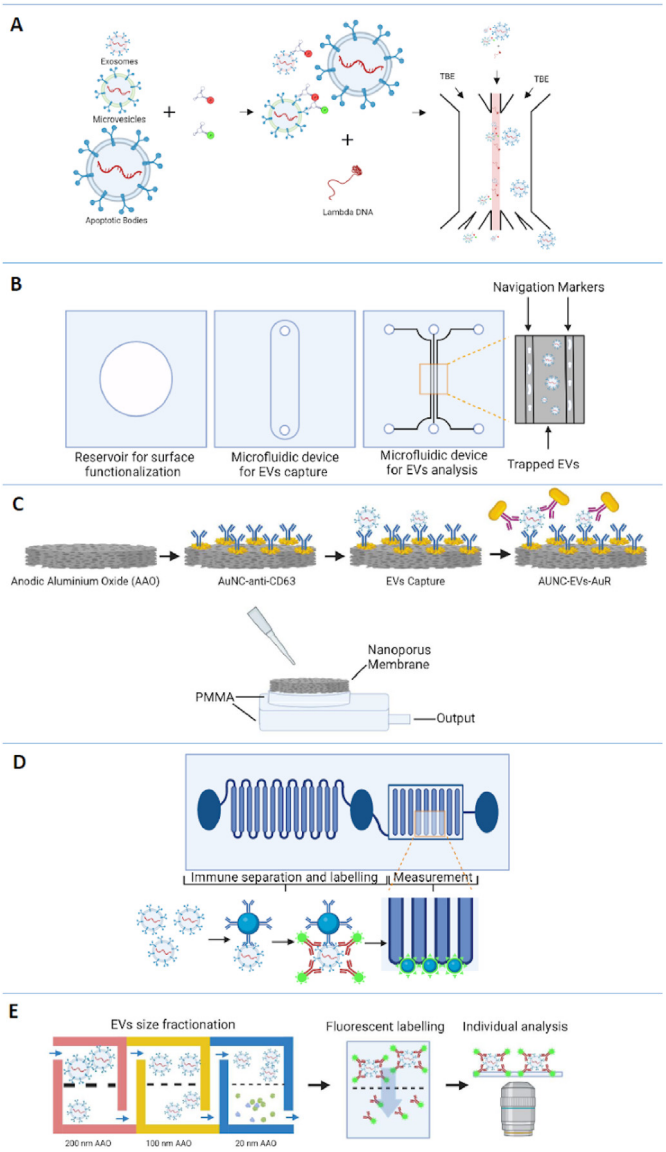

Fig. 4. Single EV analysis by identification of membrane proteins. A) Schematic representation of the work performed by Liu et al. B) Design of the device used by Beekman et al. C) Design of the platform described by Yang et al. D) Schematic representation of the work performed by Kim et al. E). Device developed by Zhang et al. EVs: Extracellular vesicles, PMMA: Polymethyl methacrylate, AAO: anodic aluminum oxide.

assembly structures to increase the reaction surface and thus improve biosensing efficiency. This multichannel microdevice divided the sample into 8 reaction areas, in which individual biomarkers could be analyzed, allowing simultaneous detection of 8 markers in bulk EVs. Interestingly, they also performed sample dilution and signal amplification with biotinylated anti-CD63 primary antibodies and streptavidin conjugated β-galactosidase, which were finally detected by chemo-fluorescence upon catalysis of fluorescein-di-β-D-galactopyranosideto perform single EV analysis [41].. Another interesting approach is the study of Chen et al. which describes a microfluidic device with a multi-layer structure for the isolation and digital counting of EVs [42]. In this chip, EVs were separated from blood components by stirring-enhanced filtration through a 0.2 μm polycarbonate membrane. Then, EVs were retained on a 20 nm blackened aluminum oxide membrane that allowed horseradish peroxidase (HRP)-labeling of EVs and washing to remove unbounded antibodies. Finally, the tyramide-tetramethylrhodamineHRP substrate produced a fluorescent product detectable by fluorescence microscopy. All the steps in this microfluidic device were managed by air-controlled valves in complex and flexible layers. Table 2 summarizes the methods based on single EV analysis by protein labeling.

Another way to perform single EV protein analysis is the encapsulation of single EVs using droplet generator microfluidic chips. With this strategy, Ko and collaborators developed an immune-droplet digital PCR amplification for the multiplex analysis of EV proteins [43]. First, EVs were incubated with antibodies barcoded with unique and amplifiable DNA sequences used as a multiplexing platform. Then, EVs were isolated by size exclusion chromatography, removing unbounded antibodies and labeled EVs were injected and encapsulated with PCR master mix to perform PCR amplification within the droplet. EV concentration was adjusted in order to limit the number of EVs per droplet to 1 or 0 and individual droplets were imaged by fluorescent microscopy or scanned to quantify EVs expressing the biomarkers of interest (Fig. 5A.) [43]. Using the same strategy, Liu and collaborators developed an ExoELISA platform for single protein analysis of single EVs encapsulated in droplets. This droplet generator mixed two hydrophilic fluids in each droplet (Fig. 5B.) [44]. First, EVs were bound to antibody-functionalized magnetic microbeads, washed, and marked with a ß-galactosidase-labeled antibody against the protein of interest. Similarly, the number of EVs was kept much lower than the number of magnetic microbeads to guarantee one or less EVs per bead. Then, the sample was washed and microbead-EV complexes were injected into the chip and mixed into the droplet with the enzymatic substrate of galactosidase. This substrate (fluorescein-di-β-D-galactopyranoside), when catalyzed by the enzyme, produced a fluorescent signal within each droplet, detectable in a single plane by fluorescent microscopy [44].

More recently, Yang and collaborators developed an ultrasensitive EV detection method of droplet digital Enzyme-Linked Immune Sorbent Assay (ddELISA). By using a droplet-based optofluidic platform, they were able to quantify specific individual EV subpopulations at high throughput (close to 20 million of droplet/min), which was 100 times greater than the methods described before [45]. They performed a droplet-based extracellular vesicles analysis (DEVA) with a microfluidic system containing 3 sections: the first composed by multiple droplet generators, the second with a long spiral that collected the droplets and stabilized them, and the third section that arrayed the droplets to be detected (Fig. 5C.) [45]. For this analysis, 10 μl of plasma were incubated with an excess of fluorescent paramagnetic microbeads, previously functionalized with EVs specific capture-antibodies. Then, using a magnet, captured EVs were washed and immunolabeled with a biotinylated antibody against the protein of interest and finally marked with HRP-streptavidin. After washing, microbeads were injected into the chip to be encapsulated one by one into droplets in conjunction with the HRP substrate. Fluorescent droplets were detected in the last section of the chip by fluorescent microscopy using a green and blue laser module and videos were obtained for the analysis of single EVs [45]. Finally, JinaKo et al. developed a system to obtain multiplex proteomic analysis of single EVs [46]. For that, droplet microfluidics were used to compartmentalize and barcode individual EVs. They employed beads and antibodies with DNA-barcoding, and after the specific recognition of proteins and DNA molecules, the DNA was sequenced to determine protein composition (Fig. 5D.) [46].

4.4. Methods based on the identification of nucleic acids

The analysis of nucleic acids on single EVs remains challenging

Table 2
Methods based on single EV analysis by protein labeling.

	Name	Target	Type	Sample	Biological application	Year	Ref.
4	λ-DNA- and Aptamer-Mediated Sorting and Analysis of Extracellular Vesicles	membrane protein/size sorting	dual	CM and blood plasma	Heterogeneity assessment and cancer typification	2019	[35]
5	Immuno-capture of extracellular vesicles for individual multi-modal characterization using AFM, SEM and Raman spectroscopy	membrane protein	single	CM	Identification, size and density surface analysis of specific EVs (EpCAM+)	2019	[36]
6	An integrative microfluidic device for isolation and ultrasensitive detection of lung cancer-specific exosomes from patient urine	membrane protein	single	Blood plasma	Isolation and biomarker identification for Lung Cancer	2020	[38]
7	Isolation and digital counting of extracellular vesicles from blood via membrane-integrated microfluidics	membrane protein	single	Blood	Isolation and quantification of specific EVs	2022	[42]
8	Integrated microfluidic system for isolating exosome and analyzing protein marker PD-L1	membrane protein	single	CM and blood plasma	Isolation and quantification of PDL1+ EVs	2022	[39]
9	EV-Ident: Identifying Tumor-Specific Extracellular Vesicles by Size Fractionation and Single-Vesicle Analysis	membrane protein	double	CM and blood plasma	Size fractionation and quantification of EVs (HER2+ and PSMA+)	2020	[40]
10	Multiplexed immunophenotyping of circulating exosomes on nano-engineered ExoProfile chip towards early diagnosis of cancer	membrane protein	multiplex	Blood plasma	Identification of 7 biomarkers for diagnosis and staging of cancer.	2019	[41]
11	Single Extracellular Vesicle Protein Analysis Using Immuno-Droplet Digital Polymerase Chain Reaction Amplification	membrane protein	multiplex	CM and blood plasma	Identification of 3 biomarkers in immune-therapy monitoring	2020	[43]
12	Single-Exosome-Counting Immunoassays for Cancer Diagnostics	membrane protein	single	Blood plasma	Identification of GPC1+ EVs for cancer diagnosis	2018	[44]
13	Ultrasensitive Single Extracellular Vesicle Detection Using High Throughput Droplet Digital Enzyme-Linked Immunosorbent Assay	membrane protein	single	CM and human plasma	Identification of tetraspanins in EVs	2022	[45]
14	Sequencing-Based Protein Analysis of Single Extracellular Vesicles	membrane protein	multiplex	CM and plasma	Identification of EV-associated signatures as disease biomarker	2021	[46]

CM: Conditioned media.

due to their small diameter and the small concentration of nucleic acids in each particle. Thus, more sensitive and reliable methods need to be developed to extract sufficient information from single EV analysis. Here we summarize the available studies on the analysis of nucleic acids on single EVs.

Zhou et al. described what they called the High-throughput Nano-bio Chip Integrated System for Liquid Biopsy (HNCIB), that in addition to nucleic acids, was able to simultaneously analyze proteins both in the lumen and on the EV surface [47]. This system was based on a glass nano-biochip that contained lipoplex particles of high density immobilized on a densely packed molecular brush layer. These lipoplex particles were positively charged and will bind and fuse to the negatively charged EVs by electrostatic interactions. As a result, these single particles combine membrane structures and cargo and allowed the detection of lumen content without the need of dissolving agents that may damage EVs. Then, fluorescent molecular beacons or antibodies hybridized to mRNAs or proteins were added and visualized in a 4-laser beam system using Total internal reflection fluorescence microscopy (TIRFM). TIRFM had a high signal-to-background ratio by acquiring just signals very low to the nano-biochip surface. Finally, imaging algorithms were applied to reduce background noise and edge delimitation, which helped colocalizing mRNAs and proteins (Fig. 6A.). Using the HNCIB, the authors were able to capture EVs and show high-resolution images of PD-L1 mRNA and miRNA-21 expression from plasma samples of 34 patients with lung cancer and 35 healthy donors (Fig. 6B.). The system was able to show a higher expression of miRNA-21 and PD-L1 mRNA in the cancer patients than in healthy donors, which was validated by RT-qPCR. This technology showed higher protein expression of PD-L1 in cancer samples that was validated by standard flow cytometry, demonstrating the accuracy of the HNCIB system. To prove the high specificity of the system to capture and analyze EVs, the authors showed high expression of EV markers such as CD9 and CD63, and low expression of non-EV markers such as albumin and apolipoprotein B. High efficiency and sensitivity was also demonstrated when detecting EVs from serial dilutions at very low concentrations and the fluorescence intensity detected was proportional to the concentration of fluorescent particles, giving a hint for quantification. The relevance of this technology is also highlighted by its potential clinical implementation, as the nanochip can be easily adapted to the multi-well plates commonly used in the laboratory routine, the low volume of plasma needed (~90 μL) and the quick turnaround time of the results (~6 h) [47].

Moreover, Zhou et al. designed a 3D microfluidic device able to perform double detection of two EV markers simultaneously [48]. This chip contained Y-shaped micropost arrays to increase the contact time with the EVs. The chip was layered with a biotinylated film and streptavidin-linked polystyrene spheres (PS) that were conjugated with specific biotinylated anti-CD63 antibodies to capture EVs. Then, quantum dots, with different emission wavelengths were labeled with specific antibodies that bind to the EVs for *in situ* detection of multiple biomarkers. On the other hand, virus-mimicking fusogenic vesicles that encapsulate different molecular beacons fused with EVs for the detection of miRNAs. Fluorescent signals were observed by confocal laser scanning microscopy. After standard curves were calculated, the limit of detection corresponded to 28 and 16 for 14, 22, and 20 EV μL^{-1} when EphA2, CA19-9, miR-451a, miR-21, and miR-10b were detected, respectively. Signals were normalized against the EV marker CD81.

Plasma from 30 pancreatic cancer patients and 10 healthy donors was used to evaluate the diagnostic accuracy of the chip. When combined, the analysis of these protein and miRNA markers in EVs was able to distinguish cancer from non-cancer patients with 100% accuracy and also showed statistical differences between patients with early or advanced stage pancreatic cancer, outperforming the current approved clinical serum marker CA19-9. However, these results need to be confirmed in independent larger cohorts. Despite not including single-EV analysis, this microfluidic technology has the potential to report individualized EV results of multiple biomarkers *in situ,* if capture and microscopic visualization were

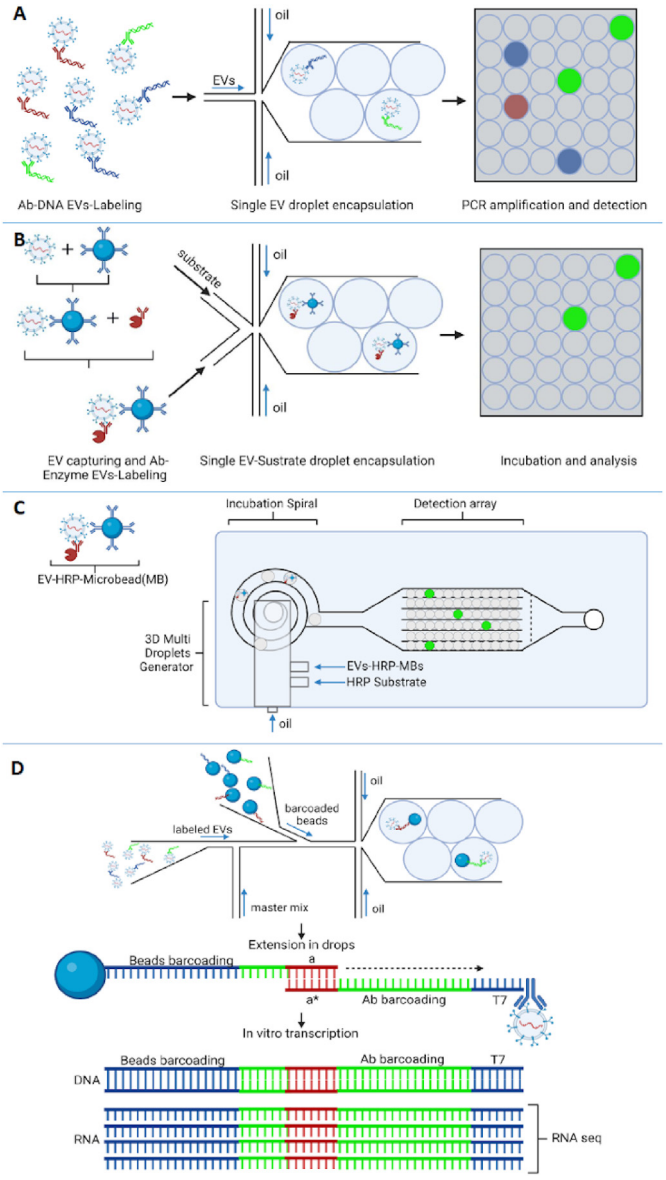

Fig. 5. Single EV analysis performed by identification of membrane proteins using droplet generators devices. A) Schematic representation of the work performed by Ko et al. B) Design of the device used by Liu et al. C) Schematic representation of the chip developed Yang et al. D) Device designed by Ko et al..Abs: Antibodies, EVs: Extracellular vesicles, HRP: HRP: Horseradish peroxidase, MBs:Microbeads.

improved. If these results were validated, this device could become a promising tool for the early diagnosis of cancer patients [48].

As a proof of concept, Cui et al. elaborated a protocol able to potentially detect single molecules of miRNA in single EVs [49]. Plasma EVs were isolated by conjugation with CD63 beads that were then included into droplets with a microfluidic device that co-flows two aqueous phases forming water-in-oil droplets. One phase contained the selected EVs and the other phase the particles encapsulating RT-PCR cocktails and lysis buffer, similarly to Ko et al. [42]. Theoretically, when a droplet encapsulates the EV and the cocktail-containing particle, retrotranscription can take place and the RT-PCR can show the signals of TaqMan probes on-plate. However, EV lysis using Triton X-100 compromised the droplet integrity, being not fully able to complete the process. Interestingly, when synthetic miRNA-21 was used in the droplets, the RT-PCR was able to detect single molecules of miRNA per droplet. If improved, this system could potentially be used to analyze miRNA expression on plasma EVs on a single-EV level.

The study by Kamyabi et al. was able to isolate plasma EVs from patients with pancreatic cancer to evaluate the KRAS mutational status in tumoral and non-tumoral EVs using the Microfluidic Isolation of Tumor-derived Extracellular Vesicles (MITEV) device [50]. This microfluidic device was composed of ~100,000 pillars, placed in a zigzag pattern that alter the flow stream, coated with biotinylated antibodies against CD9, CD63, and CD81, or EpCAM. Thus, plasma EVs positive for these common tetraspanins or tumoral-EVs expressing EpCAM were captured by these antibodies. Then, EVs were released by injecting a glycine solution and collected into PCR tubes where DNA extraction was performed. The device was able to isolate around 2−14 ng of DNA from 2 mL of plasma. Despite that no single-EV analysis was done, DNA molecules were encapsulated into droplets, which could be similar to the analysis of single-EVs. Digital droplet PCR was performed with the KRAS G12/G13 multiplex assay to detect most common KRAS mutations. In addition, the MITEV also allowed the isolation of EV DNA to perform Single Nucleotide Variant (SNV) profiling that depicted quantifiable fluorescently labeled products. Interestingly, a higher amount of DNA was isolated from common EVs (tetraspanin-positive) than from tumoral-EVs (EpCAM-positive), but a higher frequency was detected in the tumoral population, highlighting the potential application of this device for the specific study of tumoral EVs, reducing genomic background from normal EVs. With a short turnaround time of 1.5 h for EVs isolation, the MITEV device could be used in the outpatient setting to evaluate the tumor mutational burden of KRAS [50]. Thus, it could aid during early diagnosis or monitoring residual or recurrent tumor presence after therapy in cancer patients.

Later, Zhang et al. reported a configurable microwell-patterned microfluidic digital analysis platform combined with a dual-probe hybridization assay for PCR-free, single-molecule detection of specific mRNAs in EVs [51]. The microwell array of their device was configurable between the flow-through assay mode for enhanced hybridization capture and tagging of mRNAs and the digital detection mode based on femtoliter-scale enzymatic signal amplification for single-molecule counting of surface-bound targets. Also, in the same study, a dual-probe hybridization assay was described to enhance the sensitivity of the digital single-molecule detection of EV mRNAs. Combining the merits of the chip design and the dual-probe digital mRNA hybridization assay, the integrated microfluidic system demonstrated quantitative detection of synthetic GAPDH mRNA with a limit of detection of 20 aM [51].

Currently, only the HNCIB has been able to demonstrate capability for the analysis of nucleic acids in single EVs. However, other methodologies have shown promising applicability in the single EV field. In particular, simultaneous detection of different biomarkers at the DNA, mRNA, or protein level on an individual EV would open a door for a better comprehension of EV biogenesis, packaging of its cargo, and function on targeted cells but also the clinical applicability. When several biomarkers are combined, the diagnostic accuracy could be increased, highlighting the particular strength of the HNCIB technology as it allowed simultaneous detection of multiple biomarkers. Table 3 summarizes the methods based on the identification of nucleic acids.

4.5. Microfluidic flow cytometry approaches

The flow cytometer has been widely used for multi-parametric analysis of single cell heterogeneity. Similarly, it could be used to study EVs. However, EV sizes fall far below the detection limit of conventional flow cytometers, making it impossible to do single-EV analysis without significant instrument customization [52,53].

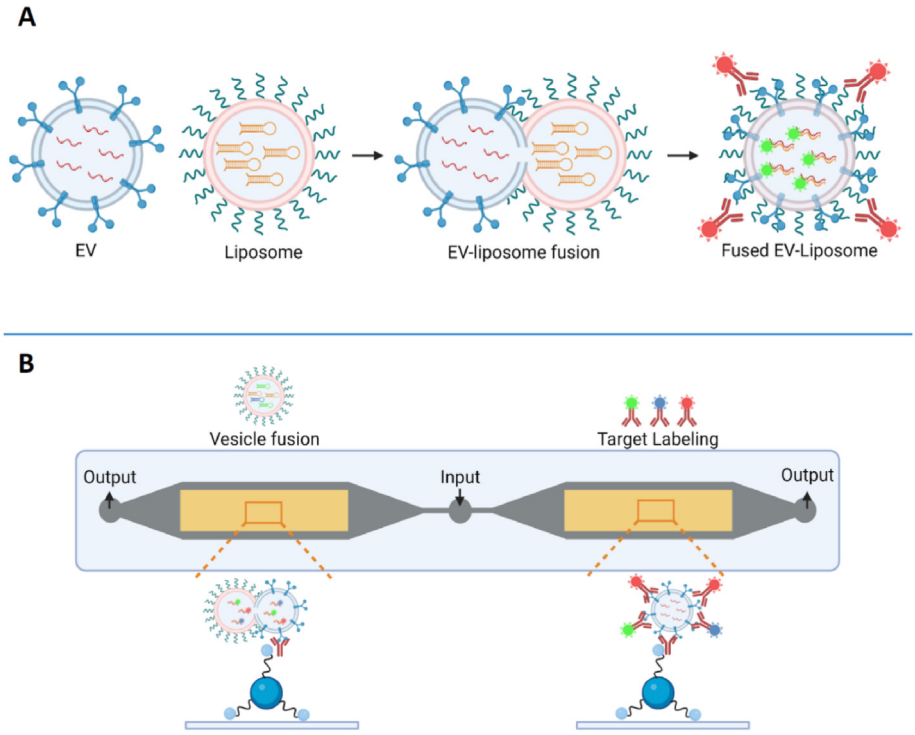

Fig. 6. Single EV analysis with identification of nucleic acids. A) Schematic representation of miRNA and mRNA labeling by liposome fusion described by Zhou et al. B) Device developed by Zhou et al. for proteomic and miRNA characterization of EVs. EVs: Extracellular vesicles.

Table 3
Applied methods on the identification of nucleic acids.

	Name	Target	Type	Sample	Biological application	Year	Ref.
15	High-throughput single-EV liquid biopsy: Rapid, simultaneous, and multiplexed detection of nucleic acids, proteins, and their combinations	mRNA	multiplex	CM and blood plasma	Identification of mRNA and protein overexpression (PD-L1+)	2020	[47]
16	Accurate Cancer Diagnosis and Stage Monitoring Enabled by Comprehensive Profiling of Different Types of Exosomal Biomarkers: Surface Proteins and miRNAs	miRNA	multiplex	CM and blood plasma	Simultaneous identification of overexpression of 3 miRNAs and 3 proteins used as biomarkers	2020	[48]
17	Isolation and mutational assessment of pancreatic cancer extracellular vesicles using a microfluidic platform	DNA	single	CM and blood plasma	Specific capture of EVs (Tetraspanin or EPCAM+) to determine KRAS mutations in DNA.	2020	[50]
18	Ultrasensitive quantification of tumor mRNAs in extracellular vesicles with an integrated microfluidic digital analysis chip	mRNA	dual	CM	Quantitative measurement of mRNA copy numbers (GAPDH and EWS-FLI1) in Ewing Sarcoma	2018	[51]

CM: Conditioned Media.

Microfluidic fabrication is a promising strategy to reduce size detection range and portability of cytometry analysis [54]. Microfluidic flow cytometers use three kinds of detection methods: optical, electrical and magnetic [55]. Among the optical methods, we can find fluorescence and imaging methods, where the excitation light hits a fluorophore, triggering an emission of photons at a different wavelength, which are captured by a detector. Additionally, these devices can have a camera to capture images of flowing particles [56]. Electrical methods are represented by impedance, which determines the perturbation of the electric current between the electrodes [57]. Magnetic methods are based on the magnetic field's perturbation when paramagnetic particles pass through it.

Here, we summarized the use of flow cytometry devices in the analysis of EVs or. As mentioned, flow cytometer configuration must be adapted to detect and measure EVs smaller than 200 nm [53]. One of the first adaptations of flow cytometry to analyze EVs was reported by Van der Vlist and collaborators by modifying a high-resolution flow cytometry called BD influx flow cytometer (BD Biosciences) [58]. Here, authors installed polyetheretherketone (PEEK) tubes to replace silicone tubing, increased the obscuration bar, and modified the laser configuration (Fig. 7A.) in order to set up an optimized protocol able to identify, quantify, and characterize fluorescently stained and antibody labeled EVs [58,59]. Another described system for EV cytometry analysis was the Nano-Flow Cytometry using CytoFLEX System (Beckman Coulter). This system allowed the modification, in a user-friendly manner, of the acquisition settings in order to be able to quantify and identify EVs subpopulations according to their protein and DNA cargo [60].

Moreover, Andronico and collaborators developed a method for EV sizing by using membrane dyes and the single molecule-sensitive flow analyzer [61]. They established a platform equipped with an inverted microscope, with two lasers for fluorescence illumination and modified pinholes, mirrors, and filters to increase the sensitivity and specificity of the collected signals. Isolated EVs flowed through a microfluidic device fabricated in PDMS/glass and images were captured by the objective (Fig. 7B.). This analysis

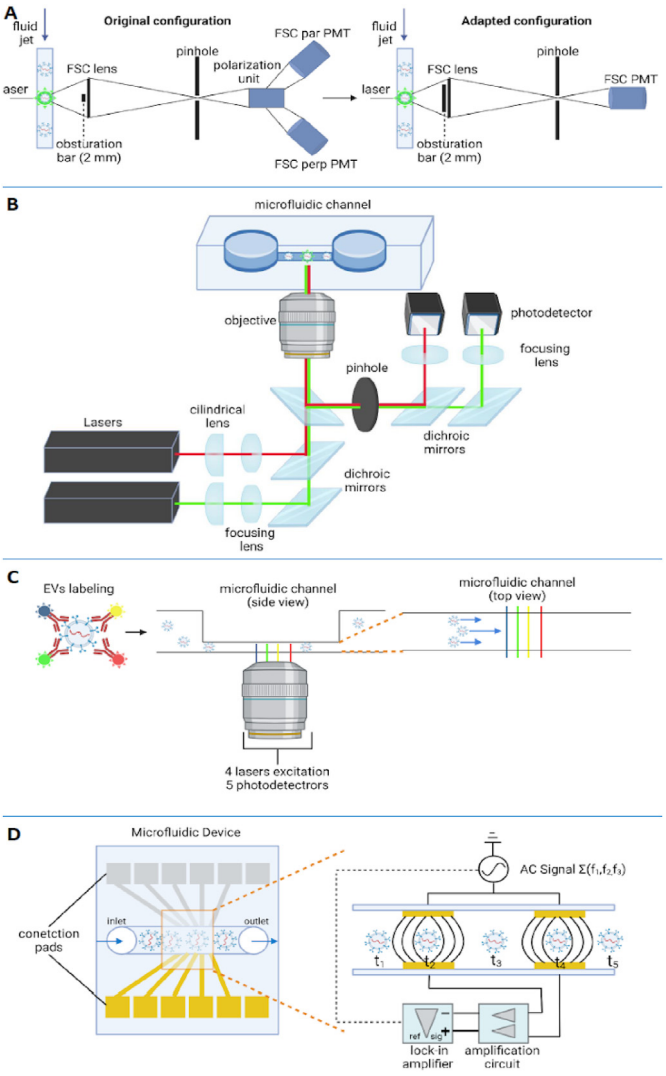

Fig. 7. Single EV analysis by flow cytometry. A) Schematic representation of cytometer modification performed by Van der Vlist et al. B) Design of the micro-flow cytometer configuration developed by Andronico et al. C) Representation of the configuration created by Jiang et al. for super resolution analysis of EVs. D) Device based on impedance sensing developed by McGrath et al.

allowed counting individual EVs and, by quantification of the fluorescent intensity, also measured the size of the EVs. Another interesting development in fluorescent micro-flow single EV analysis was the work of Jiang and collaborators [62]. They combined a single-molecule sensitive flow technique and an adaptive super-resolution imaging method. EVs stained with membrane dyes and dye-conjugated antibodies were analyzed using a microfluidic platform to determine size and protein copy number. For super-resolution mapping, EVs were labeled with novel transistor-like semiconducting polymer dots (Pdots), which exhibit spontaneous blinking. Based on the copy numbers extracted from the flow analysis, the switch-on frequency of the Pdots were finely adjusted so that structures of hundreds of EVs were obtained within 5 min. The microfluidic platform was developed based on a line-confocal design, which consisted of four spatially-separated laser lines, five detectors, and a custom-built autofocusing system (Fig. 7C.). Finally, a high-resolution image of each EV was obtained in a flow of 100 EVs per second [62]. Nowadays, picture acquisition of cytometer events is being a revolutionary way to increase the number of analytical parameters but, for single EV analysis, imaging flow cytometry require further improvement in terms of camera definition. Recent approaches have determined 200 nm as the minimum size that can be detected and only highly fluorescently stained EVs can be identified as a signal dot when they are smaller than 200 nm [63].

Microfluidic flow cytometry based on impedance detection has been recently used for single identification and counting of apoptotic bodies in pancreatic tumor cell culture [64]. To do that, the authors employed a microfluidic device with a central channel surrounded by two sets of detection electrodes [65]. By applying voltage at three discrete frequencies, the response of each frequency is correlated with the size of the analyzed particle (Fig. 7D.). Table 4 summarizes the methods based on microfluidic flow cytometry.

5. Discussion

Current clinical practice is moving towards precision or personalized medicine where clinical decisions are based on biomarker evaluation. In cancer diagnosis, tissue biopsy analysis can offer great information to stratify patients and lead to proper treatments, however tissue heterogeneity is still an issue, biomarkers are limited, monitoring by resampling is not advisable, thereby longitudinal monitoring of the disease is hindered. Liquid biopsy is an fascinating approach that has recently emerged to face these limitations in the management of tumors and other diseases. The study of EV has been growing exponentially during the last years. Recent advances in the field suggest that EV multiparametric analysis can be a successful strategy to identify accurate biomarkers for clinical practice. Thus, implementation of single EV analysis in liquid biopsy was presented as a potential solution for disease identification and monitorization, improving the understanding of spatial and temporal tissue heterogeneity, as well as the early diagnosis and recurrence identification in a single blood test. As we presented in this review, single EV analysis is raising the interest of multidisciplinary researchers, and the development and application of microfluidic systems have allowed to perform many studies in the last 2 years.

In this review, we have observed several strategies to identify and analyze single EVs by microfluidic systems. Most works have been carried out by membrane protein identification, and by singleplex or by a limited number of targets. However, thanks to the proteomic approach by sequencing of DNA-barcode labeled antibodies [46], the study of proteomic signatures of EV subpopulations can be a powerful tool to face the challenges in the field of liquid biopsy [66]. Particularly, these challenges are mainly related to the need to identify specific EV characteristics in EVs produced by pathological cells. Key information on EVs includes tissue-specific biomarker identification that can help localize the disease or specific molecules that can indicate resistance to treatment or disease progression, among others. For example, as abovementioned, expression of miR-21 or PD-1/PD-L1 on single EVs have been proposed as biomarkers for diagnosis treatment response in lung cancer [20,22].

On the other hand, there is still a strong interest in developing a strategy for nucleic acid characterization in single EVs, but unfortunately, similarly to EV proteins, their identification must be performed after EV lysis. EV components of lysed EVs are mixed with EVs from other sources, raising the difficulty to distinguish the source of the proteins, DNA, RNA, or miRNA expression. Most of the works in this area performed single molecule analysis by digital PCR, getting close to single EVs analysis. Interestingly, we only found two studies that specifically performed single EV mRNA [47] and miRNA [48] analysis by hybridization of fluorescent probes

Table 4
Methods based on microfluidic flow cytometry.

	Flow Cytometry				
	Methods based on protein identification				
	Name	Target	Type	Year	Ref.
19	Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry	membrane protein	multiplex	2012	[58]
20	Comparison of extracellular vesicle isolation and storage methods using high-sensitivity flow cytometry	membrane protein	multiplex	2021	[59]
21	Mapping Subpopulations of Cancer Cell-Derived Extracellular Vesicles and Particles by Nano-Flow Cytometry	membrane protein	multiplex	2019	[60]
22	High-Throughput Counting and Super resolution Mapping of Tetraspanins on Exosomes Using a Single-Molecule Sensitive Flow Technique and Transistor-like	membrane protein	multiplex	2021	[62]
	Methods based on physical characteristics				
	Name	Target	Type	Year	Ref.
23	Apoptotic Bodies in the Pancreatic Tumor Cell Culture Media Enable Label-Free Drug Sensitivity Assessment by Impedance Cytometry	impedance	single	2021	[64]

transported by liposomes that fuse with EVs. The combination of this method with membrane protein analysis could be an interesting approach to evaluate gene expression from specific EV subpopulations of interest.

In addition, we classified the measuring methods into statics or flow cytometry approaches. Flow cytometry is the most powerful technique to obtain a high throughput analysis of these EVs, but multiplexing is limited due to spectral overlap, as in fluorescent microscopy. There is high interest in EV cytometry analysis and an increasing number of studies have been performed with clinical samples [67]. Although this type of evaluation is the most promising strategy, analysis with conventional cytometers produces high loss of information from small EVs. Thus, it is imperative to perform critical modifications of the flow cytometer system or to design new micro- or nano cytometers to obtain precise and robust results.

Finally, it is important to highlight that an important number of these reviewed articles applied methods already used in batch EV analysis, which have been modified to reach single particle analysis. In all the reviewed works, dilution of the sample is critical and, generally, all authors agreed in the use of concentrations lower than 10×10^4 EVs uL^{-1} [39,42], and specially in articles with encapsulation of single EVs into droplets, that concentration must be even lower; ensuring that in each droplet there is one or zero EV. Additionally, to visualize individual nanosized particles, an increase of signal is needed in some cases. In this context, several strategies have been applied, for example, Riazanski et al. [30]increased the signal by performing all membrane and cytosolic staining instead of just the membrane biomarker. Other promising option for increasing immune staining signal is using secondary antibodies conjugated with nanorods, which showed a high specific signal [38], using biotinylated primary antibodies and streptavidin conjugated β-galactosidase for chemo-fluorescence detection [42] or, with DNA barcode-labeled antibodies and PCR amplification or sequencing [43–46]. Finally, it is important to highlight the potential to reduce the diameter of the microfluidic channel to decrease the number of flowing particles and increase their alignment. This allows to transform single cell or bulk EVs technologies into single EV analysis as performed by Rodriguez-Quijada work [31] or in the mentioned cytometry studies [59,60,62].

6. Conclusion

Single EV analysis is a field with high interest and promising future. Microfluidic systems are key to perform EV analysis with high sensitivity, robustness, and specificity as well as lower sample and reagents. Significant advances in the study of single EV analysis have been made, facing a big number of challenges with creative strategies for EVs isolation, labeling, manipulation, and sensing. The works reported in this review are the first approaches in the characterization of single EVs and open the window for further studies on biomarkers and liquid biopsy with promising diagnostic, monitoring and prognostic potential.

Author contributions

Francisco G. Ortega Sánchez: Conceptualization, Methodology, Formal analysis, Writing - Original draft preparation, Supervision. Teresa Valero: Data curation, Writing. Reviewing and Editing. Thomas Widmann: Writing - Reviewing and Editing. Matias Regiart: Reviewing and Editing. Maria T. Jerez-Salcedo: Data curation and Reviewing. Martín A. Fernandez-Baldo: Conceptualization and Supervision. Diego de Miguel Perez: Writing, Formal analysis and Original draft preparation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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