



Single-cell extracellular vesicle analysis by microfluidics and beyond

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ABSTRACT

Extracellular vesicles (EVs) are membrane vesicles encapsulated by lipid membranes present in most body fluids. These EVs contain abundant biomolecular composition, including proteins, nucleic acids, lipids, metabolites, etc., and thus play an important role in intercellular communication. However, EVs are highly heterogeneous in terms of physical properties and biomolecular composition. Therefore, it is crucially important to study EV on single cell and single particle level for precise analysis and functional evaluation. Microfluidic platforms provide the possibility for single-cell EV analysis by integrating cell manipulation, EV enrichment, sensitive nucleic acid and protein detection together. In this review, we have summarized current status in single-cell EV and single EV particle study by microfluidic platform with advanced detection tools. The importance of single-cell EV on heterogeneity and future development are also discussed and prospected.

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1. Introduction

Extracellular vesicles (EVs) are membrane vesicles encapsulated by lipid membranes present in almost all body fluids [1]. EVs can be classified into exosomes (40–160 nm), microvesicles (100–1000 nm) and apoptotic bodies (100–5000 nm) according to their size distribution and biogenesis [2–4]. Growing body of research shows that EVs are rich in biomolecular components, including proteins, nucleic acids, lipids, metabolites, etc. [5], and thus play an important role in intercellular communication [6]. Therefore, the classification toward the protein composition on the surface of EV or the biological functionality are vitally significant. The highly heterogeneous EV cargo and membrane composition provide the possibility along with multiple challenges for applications in disease diagnosis [7], therapy [8], and drug delivery [9].

The heterogeneity of EV is mainly reflected in the different biogenesis mechanisms and biophysical properties of each subgroup [10–12]. 1) In terms of biogenesis, exosomes are small lipid membrane vesicles produced via the endocytic pathway. As shown in Fig. 1, exosome formation involves early endosomes (EEs), late

endosomes, and multivesicular bodies (MVBs). However, the biogenesis of microvesicles is not as complex as that of exosomes, which are non-apoptotic EV, formed directly through the plasma membrane cytosol. In addition, apoptotic vesicles are excreted by apoptotic cells through outward blistering and fragmentation of the cell membrane. 2) The heterogeneity in biophysical properties, including size, surface composition, contents and functionality, etc. For size, exosomes are approximately 40–160 nm. However, microvesicles or apoptotic particle size can range from a few microns to as small as exosomes, or even smaller than exosomes [1]. The EV surface proteome (surfaceome) is the key to signal transmission. Greening et al. [13] analyzed large EV surface proteins by combining biotin capture and mass spectrometry, and revealed surfaceome heterogeneity between the two EV subtypes. The category and amount of EVs contents largely determine their function in the organisms. Different subtypes of EVs, whether proteome [14], genome [15] or metabolome [16], have been demonstrated to be heterogeneous and thus they are functionally heterogeneous [17]. Collectively, these heterogeneities are mainly attributed to the fact that the parent cells are inherently different and follow different mechanisms for the formation and release of EVs regarding their subtypes [1]. In addition, the influence of culture conditions on the heterogeneity of EVs is also not negligible [18].

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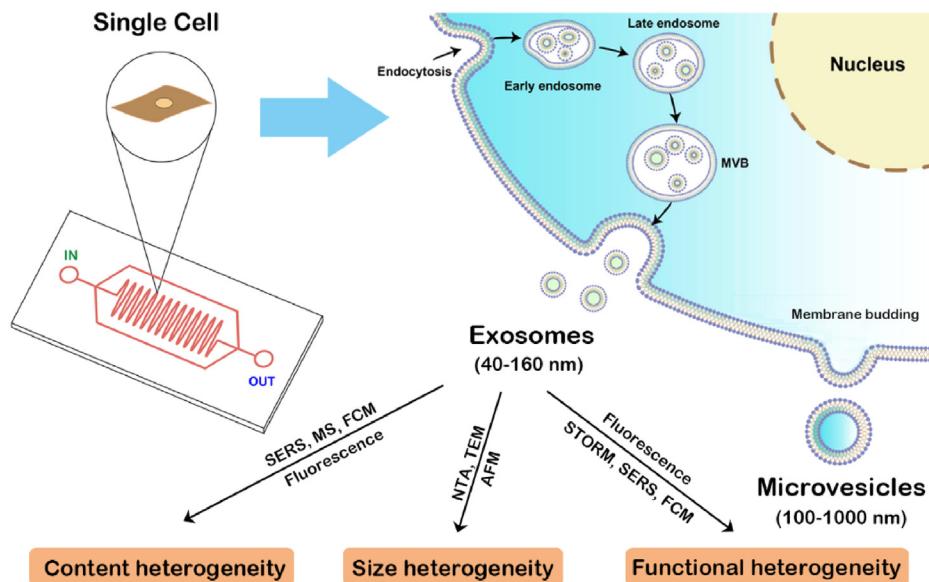


Fig. 1. The schematic of biogenesis mechanisms and heterogeneity for extracellular vesicles. Modified from the illustrations from Ref. [4] with permission from the American Association for the Advancement of Science, Copyright 2020.

Common methods for EVs isolation, like ultracentrifugation [19], precipitation [20] and composition analysis, like western blotting [21,22], enzyme linked immunosorbent assay (ELISA) [23] and real-time polymerase chain reaction (RT-PCR) [24] are employed at the bulk level, which may lose critical information. For example, in disease diagnosis, the heterogeneity of EV implies that each EV may carry different markers and analysis at the bulk level will lose important disease-related markers. In other words, obtaining rich and independent information of EV cargos will contribute to the advancement of EV basic research and the major applications of EV in the clinic. Therefore, it is necessary to explore EV at single cell and single particle level to obtain precise information. In addition, traditional EV isolation and analysis methods are not so efficient to precisely analyze single-cell EV studies with small sample size.

Microfluidic platform, called “lab-on-a-chip” may offer numerous advantages over traditional EV research methods, such as low sample consumption, high sensitivity, low cost, and high throughput [25–28] due to the capability to achieve multiple research goals on a single device [29], meeting the requirements of single-cell or single EV research. Therefore, microfluidic platform has great potential in this field. In this review, we summarized recent progress on microfluidic-based platform in the field of single-cell EV regarding three aspects: separation, characterization, and analysis. The advantages of single-cell strategy used to the investigation of heterogeneity on EV were also discussed.

2. Microfluidics-based platform for single-cell EV analysis

2.1. Isolation of single cell

Obtaining single cells and culturing them is a prerequisite for achieving single-cell EVs research. Many techniques have been developed for single cell isolation, such as limiting dilution, gradient density centrifugation, manual pipetting, magnetic cell sorting and flow cytometry [30], but these methods usually associated with numerous disadvantages that are not suitable for single-cell EVs studies, such as high sample consumption, tedious and prone to contamination. In recent years, single cell isolation

and culture based on microfluidics has gained increasing popularity. Microfluidics not only enables the isolation of single cells in microscopic samples but also allows a more intuitive and accurate assessment of cell or EV characteristics. The unique advantages of microfluidics for single cell isolation and diverse designs for single cell manipulation are introduced.

Compared with conventional single cell isolation techniques, microfluidic chips with micrometer dimensions are suitable for introducing single cells fixing, culturing, assaying and analyzing them. Thus, microfluidic technology shows unique advantages in single cell subculture. Firstly, multiple compartments can be integrated in the microfluidic device, which allows them to require only small sample and reagent volume [31], which is consistent with the small sample volumes of single-cell EV subjects. Secondly, Several valves can be incorporated into the device to enable simultaneous regulation of the flow, sorting and capture process of multiple single cells, thereby reducing labor costs and minimizing sample contamination, which implies that high-throughput single-cell EVs analysis can be performed using microfluidics [31]. In addition, microfluidics can also be used in conjunction with real-time detection techniques which may offer recordings to detect the characteristics of individual cells or EV [32–34]. Notably, microfluidics can apply micromanipulators and reverse hydrodynamic forces to recover single cells for further downstream analyses [35,36].

Common microfluidics-based single cell separation techniques are droplet-based microfluidics, trap-based microfluidics, and valve-based microfluidics. In this section, the principles of these types of microfluidic techniques for separating single cells are described in details.

2.1.1. Droplet-based microfluidics

Droplet microfluidics is a technique that confines the reactions to nano- or picolitre volumes [37], and it is one of the techniques commonly used to accurately obtain individual cells for single cell culture and various downstream analyses [30]. For example, Hattori et al. developed a droplet array-based method to culture single cells and to dynamically characterize the secretion of EVs from single cells by optical quantitative analysis [38]. The common dispersions

used to generate droplets can be cultured of suspended cells, and droplets containing individual cells can be incubated *in situ* within the microfluidic device or transferred to off-chip culture [39].

There are various microfluidic droplet generation techniques, which are broadly classified into two main categories: passive and active encapsulation. The former can be divided into T-junction structure, flow-focusing structure, and coaxial focusing structure according to the device structure. During passive cell encapsulation, droplet formation is primarily based on the interaction between flow shear and surface tension splitting the continuous fluid into small discrete droplets of nanoscale volume. The fluid in the microchannel is precisely manipulated by modifying technical parameters such as fluid flow rate, viscosity, and microchannel geometry to produce fast, stable, and efficient droplets of uniform size [40,41]. Although passive encapsulation has many advantages, such as simplicity of equipment and ease of operation, the random encapsulation process results in a low percentage of microdroplets obtained that contain single cell. In order to improve the efficiency of encapsulation, active encapsulation that combines acoustic forces, electrical, magnetic, optical with passive encapsulation to achieve controllability which has gained the attention of researchers. Acoustic-based droplet ejection technology has emerged as a powerful tool for precise control of droplet size and has been widely used in many biomedical applications. Lagerman et al. [42] combined flow focusing with ultrasonic vibration for high-throughput production of size-tunable single-cell encapsulated droplets (Fig. 2A). During platform operation, ultrasound energy regulates droplet size by controlling the break point of the droplet. The results showed that the ultrasonic pressure wave enhances control of droplet size and the encapsulated Jurkat cells maintained good cell viability in the droplet, which allowed further single-cell analysis. In addition, electric fields are widely used for droplet generation due to their fast response time and good compatibility with microchannel structures. Wu et al. [43] developed a fully integrated paper-based droplet microfluidic platform for droplet generation and encapsulation of yeast cells by adding an electric field in separate droplets dispersed in carrier oil (Fig. 2B). The size of the droplets produced by the platform is decreased by increasing electric field, allowing smaller droplets to be produced at higher voltages. Similar to electric fields, magnetic fields are used for droplet generation. It has revealed many advantages, such as simple operation and no heat generation. In addition, magnetic fields are less affected by liquid pH, temperature, etc., showing potential in microfluidic droplet technology. Navi et al. [44] combined magnetic fields and microfluidics to isolate single MCF-7 cell encapsulated water-in-water droplets and achieve 100% purity in a single operation (Fig. 2C). Because the system operates with permanent magnets and the magnetic force does not affect cell viability within the droplet, this simple and biocompatible microfluidic platform has the potential to be used for single-cell EVs studies. Moreover, optical has also been used for single cell droplet formation due to its high sensitivity and no need to incorporate additional substances to the cell culture medium. Hu et al. [45] developed a fluorescence-activated droplet formation method for encapsulating micro-droplets of HeLa single cells (Fig. 2D). The platform is based on the principle that droplets containing single cells are generated by opening/closing a two-phase hydrodynamic gate valve during optical detection of single cells. In experiments to encapsulate HeLa cells, the platform showed efficient single-cell encapsulation efficiency, with 82.5% of the droplets occupied by single cells. Although the incorporation of acoustic forces, electrical, magnetic, optical can effectively control droplet formation, these components increase the cost and complexity of microfluidic device fabrication. Mechanical vibration has potential for cell encapsulation because only a simple external vibration excitation device is required. In addition

the device does not contaminate the cells because it is external to the microfluidic control [46]. In general, droplet technology has become an important branch of single-cell acquisition based on microfluidics due to its outstanding advantages [40].

2.1.2. Trap-based microfluidics

Cell trap-based microfluidic chips are also widely used for single-cell studies due to their ability to efficiently trap individual cells and culture them with low risk of contamination. Studies have shown that trap-based microfluidics has the highest efficiency as compared to other cell separation methods, with up to 97% of traps filled with individual cells [47]. Cell trap-based microfluidic chips can be broadly classified into contact capture (e.g. hydrodynamic method, etc.) and contactless capture (e.g. dielectrophoresis (DEP) method, etc.) according to the contact between cells and the surface during capture [48–50]. Among them, hydrodynamic traps have received more attention, it is passive structures in the microfluidic channel that allow only one cell to enter into the trap, which increases the capture efficiency of a single cell.

In a typical hydrodynamic trap-based microfluidic chips, single cells are separated from the continuous phase by an array consisting of many mechanical traps (including walls or pores of various shapes). Since the size of trap can be changed at design time, it is adjusted according to the diameter of the cells to be studied to achieve high throughput cell manipulation [51]. The type of trap also has a significant impact on the capture efficiency of single cells. A comparative study of the efficiency of different microfluidic-based common trap types (shortcut structure, U-shaped structure, microcavity structure) for capturing single cells has been done [48], and the results showed that the most efficient microfluidic chip structure for single-cell capture is U-shaped structure, which is not only effective in capturing single cells, but also enables long-term continuous culture and single-cell observation of captured cells consuming less sample and reagents. Moreover, cell traps should be designed regarding subsequent cell culture and analysis. Combining culture chamber with a trap can reduce cell loss during transfer. Wessel et al. [52] designed a trap-based microfluidic perfusion platform that can load, visualize, process, and efficiently record single oocytes and embryos, and implement the cell incubation process within the device with reduced sample loss caused by cell transfer (Fig. 2E). This strategy provided a new platform for the application of microfluidic systems in biology. Sophisticated trap design, appropriate single cell concentration and fluid flow rate, etc., enables hydrodynamic traps to achieve high cell capture efficiency and yield.

In addition to hydrodynamic traps, as a contactless capture method, DEP is also one of the commonly used capture cell methods, which achieves cell trapping by applying DEP forces to the cells. Bai et al. [35] developed an integrated DEP capture method and the results showed single mouse embryonic fibroblast cells (NIH 3T3) capture rate of 91.84% as observed using microscopy in real-time imaging, which breaks the Poisson limit for the capture of cells (Fig. 2F). In this platform, appropriate parameters such as flow rate, voltage, and electric wave are crucial for efficient cell separation.

Many novel ideas have emerged in chip development. Zhang et al. [53] integrated the hydrodynamic U-shaped trap into a hand-held single cell pipette (HSCP), which allows for the rapid and efficient separation of single cells from cell suspensions without the need for microscopic manipulation under a microscope. The traps in conventional working chips are pre-formed before conducting experiments. Interestingly, Liu et al. [54] proposed a new *in-situ* digital projection lithography technique that integrated real-time *in-situ* generation of digital masks for particle processing and fluid control into conventional digital micromirror devices (DMD)-

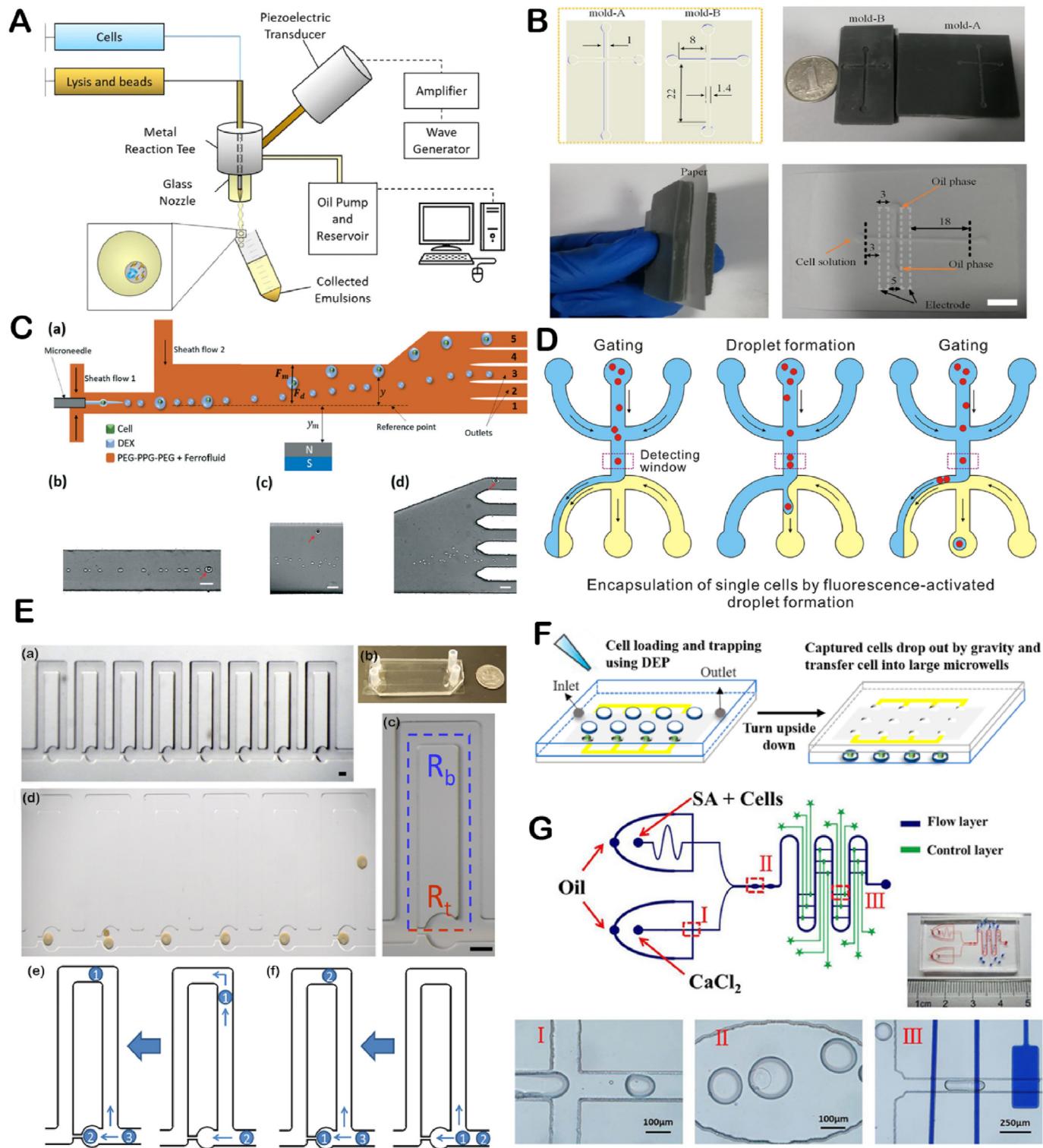


Fig. 2. Microfluidics-based single cell isolation methods. **A)** Acoustic-based droplet technology microfluidics for single cell analysis. Adapted from Ref. [42] with permission from the Elsevier, Copyright 2019. **B)** Controlled generation of droplets using an electric field for single cell isolation. Adapted from Ref. [43] with permission from the Wiley-VCH, Copyright 2022. **C)** Magnetic fields combined with microfluidics to isolate single cell. Adapted from Ref. [44] with permission from the Royal Society of Chemistry, Copyright 2018. **D)** Encapsulation of single cell into droplets by optical. Adapted from Ref. [45] with permission from the Elsevier, Copyright 2016. **E)** Hydrodynamic trap-based microfluidic chips to isolate single cell. Adapted from Ref. [52] with permission from the Elsevier, Copyright 2015. **F)** Single cell was trapped by applying dielectrophoresis (DEP) force. Adapted from Ref. [35] with permission from the American Chemical Society, Copyright 2020. **G)** A valve-based microfluidic device for on-chip single cell treatments. Adapted from Ref. [56] with permission from the Wiley-VCH, Copyright 2019.

based projection lithography. By combining controlled fluid flow with DMD lithography, precise micro-traps or micro-filters can be rapidly generated at pre-designed locations to capture the corresponding particles, which provided a new idea for the development of trap-based microfluidic platform. Overall, trap-based microfluidics has great potential for single cell isolation and culture due to its simple design and ease of operation.

2.1.3. Valve-based microfluidics

Valve-based microfluidic devices are another commonly used method for single-cell collection in addition to the two devices mentioned above. The valve-based microfluidic device is smartly divided into several compartments by means of valves, each of which can carry out independent reactions. The valves can be opened and closed as required, allowing complex tasks like capturing and culture cells to be performed. In this device, the design of the valve structure and fluid flow rate, viscosity, etc. are the key technology for efficient cell separation. In addition, the valve-based microfluidic system allows automated operation in the chip, greatly helping researchers to achieve various experimental functions required for single-cell analysis. Valve-based microfluidics thus helps to accurately simulate the dynamics of the cellular microenvironment with high precision and controllability [55].

Currently, microfluidic devices used for single cell analysis are unable to fix cells for bioassays while manipulating the reagents. Zhao et al. [56] designed a microfluidic feeding platform based on a double-layer pneumatic valve, which allowed simultaneous cell fixation and processing on the sheet and non-destructive collection of the processed cells for further analysis (Fig. 2G). The high throughput analysis should be considered while designing the microfluidic chips. The low throughput microfluidic chips cannot meet the advances in scientific research, thus posing a higher challenge to the throughput of microfluidic chips. The improvement in microfluidic chip throughput is of significant importance in single cell analysis, especially in drug discovery and therapeutic personalization. Wang et al. [57] applied the relationship between geometry, valve size and sealing pressure in exploring the design and optimization of microfluidic platforms to fabricate microfluidic devices consisting of up to 5000 hydrodynamic traps and corresponding microvalves for high-throughput single-cell compartmentalized protein expression quantification. This work provided an inspiration for designing high-throughput analytical chips.

Although valve-based microfluidics can overcome the drawbacks of limited operability, the fabrication and complex operation of the device add to the cost of the device. Moradi et al. [58] reported a fault-tolerant design of a microfluidic crossbar for droplet barcoding in single-cell analysis improved fault tolerance to single-sided faults by increasing the number of valves on the crossbar, maintaining the separation efficiency of the device. The development of this device validates the promising application of valve-based microfluidics for single-cell analysis.

Various techniques for single cell isolation have been described above, and they have their respective advantages and disadvantages for cell acquisition and culture. For example, the droplet-based microfluidics is simple and efficient for encapsulation, however produces microdroplets that are not suitable for long-term culture of single cells. The trap-based microfluidic chips, although simple in design and high throughput methodology, obtains single cells that sometimes require translocation for culture. The valve-based microfluidic device, although they can be precisely controlled to smartly divide into several compartments may be more suitable, are complex in design and costly in price. In views of the limited number of studies on single-cell EVs by microfluidics, it's necessary for the investigation of suitable form on cell acquisition

toward single-cell EV analysis.

2.2. Capture of single-cell EVs on microfluidic device

Isolation of EVs is a critical step in realizing single-cell EV level study. In other words, the quality of EV separation affects subsequent EV research. A variety of separation methods including ultracentrifugation, precipitation, size exclusion chromatography and well-established procedures have been developed for the separation of EVs [59]. However, these techniques require large sample volume which is not suitable for the isolation of EVs secreted from single cell in combination with microfluidic technology. In contrast, immune-isolation methods require no limit to the minimum isolated sample volume and the antibodies can be well combined with microfluidic technology. Therefore, immune-based capture is currently the main EV isolation method in single-cell EV research based on microfluidics. The principle of this method is that specific marker proteins (e.g. CD9, CD63, CD81, etc.) present on the surface of EVs can specifically bind to the corresponding antibodies immobilized on glass sides or microchannels, thus enabling the specific enrichment and isolation of EVs for subsequent characterization. For instance, Son et al. [60] modified the anti-CD63 Ab on the microbeads to captured the exosomes in microfluidic device for dynamic characterization of exosomes released from single HepG2 cells (Fig. 3A). In addition, the antibodies to marker proteins are also smeared on glass plates. Chiu et al. [61] immobilized anti-CD63 Ab on the glass slide to enrich MCF-7 cell and MDA-MB-231 cell-secreted exosomes (Fig. 3B). Similar EV capture principle was also applied in Cai's study [62]. In order to comprehensively capture the exosome, Ji et al. coated capturing antibodies (e.g., anti-CD63, anti-CD81, anti-CD9) in microarray to form antibody barcodes on the glass plate for EVs capture and characterization from human oral squamous cell carcinoma (OSCC) cell lines (Fig. 3C) [63]. The ring shaped region between the two concentric valves composed of PDMS, coated with CD81 antibodies against epitopes in the EV membranes for capture single MCF-7 cell secreted EVs in the study by Nikoloff et al. (Fig. 3D) [55].

Aptamer technology works in similar fashion to antibodies, also has great potential in single-cell EV isolation. Aptamers are short, single-stranded oligonucleotides that exhibit high affinity and selectivity for specific targets, such as EV surface proteins (CD9, CD63, CD81, etc.) [64]. Compared with antibodies, aptamers have many advantages, such as low manufacturing cost, easy modification, relatively mild elution conditions and release EVs in their native form [65]. They may also be modified on the surface of microfluidic devices like antibodies to perform separation tasks. At present, a variety of nucleic acid aptamers for EV isolation have been developed. For example, Qan et al. [66] modified microchannels with aptamer in a microfluidics system for rapid and efficient isolation of exosomes by targeting exosome-carrying CD63 and PTK7 (Fig. 4A). In addition to the aptamers being immobilized in the microchannel, it can also bind to the EVs in free form. For example, Tan et al. reported that nucleic acid aptamers can inter-recognize surface proteins of extracellular vesicles using seven nucleic acid aptamers [67]. It was further modified by fluorescent inducers to label serum EVs in free form, which could be detected and classified by thermal enrichment and linear discriminant analysis of cancer. Recently, Yao et al. [68] developed a microfluidic chip based on high affinity between EV membrane protein (CD63) and to achieve rapid enrichment and sensitive detection of EVs in serum (Fig. 4B).

In the field of microfluidic-based platforms for EV research, several separation approaches based on EV physical characteristics have also been developed in addition to enrichment methods based on EV surface markers, including: microfluidic filtering,

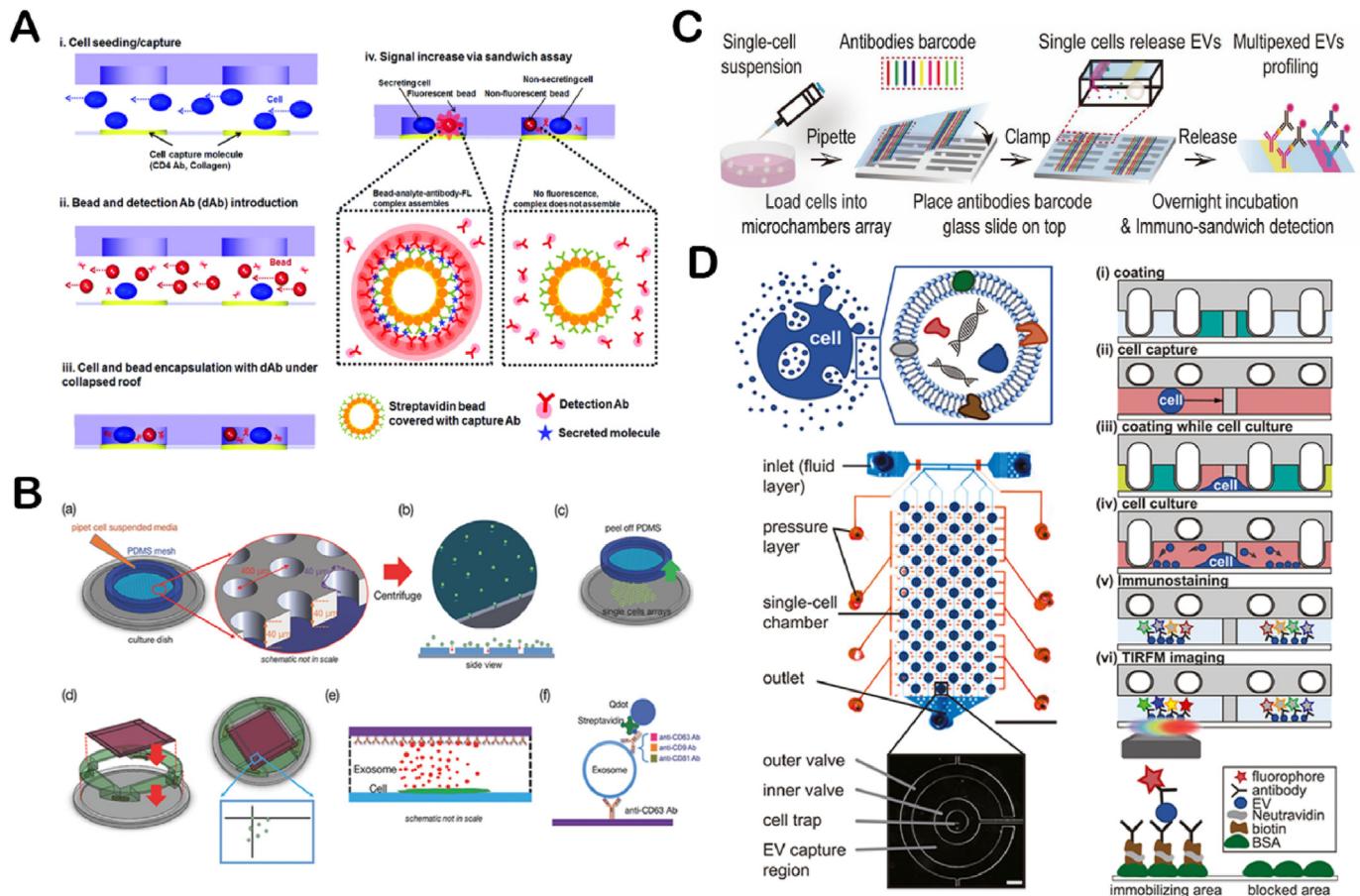


Fig. 3. Capture of single-cell EVs based on immune-isolation on microfluidic device. **A)** A reconfigurable microfluidic device for dynamic monitoring exosome release from single cells. Adapted from Ref. [60] with permission from the Royal Society of Chemistry, Copyright 2016. **B)** PDMS to the analysis of single-cell exosomes microfluidic-based. Adapted from Ref. [61] with permission from the Wiley, Copyright 2016. **C)** Platform for multiplexed profiling of single-cell EV secretion. Adapted from Ref. [63] with permission from the National Academy of Sciences, Copyright 2019. **D)** The device with 72 integrated two concentric valves composed of PDMS. Adapted from Ref. [55] with permission from the National Academy of Sciences, Copyright 2021.

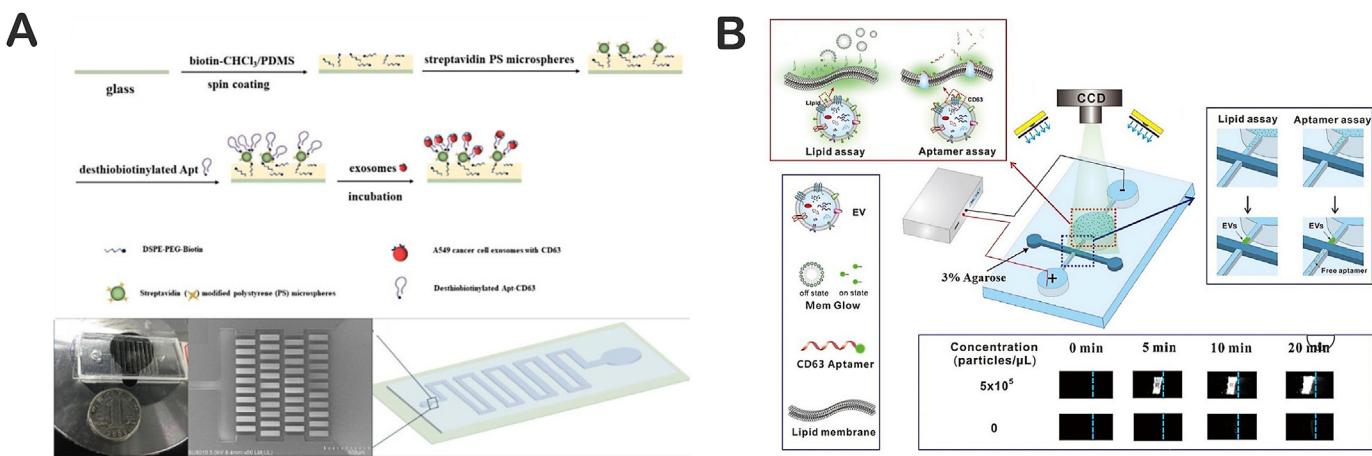


Fig. 4. Capture of single-cell EVs based on aptamer technology on microfluidic device. **A)** The aptamer being immobilized in the microchannel for isolation of exosomes. Adapted from Ref. [66] with permission from the Multidisciplinary Digital Publishing Institute, Copyright 2022. **B)** The aptamer was bind to the EVs in free form to achieved rapid enrichment and sensitive detection of EVs in serum. Adapted from Ref. [68] with permission from the Elsevier, Copyright 2022.

deterministic lateral displacement (DLD), etc. [27]. The operating principle of microfluidic filtering is just like common filtration to achieve isolation based on the size of EVs [69]. Similarly, the DLD is also based on the diameter of the EVs to achieve separation and

sorting. Specifically, particles of different sizes will flow out from different outlets due to the larger diameter particles have lateral displacement on the specific column array microfluidic platform compared to smaller particles [70]. Furthermore, some methods

Table 1

Advanced detection tools for single EV or single-cell EV analysis.

Techniques	Principle	Advantages	Disadvantages	Refs.
Fluorescence	The recording substance emits light at a higher wavelength than the excitation wavelength	High sensitive, easy operation	Photobleaching of the fluorescence	[55,60,61,63]
Raman spectroscopy	Analyzing scattering spectra with frequencies different from the incident light	High resolution, high sensitivity	Stability is difficult to control	[77]
Mass spectrometry	Analysis of the mass ratio of charges generated by each component	High sensitivity, specificity and high-throughput, obtain structural information	Harsh sample preparation	[88]

based external force field, such as acoustofluidic technology, dielectrophoretic (DEP) separation, have also been used for the isolation of EVs [27]. Among them, the separation principle of acoustic fluidics is based on the fact that different particles are subjected to different forces in the acoustic field. In particular, as larger particles are subjected to stronger forces, they are deflected over larger distances on the microfluidic platform, and conversely, smaller diameter particles are deflected over smaller distances [71]. Unlike acoustofluidic, the principle of DEP separation of EVs is based on the fact that different EVs are subjected to different electric field forces in the DEP field due to their different dielectric properties [72]. Overall, these methods are expected to inspire the design of microfluidic platforms for EV isolation and have potential in the field of single-cell EV research.

3. Advanced detection tools for single-cell EV analysis

Precise analysis of the obtained EVs is another key step in the study of single-cell EVs, which helps to researchers understand the heterogeneity of membrane composition and content of EVs from different single cells. There are many advanced detection tools (e.g., fluorescence, Raman spectroscopy, mass spectrometry) being used for EVs analysis. The description and comparison of different techniques are summarized in Table 1.

3.1. Fluorescence

Fluorescence technique is one of the highly sensitive and commonly used EV analysis techniques [73], which works on the principle that a substance is excited by a specific wavelength of light and the substance emits the light of higher wavelength than that of excitation wavelength for a very short period of time. Son et al. [60] developed a reconfigurable microfluidic device for dynamic characterization exosome release from single HepG2 cells. The microfluidic device is integrated with a fluorescence microscope to realize the detection and counting of single cell secreted exosomes. The simultaneous multiplex analysis of multiple single cells by microfluidic devices is a prerequisite for achieving high throughput analysis of single cells. Ji et al. [63] used photolithography to create strip molds and then poured polydimethylsiloxane (PDMS) prepolymer onto the molds to form PDMS microchips. Moreover, capture antibodies are coated in another microarray to form antibody barcodes on the glass plate. It was then placed on top of the microarrays and co-incubated overnight for EVs capture. The captured EVs were then fluorescently labeled using a detection antibody (biotin-CD63) and streptavidin-APC or streptavidin-PE form an immuno-sandwich to facilitate analysis using a fluorescence scanner.

Continuous monitoring of cell behavior is critical in single-cell studies, and most microfluidic devices can only perform snapshot tests at a single time point. Therefore, a device is urgently needed that can support time-lapse studies. Chiu et al. [61] applied PDMS to analyze single-cell exosomes employing a microfluidic-system. The device is capable of non-invasively collecting and

investigating single-cell secretory EVs at different time points, and subsequently obtaining quantitative information on single-cell behavior based on the biomarker signal pairs of individual cells under a given perturbation. Specifically, the two-dimensional array of through holes fabricated using PDMS were adhered to glass plates to form microwells for single cell collection and culture. The glass slide was immobilized with anti-CD63 Ab, which can be used to enrich cell-secreted exosomes and incubation of the collected exosomes with marker proteins antibodies (anti-CD63, anti-CD9, or anti-CD81). Subsequently, the glass slide was incubated in streptavidin-coated quantum dots (Qdots). The whole integrated system facilitated the imaging and counting using inverted fluorescence microscopy and specific software. Moreover, further results of counting analysis implied that tetraspanins CD9 and CD81 may be related to malignancy of cancer cells. The four-color total internal reflection fluorescence microscopy (TIRFM) is an excellent fluorescence tool for multiple analysis. Nikoloff et al. [55] combined microfluidics with fluorescence technology to design a device with 72 integrated two concentric valves composed of PDMS for the identification of single-cell EV secretions. Single-cell secreted EVs were captured by CD81 antibodies modified on the luminal surface and then immunologically stained by anti-CD63, anti-HSP70, anti-TSG101 and anti-Annexin V. The TIRFM was used to reveal the location of EVs based on the fluorescence signal of EVs surface marker proteins. The result showed that the proposed technique is sensitive enough to differentiate between the number of EVs secreted by one, two, and three or more cells in a well. In general, fluorescence technology has many advantages in microfluidic-based exosome detection, such as high accuracy and sensitivity.

3.2. Raman spectroscopy

Raman spectroscopy (RS) can provide information on molecular vibrations and rotations by analyzing scattering spectra with frequencies different from the incident light, and is applied to the study the chemical structures, crystallinity and molecular interactions. Laser tweezers Raman spectroscopy (LTRS) is a derivative technique of micro-RS that is especially suitable for single-cell detection by trapping the particles with laser tweezers and collecting the scattered light with a Raman spectrometer [74]. Qiu et al. [75] used LTRS to monitor internal structural changes and chemical modifications in NPC cells after exposure at a clinical dose to X-ray irradiation (IR) at a single-cell level (Fig. 5A). For vesicles characterization, LTRS is capable of trapping single vesicles at the laser's focal point using a tightly focused laser beam and providing the global chemical composition of single EVs without using any exogenous label. Sergei G et al. demonstrate the specificity and potential of LTRS for single EV measurements using examples of mixed samples of DOPC liposomes, exosomes from human urine and rat hepatocytes etc. [76]. Specifically, LTRS can provide global signatures of biomolecular composition for each subpopulation at the level of individual EVs or very few EVs, enabling the characterization of these subpopulations.

Since the Raman scattering signal is relatively weak, surface-

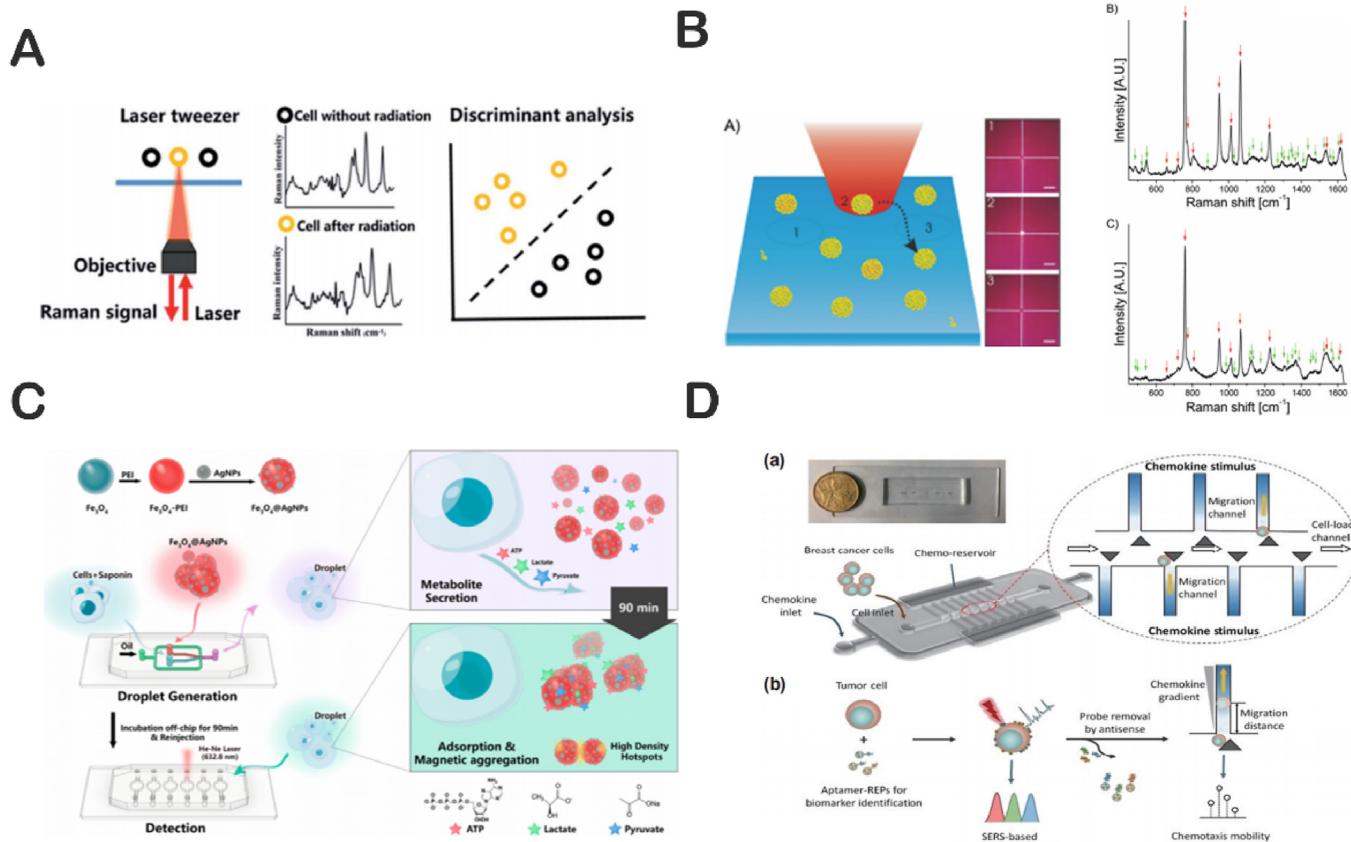


Fig. 5. Raman spectroscopy applied to characterize EVs. **A)** Schematic of the home-made laser tweezers Raman spectroscopy system. Adapted from Ref. [75] with permission from the Royal Society of Chemistry, Copyright 2020. **B)** Schematic representation of the SERS measurements of AuNP-coated EVs. Adapted from Ref. [77] with permission from the Wiley, Copyright 2016. **C)** Overview of workflow of SERS-microfluidic droplet platform for single cell encapsulation and simultaneous detection of three metabolites produced by a single cell. Adapted from Ref. [78] with permission from the American Chemical Society, Copyright 2019. **D)** 2D profiling of tumor chemotactic and molecular phenotype at single cell resolution using a SERS-microfluidic chip. Adapted from Ref. [79] with permission from the Springer, Copyright 2022.

enhanced Raman scattering (SERS) was introduced and is generally used for the characterization of single cells. SERS can enhance the Raman scattering of molecules or nanostructures adsorbed on rough metal surfaces. Stephan et al. [77] proposed a new approach for single exosome identification based on SERS for diagnostic applications. EVs can be functionalized with gold nanoparticles (AuNPs) on their surface, forming an irregularly shaped nanoshell that enables the generation of an enhanced Raman signal while maintaining a colloidal suspension of individual vesicles. The spectra are recorded by individual AuNP-coated EVs adsorbed on the quartz surface. Peaks from exosomal biomolecules could be clearly identified in the spectra from B16–F10 melanoma-derived vesicles and RBC-derived vesicles (Fig. 5B). It is considerable that EVs without AuNP coating could not generate a clear Raman signal under the same conditions, underscoring the importance of surface-enhanced Raman scattering for enhancing the signal of single vesicles.

Microfluidics combined with SERS enables the analysis of events occurring in extremely small volumes at ultrasensitive intensities. Sun et al. [78] presented a SERS-microfluidic droplet platform to realize the label-free simultaneous analysis of multiplexed metabolites at the single-cell level via a versatile magnetic SERS substrate composed of silver nanoparticles (AgNPs, 30 nm)-decorated 400 nm Fe_3O_4 magnetic microspheres. Formation of water-in-oil droplets containing single cells and (Fe_3O_4 @AgNPs) in a microfluidic chip, within the off-chip cell incubation, a variety of metabolites secreted by this cell were directly adsorbed on these

Fe_3O_4 @AgNPs, and they can be spontaneously collected together in droplets due to the robust magnetism produced by Fe_3O_4 (Fig. 5C). When the dynamic adsorption of metabolites reached an equilibrium, the SERS signal of metabolites on Fe_3O_4 @AgNPs in the aggregated state can be acquired. Zhang et al. [79] proposed a single-cell microfluidic chip that integrates the measurement of chemotactic behavior and the expression of surface proteins. In this platform, breast cancer cells were isolated in fluidic channels and then labeled with three SERS nanovectors. The results showed that the platform was capable of analyzing surface protein expression at single-cell resolution (Fig. 5D). Above examples suggested the possibility of using RS to characterize and analyze individual EVs. In future studies, we expect the integration of microfluidics with RS to probe the molecular characteristics of single-cell EV/single EV for understand the heterogeneity.

3.3. Mass spectrometry

Mass spectrometry (MS) has been emerging into a common tool for identifying structures and performing quantitative analysis. Such as, it enables precise determination of the protein expression levels as well as their amino acid sequence and post translational modifications. Particularly, it has been demonstrated that liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) is an effective method for EV analysis [80]. The proteome and metabolome are receiving increasing attention, especially the latter, due to its involvement in multi-omics molecular information

[81]. Mass spectrometry platform is an important tool for studying the proteome and metabolome in the field of EV. It is an effective tool for identification and quantitation of peptides or metabolites owing to its high sensitivity, specificity and high-throughput [82]. The reported studies mainly focus on proteomic or metabolomic identification of EVs in the level of bulk by mass spectrometry-based approaches. More recently emerging techniques are reported for single cell proteomics/metabolomics. However, the EVs from single cell are far more challenging than the cell itself due to their limited content of proteins and metabolites. Since the amount of EVs secreted by a single cell is limited and requires much high sensitivity of MS for their analysis. Fortunately, combining microfluidics with mass spectrometry allows high throughput at the single cell level of multiplex analysis [81,83–85], which gives great encouragement to workers who want to engage in EV proteomic and metabolomic studies at the single cell level. For instance, combining microfluidics with mass spectrometry allows high throughput at the single cell level of multiplex analysis. Xu et al. [86] combined a serpentine channel microfluidic device with pulsed electric field-induced electrospray ionization high-resolution mass spectrometry (PEI-ESI-HRMS) to achieve single yeast single-cell high-throughput analysis and annotation of multiple metabolites. However, media and intracellular components can produce matrix interference effects that can affect the sensitivity and accuracy of single-cell analysis results. To address this issue, Zhang et al. [87] developed a method that combines droplet-based microextraction with single-cell mass spectrometry. The method not only enables matrix-free, selective and sensitive detection of single-cell metabolites, but also has the capability of reliable, high-throughput single-cell analysis. Recently, Dmitriy et al. [88] developed a technique based on secondary ion mass spectrometry with nanoparticle projectiles (NP-SIMS) to characterize the surface protein content of single EVs. Specifically, this technique determines the relative abundance of two common surface proteins (CD63 and CD81) in urinary EVs by labeled antibodies, and only requires a few hundred or thousand EVs in the analysis region to detect the presence of the tagged antibodies.

4. Single-cell EV/single EV particle characterization

Another critical aspect of EVs is physical characterization, which includes size, morphology, quantity, and mechanical properties. Common characterization techniques for EVs include flow cytometry, nanoparticle tracking analysis, super-resolution and atomic force microscopy, etc. The working principles, advantages and disadvantages of these methods are summarized in Table 2.

4.1. Flow cytometry

Flow cytometry (FCM) seems to be the efficient strategy for single EV analysis since it enables the evaluation and classification of individual particles. FCM has been very widely used at the single

cell level. However, for conventional FCM it is difficult to detect vesicles smaller than 300 nm in size, and individual EV contain low levels of markers, which are difficult to analyze by conventional FCM. Shen et al. [89] reported a single-EV flow cytometry analysis technique that enabled single-EV counting and phenotyping on conventional flow cytometry for the first time by target-initiated engineering (TIE) of DNA nanostructures on each EV. The principle of this technology was to use conformationally switchable DNA probes to specifically bind to EV surface protein markers and to scale the overall size of individual EVs to more than 500 nm through a hybridization chain reaction (HCR) for the analysis (Fig. 6A). However, the method has a long pre-processing time and requires more workload to design more different probes and extension hairpins for high-throughput analysis on single EVs. Others have improved the sensitivity of ordinary flow cytometers for forward scattering (FSC) and side scattering (SSC) by optimizing the optical configuration and fluid settings to detect single EV [90,91]. However, all these methods can only count EV but not analyze it. Tian et al. [92] developed a high sensitivity flow cytometer (HSFCM) by reducing probe volume to reduce background, extending particle transport time to increase photon production, and photon burst detection. The instrument allowed simultaneous three-channel detection, both by side scattering to analyze EV size and by two fluorescence to detect biomarkers of EV. HSFCM enables protein analysis and size analysis of single EVs with size ≥ 40 nm (Fig. 6B). However, the home-made instrument was not available for other laboratories, limiting the wide use of this method. Commercial nano flow cytometry (NanoFCM) has become available for single EV analysis. In contrast to conventional flow cytometry, NanoFCM allows the characterization of individual particles, as the resolution limit is reduced to 40 nm. Thus, small EVs (40–200 nm) can be analyzed in concentration and size in addition to fluorescence-based marker detection. Rak et al. [93] used nanoflow cytometry to map subpopulations of cancer cell-derived extracellular vesicles. This implies the potential of nanoflow cytometry in characterizing EV as depicted in Fig. 6C. In addition, Viviane Ponath et al. purified EVs from cells overexpressing BAG6 and B7–H6 (two ligands of NKp30), respectively, and single EV phenotypic analysis was performed by applying NanoFCM based on four EV markers (Hsp 70, CD9, CD63, and CD81) [94]. Currently, the NanoFCM have already analyze and sort single EVs [95,96]. In the future, it will be expected to be promising in the field of single-cell EV in combination with microfluidic technology.

Imaging flow cytometry (IFCM) is a method combining flow cytometry with imaging, which enables visualization and direct quantification of the acquired images. IFCM is reported to be sufficiently applicable and sensitive to detect hematogenous particles/vesicles with diameters above 200 nm, but its potential for detecting individual EV needs to be further explored. Görgens et al. [97] was able to define and optimize IFCM acquisition and analysis parameters on the instrument for the detection of single EVs by using CD63eGFP-labeled single EVs as biological reference

Table 2
The techniques of characterization for single EV or single-cell EVs.

Techniques	Principle	Advantages	Disadvantages	Refs.
Flow cytometry	Measurement of scattered light from particles or fluorescence marked on the particles	Easy operation, concentration and size information can be obtained at the same time	Free dyes can cause interference	[89]
Super-resolution microscopy	Exploit photo switching of fluorescent probes or a nonlinear response in the fluorescence emission	High resolution, high sensitivity	Expensive instrumentation, low throughput	[33,104]
Atomic force microscopy	Determining the atomic interaction forces between the molecule and force sensitive elements to molecular characterization	Simple sample preparation, provides three-dimensional information and mechanical properties of EV particles	Low throughput, limited information	[113,114]

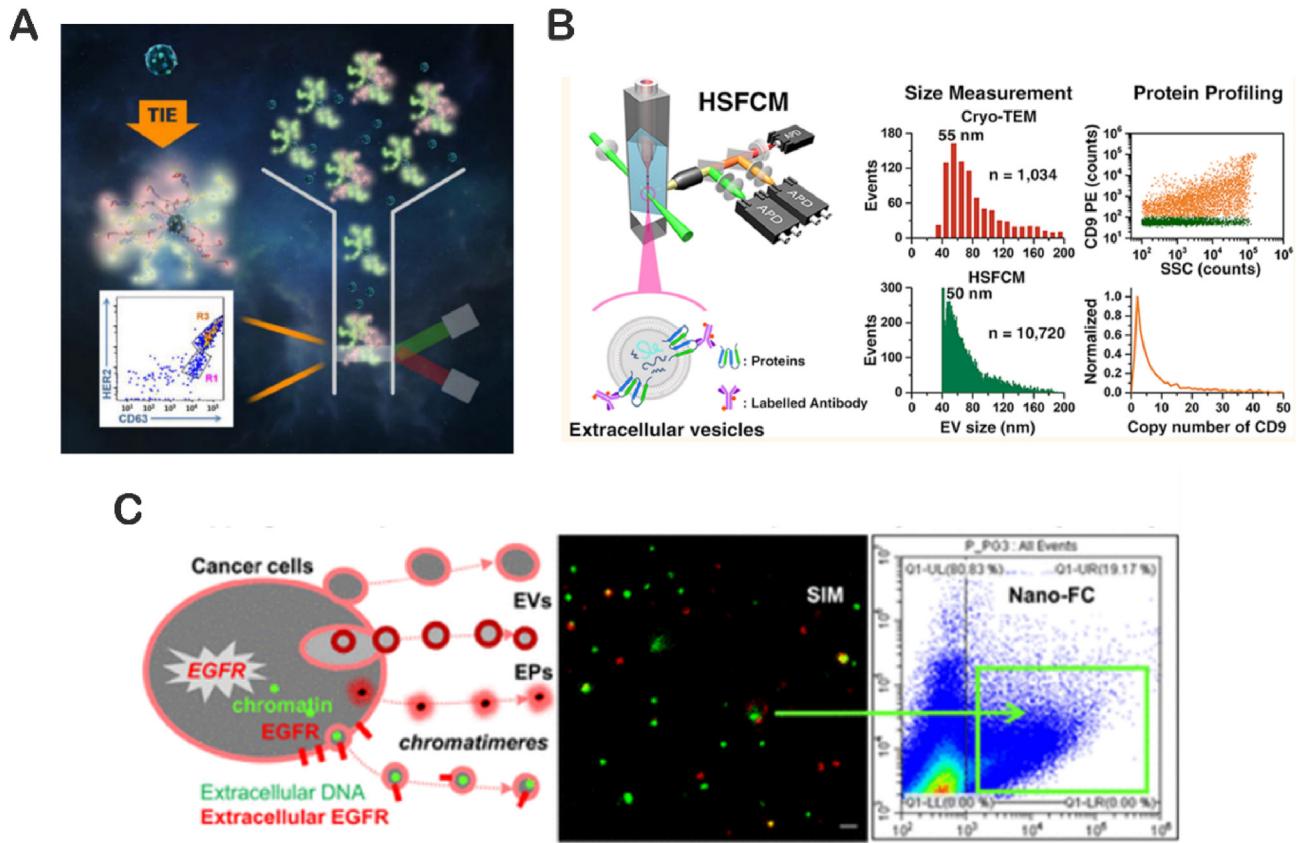


Fig. 6. Flow cytometry applied to characterize EVs. **A**) Single EV flow cytometry analysis enabled by target-initiated engineering (TIE) of DNA nanostructures. Adapted from Ref. [89] with permission from the Wiley, Copyright 2018. **B**) The working principle of high sensitivity flow cytometer (HSFCM). Adapted from Ref. [92] with permission from the American Chemical Society, Copyright 2018. **C**) Mapping landscapes of extracellular vesicle and particles by nano-flow cytometry. Adapted from Ref. [93] with permission from the American Chemical Society, Copyright 2019.

materials. Ricklefs et al. [98] optimized the instrument for multi-parametric IFCM analysis by integrating different software settings and tested it in the surface characterization of four proteins (CD9, CD63, CD81) in human and mouse cell cultures and plasma samples. The results show that IFCM can achieve robust, practical and simple single EV characterization. This implies the potential of IFCM for future functional studies and clinically relevant applications.

4.2. Super-resolution microscopy

Super-resolution microscopy has the spatial resolution beyond "the diffraction limit" [99], with great potential for EV research, especially for single cells or single EV. At present, common members of the super-resolution microscope including photoactivation localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), structured illumination microscopy (SIM), stimulated emission depletion (STED) microscopy, saturated excitation (SAX) microscopy, etc. [99–101]. Chen et al. [102] used PALM and STORM for imaging and intracellular tracking of cancer-derived exosomes. The results suggested that super-resolution microscopy-based EV imaging and tracking have great potential for investigating the mechanisms of EV-mediated cancer metastasis. Similarly, Nizamudeen et al. [103] performed accurate analysis and live imaging of stem cell-derived EVs by direct-STORM (Fig. 7). The results further demonstrated the advantages of super-resolution microscopy with higher sensitivity and lower variability in detection of EVs down to 20–30 nm size in comparative experiments

with traditional characterization techniques. The dSTORM was also used to perform 3-D imaging of the surface microdomains of individual extracellular vesicles [104]. Moreover, super-resolution microscopy was also used to revealed the heterogeneity in EVs secretion by single human monocyte-derived macrophages (M0-, M1- or M2-like) [33]. In general, super-resolution microscopy has become an emerging tool for single EV characterization [105–107]. Therefore, the scope of application for super-resolution microscopy in EV research is expected to be further developed, for example, it can be combined with microfluidics to take full advantages of both platforms in order to advanced more challenging and meaningful studies of single-cell EVs or single EVs.

4.3. Atomic force microscopy

Atomic force microscopy (AFM) was first introduced in 1986 [108], which works by determining the atomic interaction forces between the molecule and force sensitive elements to molecular characterization [109]. Unlike electron microscopy, which provides only two-dimensional images, AFM provides a true three-dimensional view of the surface and also the mechanical properties of EV particles [110]. AFM allows the determination of size distribution and analysis of membrane protein composition while characterizing the mechanical properties of EVs [111]. Some immobilization methods [111] and imaging modes [109] for EV visualization by AFM are summarized in Fig. 8. Specifically, the workflow of EV imaging using AFM was well described in this report [112]. LeClaire et al. [113] directly compared the structure

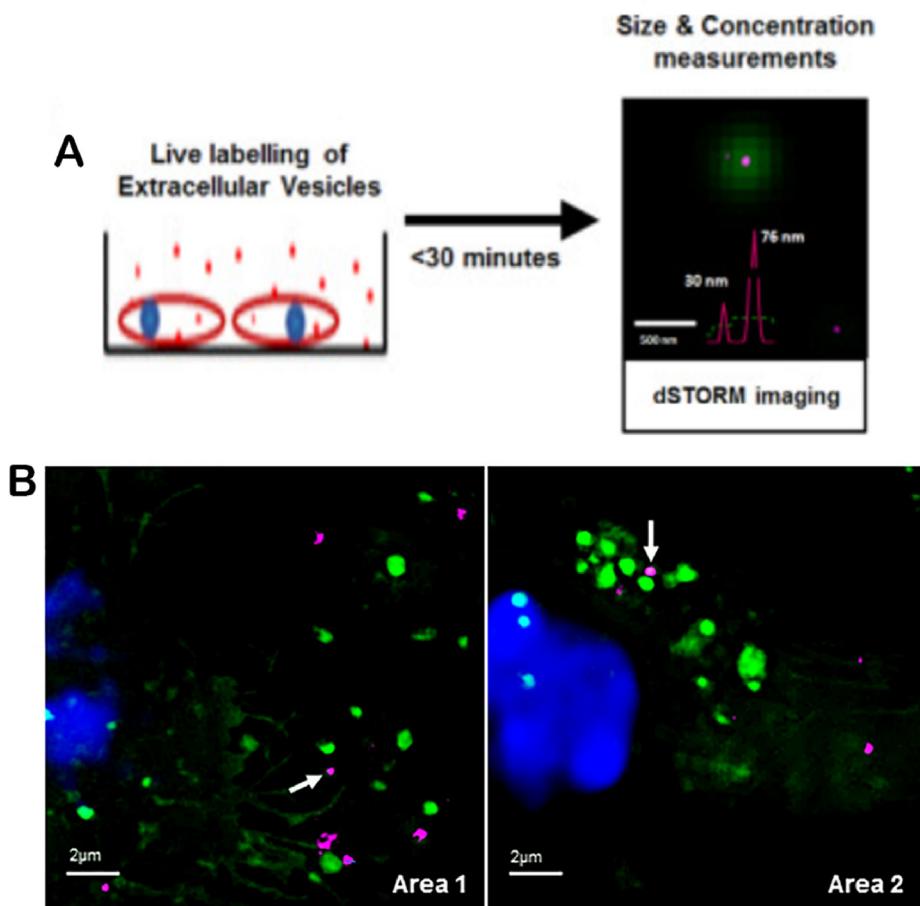


Fig. 7. Rapid and accurate analysis of stem cell-derived EVs with super resolution microscopy and live imaging. **A)** The workflow of analysis and live imaging. **B)** Imaging of EVs (red) uptake by neural stem cells (green). Adapted from Ref. [103] with permission from the Elsevier, Copyright 2018.

and mechanical properties of breast cancer cell-derived EVs and parent cells by AFM, which shows the application potential of AFM in single EV characterization. Similarly, Ye et al. [114] established relationship between the nanomechanical signature of the EV and tumor malignancy after using AFM to quantitatively investigate the nanomechanical properties of human breast cancer cell-derived EVs at single vesicle level. These findings pave the way for better understanding cancer mechanobiology. Moreover, AFM also were used to reveal the biomechanical properties of EV from blood plasma [115] at single particle level in air and in liquid medium. In short, AFM is the main characterization tool for obtaining EV mechanical properties at single vesicle level.

4.4. Other techniques

Electron microscopy (EM) is a non-optical method to analyze the size and morphology of EV sample. Since microscopic particles have wave-particle duality, the motion of electrons in a magnetic field is similar to the propagation of light in a medium. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are commonly used to characterize EVs, and TEM can also be combined with immunogold techniques for the identification of proteins on the surface of EVs. Under SEM, TEM, the single EVs show the classical cup-shape morphology [116–118]. SEM is based on the electron beam to scan the surface of the sample, generating feedback signals through the interaction between electrons and atoms in the sample, providing information on the elemental

composition of the sample surface and the three-dimensional morphology with high resolution. TEM reflects the information of the sample through the ultra-thin sample by electron beam, and can analyze the internal ultrastructure of the sample, giving a 2D projection of the internal structure. Although these two electron microscopes have high resolution, they can only observe conductive samples in vacuum, and the sample preparation process must be fixed, dehydrated, and stained, which will affect the EV morphology to a certain extent and cannot present the natural morphological characteristics of EV in full, and cannot observe EVs in special environments such as liquids. With the development of technology, cryoelectron microscopy (CEM) allows the analysis of frozen sample structures at ultra-low temperatures, enabling direct observation of liquid, semi-liquid and electron beam sensitive samples. The EVs characterized by cryoelectron microscopic method show a circular structure, indicating that dehydration can have an effect on the morphology of EVs [119]. Nanoparticle tracking analysis (NTA) is a method commonly used to rapidly assess the size and concentration of EVs, and works by focusing the EVs on a laser beam to produce scattered light, which is focused by the microscope onto the camera's sensor tracking each particle and calculates the particle size using a special formula [120]. However, NTA has the disadvantage of non-specificity in EV characterization in that it cannot differentiate all particles even other than EVs, whose size is within the detection range. The advent of nanoparticle tracking analysis with fluorescence has made it possible to solve this problem. Park et al. [121] demonstrated a fluorescence-

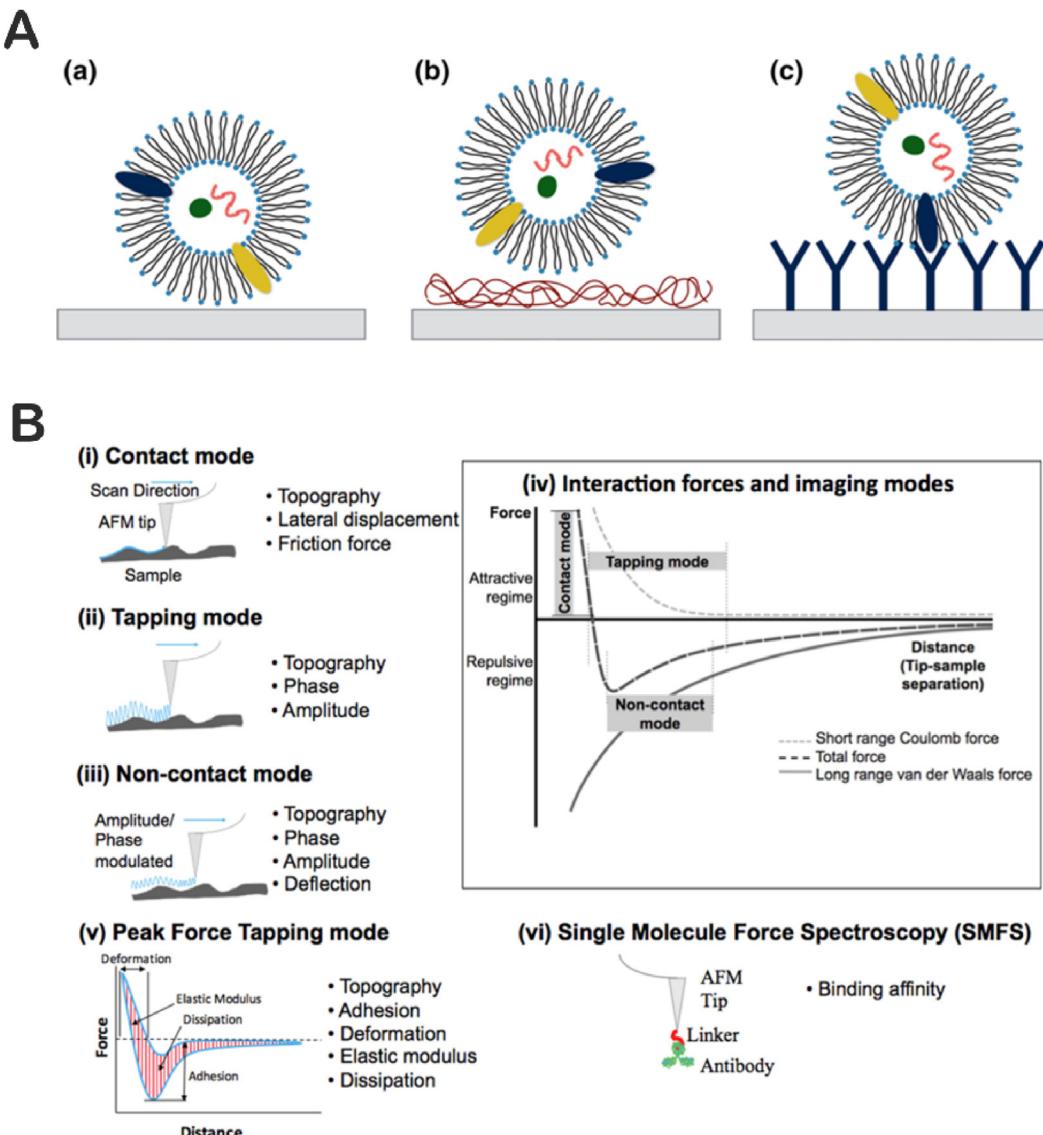


Fig. 8. Some immobilization methods and imaging modes for EV visualization by AFM are summarized. **A)** The immobilization methods for EV visualization by AFM. Adapted from Ref. [111] with permission from the Springer, Copyright 2017. **B)** Some commonly used AFM imaging modes. Adapted from Ref. [109] with permission from the IOPscience, Copyright 2018.

based NTA system for the characterization of both the size and membrane protein expression of single EV.

5. Single-cell strategy for the study of heterogeneity on EVs

The progress of EV research is hampered by heterogeneity; nonetheless, revealing heterogeneity between EV individuals might clarify the physiological activities of EVs *in vivo*. This will further promote the progress of basic research on EVs and accelerate the development of EVs for clinical applications. For cancer, the study of EVs heterogeneity has great significance for both diagnosis and therapy. Specifically, in clinical cancer diagnosis, clear information on EVs heterogeneity can help to accurately use biomarkers to improve diagnostic accuracy and implement good prognostic monitoring. In addition, in clinical cancer treatment, clear information on heterogeneity can help to select appropriate drugs to improve treatment efficiency and reduce cancer recurrence. Tan et al. [67] investigated serum EV heterogeneity using nucleic acid aptamers for surface proteome profiling. Although this

experiment has been used in elucidating heterogeneity, the EVs in the study were in a mixed state and it was not clear that each EV was secreted from which cell. Heterogeneity exists between EVs of different cell sources. For example, proteomic and lipidomic analysis of exosomes and microvesicles from U87 glioblastoma cells, Huh 7 hepatocellular carcinoma cells and human bone marrow-derived mesenchymal stem cells (MSCs) were different [122]. In addition, heterogeneity exists between EVs of different cell subtype. For example, M0-, M1- or M2-like macrophages, three subtypes of monocyte-derived macrophages (MDMs), secreted EVs behave differently in terms of both size characterization and proteomic analysis [33]. Therefore, there is a great need to study EV heterogeneity based on single cell strategy. Moreover, heterogeneity exists between different EV subtypes. For example, small-EVs contain higher content of phosphoglycerolipids, glycerolipids and fatty acids than large-EVs. On the contrary, large-EVs were comprised of more sphingolipids, amino acids and derivatives, organic acids, steroids, amides and others compared to small-EVs [16]. This implies that further studies at the single EV level in

single cells are of great importance.

Microfluidic chips are suitable for single-cell-based heterogeneity studies [123], because they can isolate and culture single cells to achieve the separation and analysis of EVs in microfluidics for precise information. Han et al. [124] developed an integrated microfluidic platform consisting of a single cell capture chip and a spatially encoded EV antibody barcode chip, which identified a specific functional cell subpopulation of unique phenotypic exosomes (HSP70, EPCAM) within ovarian tumor cells. This implies that this platform has great potential in the exploration of single cell biomarkers in biological and clinical research (Fig. 9). This integration strategy also emerged in Lu's study [63]. They revealed the complex heterogeneity of EV secretion by profiling EVs secreted from single human OSCC (SCC25) cell using a self-developed microfluidic platform. Specifically, they performed a statistical analysis of EVs secreted from single cell using EV surface marker proteins (CD63, CD9, CD81, EpCAM, and HSP70). The results suggest that the complex heterogeneity of EV secretion. Moreover, Dittrich et al. [55] also investigated the heterogeneity of EVs' secretion by the single-cell EV microfluidic platform. The results suggest that single cells prefer to secrete certain subtype of EVs. For example, the ANXA5⁺HSP70⁺TSG101⁺CD63⁺ subtype accounted for the largest proportion, followed by the CD81⁺CD63⁺ subtype. This result reinforces the existence of heterogeneity in EV secretion. In general, although EVs have great potential in both basic research and clinical applications, EV heterogeneity is now a challenge in the EV field. Studying EV heterogeneity using a single-cell strategy will yield precise biological information.

6. Conclusion and perspective

Since the field of single-cell EV research is in infancy, the current research methods and technologies in this field are in rapid development. The characteristics of the microfluidic platforms are

very consistent with single-cell research, holding great potential in the field of single-cell EVs. In this review, we have surveyed microfluidic methods for single-cell isolation and culture, and highlighted the numerous advantages of microfluidics and a summary of single-cell EV isolation methods aimed at providing a microfluidic platform design inspiration for the beginners in this field. In order to study single-cell EVs, it is an important step to isolate individual cells and maintain normal physiological activity for a specific period of time. Microfluidics allow cells to exist in cubicles and secrete EVs. In order to ensure that there is as much as one cell in each compartment, it is also necessary to control the culture time to prevent cell proliferation. The immunoaffinity may be used to capture EVs, but it has certain disadvantages. Firstly, the isolation purity is low, and secondly, the binding of EVs to antibodies also takes longer time and relatively expensive approach. In addition, some EVs do not have characteristic surface markers, they may not be captured, thus potentially causing EV loss. Hopefully, there will be more methods for capturing single-cell EVs according to the physical characteristics of EVs, or combining physical and chemical methods. Once a single-cell EV has been captured, its physical and chemical characteristics are studied.

Subsequently, state-of-art technologies were reviewed for single-cell and single particle EV study e. g. fluorescence, super-resolution microscopy (STED, PALM, STORM), RS, AFM, NanoFCM. The combination of these techniques with microfluidics will achieve the integration of single cell culture, EV collection, and EV characterization. Some detection methods, such as fluorescence techniques, have high-sensitive but photobleaching of the fluorescence probes during analysis. AFM, can detect very small particles, but the process is cumbersome, difficult to operate and requires professional operation, but it can expose the physical properties of individual EVs. Although it cannot perform high-throughput detection, it is a more suitable method for studying single-cell exosomes because of the limited amount of EV secretion.

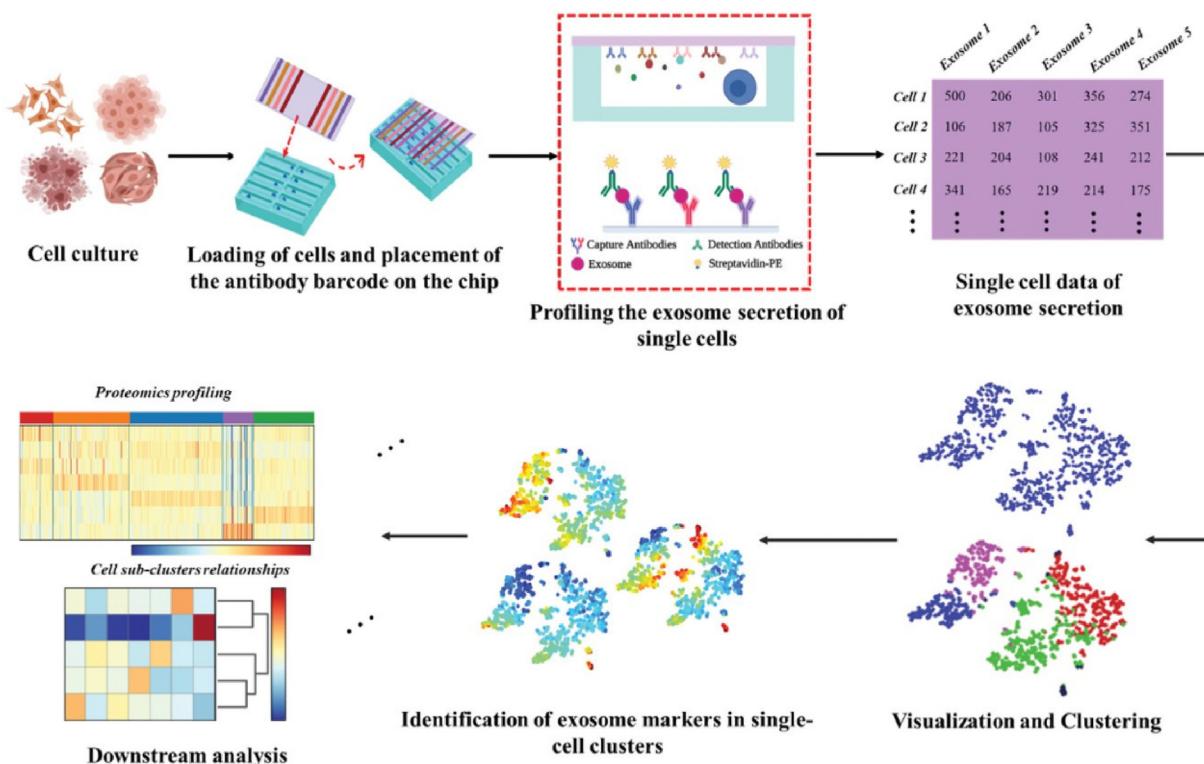


Fig. 9. Single-cell exosome analysis and exosome-based single-cell heterogeneity analysis. Adapted from Ref. [124] with permission from the Wiley, Copyright 2022.

Notably, the quality of the sample preparation has a considerable impact on the results of EVs analysis. RS is label-free, noninvasive, highly sensitive and specific tool and its coupling with microfluidics to analyze single-cell EVs is a very promising research field. After fixing a single-cell EV, it is irradiated with a Raman laser to detect the Raman signal of a single particle and characterize it, but the single EV is small and the marker content is low, so the Raman signal is needed to be enhanced. NanoFCM enables accurate counting of single EVs, as well as analysis and sorting of EV sub-populations, for high-throughput, multi-parameter detection of EVs. Mass spectrometry has the potential to analyze the biochemical components of EVs from single-cell due to its high sensitivity and ability to obtain structural information about the substance, however, ideal analytical results require sophisticated miniaturized sample preparation and advanced mass analyzer. Finally, we investigated the current status of heterogeneity studies of single-cell EV based on microfluidics platform. Studying EV at the single-cell level makes it easier to understand the intercellular heterogeneity of evictions numbers and phenotypes in different cells, which will help drive EVs research and applications. We expect more scholars to study the heterogeneity of single-cell EVs through microfluidic methods, comprehensively characterize single-cell EVs with a variety of methods, learn from each other's strengths, take the advantages of existing methods, and improve traditional methods. For the strategy, EV are loaded with rich biological information, such as miRNAs, mRNAs, metabolites, and proteins. Currently, most studies on single-cell EVs focus on the analysis of surface proteins [55,60,61,63], and although the identification of surface proteins of EVs is an important step in understanding heterogeneity, it is not sufficient to comprehensively evaluate EVs. Therefore, multi-dimensional analysis of EVs is one of the future directions of single-cell EVs research based on microfluidic platform.

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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References

- [1] M. Colombo, G. Raposo, C. Théry, Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles, *Annu. Rev. Cell Dev. Biol.* 30 (2014) 255–289. <https://doi.org/10.1146/annurev-cellbio-101512-122326>.
- [2] M. Mathieu, L. Martin-Jaulard, G. Lavieu, C. Théry, Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication, *Nat. Cell Biol.* 21 (2019) 9–17. <https://doi.org/10.1038/s41556-018-0250-9>.
- [3] E. van der Pol, A.N. Böing, P. Harrison, A. Sturk, R. Nieuwland, Classification, functions, and clinical relevance of extracellular vesicles, *Pharmacol. Rev.* 64 (2012) 676–705. <https://doi.org/10.1124/pr.112.005983>.
- [4] R. Kalluri, V.S. LeBleu, The biology, function, and biomedical applications of exosomes, *Science* 367 (2020) aaau6977. <https://doi.org/10.1126/science.aaau6977>.
- [5] C. Théry, K.W. Witwer, E. Aikawa, M.J. Alcaraz, J.D. Anderson, R. Andriantsitohaina, A. Antoniou, T. Arab, F. Archer, G.K. Atkin-Smith, D.C. Ayre, J.M. Bach, D. Bachurski, H. Baharvand, L. Balaj, S. Baldacchino, N.N. Bauer, A.A. Baxter, M. Bebawy, C. Beckham, A. Bedina Zavec, A. Benmoussa, A.C. Berardi, P. Bergese, E. Bielska, C. Blenkiron, S. Bobis-Wozowicz, E. Boillard, W. Boireau, A. Bongiovanni, F.E. Borràs, S. Bosch, C.M. Boulanger, X. Breakefield, A.M. Breglio, M. Brennan, D.R. Brigstock, A. Brisson, M.L.D. Broekman, J.F. Bromberg, P. Bryl-Górecka, S. Buch, A.H. Buck, D. Burger, S. Busatto, D. Buschmann, B. Bussolati, E.I. Buzás, J.B. Byrd, G. Camussi, D.R.F. Carter, S. Caruso, L.W. Chamley, Y.T. Chang, A.D. Chaudhuri, C. Chen, S. Chen, L. Cheng, A.R. Chin, A. Clayton, S.P. Clerici, A. Cocks, E. Cocucci, R.J. Coffey, A. Cordeiro-da-Silva, Y. Couch, F.A.W. Coumans, B. Coyle, R. Crescitelli, M.F. Criado, C. D'Souza-Schorey, S. Das, P. de Candia, E.F. De Santana, O. De Wever, H.A. del Portillo, T. Demaret, S. Deville, A. Devitt, B. Dhondt, D. Di Vizio, L.C. Dieterich, V. Dolo, A.P. Dominguez Rubio, M. Dominici, M.R. Dourado, T.A.P. Driedonks, F.V. Duarte, H.M. Duncan, R.M. Eichenberger, K. Ekström, S. El Andaloussi, C. Elie-Caille, U. Erdbrügger, J.M. Falcón-Pérez, F. Fatima, J.E. Fish, M. Flores-Bellver, A. Förösöni, A. Frelet-Barrand, F. Fricke, G. Fuhrmann, S. Gabrielson, A. Gámez-Valero, C. Gardiner, K. Gärtner, R. Gaudin, Y.S. Gho, B. Giebel, C. Gilbert, M. Gimona, I. Giusti, D.C.I. Goberdhan, A. Görgens, S.M. Gorski, D.W. Greening, J.C. Gross, A. Gualerzi, G.N. Gupta, D. Gustafson, A. Handberg, R.A. Haraszti, P. Harrison, H. Hegyesi, A. Hendrix, A.F. Hill, F.H. Hochberg, K.F. Hoffmann, B. Holder, H. Holthofer, B. Hosseinkhani, G. Hu, Y. Huang, V. Huber, S. Hunt, A.G.E. Ibrahim, T. Ikezu, J.M. Inal, M. Isin, A. Ivanova, H.K. Jackson, S. Jacobsen, S.M. Jay, M. Jayachandran, G. Jenster, L. Jiang, S.M. Johnson, J.C. Jones, A. Jong, T. Jovanovic-Talisman, S. Jung, R. Kalluri, S. ichi Kano, S. Kaur, Y. Kawamura, E.T. Keller, D. Khamari, E. Khomyakova, A. Khorrova, P. Kierulf, K.P. Kim, T. Kislinger, M. Klingeborn, D.J. Klinke, M. Kornek, M.M. Kosanović, Á.F. Kovács, E.M. Krämer-Albers, S. Krasemann, M. Krause, I.V. Kurochkin, G.D. Kusuma, S. Kuypers, S. Laitinen, S.M. Langevin, L.R. Languino, J. Lannigan, C. Lässer, L.C. Laurent, G. Lavieu, E. Lázaro-Ibáñez, S. Le Lay, M.S. Lee, Y.X.F. Lee, D.S. Lemos, M. Lenassi, A. Leszczynska, I.T.S. Li, K. Liao, S.F. Libregts, E. Ligeti, R. Lim, S.K. Lim, A. Liné, K. Linnemannstöns, A. Llorente, C.A. Lombard, M.J. Lorenzen, Á.M. Lörincz, J. Lötvall, J. Lovett, M.C. Lowry, X. Loyer, Q. Lu, B. Lukomska, T.R. Lunavat, S.L.N. Maas, H. Malhi, A. Marcilla, J. Mariani, J. Mariscal, E.S. Martens-Uzunova, L. Martin-Jaulard, M.C. Martinez, V.R. Martins, M. Mathieu, S. Mathivanan, M. Magueri, L.K. McGinnis, M.J. McVey, D.G. Meckes, K.L. Meehan, I. Mertens, V.R. Minciachchi, A. Möller, M. Møller Jørgensen, A. Morales-Kastresana, J. Morhayim, F. Mullier, M. Muraca, L. Musante, V. Mussack, D.C. Muth, K.H. Myburgh, T. Najrana, M. Nawaz, I. Nazarenko, P. Nejsum, C. Neri, T. Neri, R. Nieuwland, L. Nimrichter, J.P. Nolan, E.N.M. Nolte-t Hoen, N. Noren Hooten, L. O'Driscoll, T. O'Grady, A. O'Loughlin, T. Ochiya, M. Olivier, A. Ortiz, L.A. Ortiz, X. Osteikoetxea, O. Ostegard, M. Ostrowski, J. Park, D.M. Pegtel, H. Peinado, F. Perut, M.W. Pfaffl, D.G. Phinney, B.C.H. Pieters, R.C. Pink, D.S. Pisetsky, E. Pogge von Strandmann, I. Polakovicova, I.K.H. Poon, B.H. Powell, I. Prada, L. Pulliam, P. Quesenberry, A. Radeghieri, R.L. Raffai, S. Raimondo, J. Rak, M.I. Ramirez, G. Raposo, M.S. Rayyan, N. Regev-Rudzki, F.L. Ricklef, P.D. Robbins, D.D. Roberts, S.C. Rodrigues, E. Rohde, S. Rome, K.M.A. Rouschop, A. Rughetti, A.E. Russell, P. Saá, S. Sahoo, E. Salas-Huenuleo, C. Sánchez, J.A. Saugstad, M.J. Saul, R.M. Schiffeler, R. Schneide, T.H. Schøyen, A. Scott, E. Shahaj, S. Sharma, O. Shatnyeva, F. Shekari, G.V. Shelke, A.K. Shetty, K. Shiba, P.R.M. Siljander, A.M. Silva, A. Skowronek, O.L. Snyder, R.P. Soares, B.W. Sódar, C. Soekmadji, J. Sotillo, P.D. Stahl, W. Stoovogel, S.L. Stott, E.F. Strasser, S. Swift, H. Tahara, M. Tewari, K. Timms, S. Tiwari, R. Tixeira, M. Tkach, W.S. Toh, R. Tomasini, A.C. Torrecillas, J.P. Tosar, V. Toxavidis, L. Urbanelli, P. Vader, B.W.M. van Balkom, S.G. van der Grein, J. Van Deun, M.J.C. van Herwijnen, K. Van Keuren-Jensen, G. van Niel, M.E. van Royen, A.J. van Wijnen, M.H. Vasconcelos, I.J. Vechetti, T.D. Veit, L.J. Vella, E. Vélot, F.J. Verweij, B. Vestad, J.L. Vinas, T. Visnovitz, K.V. Vukman, J. Wahlgren, D.C. Watson, M.H.M. Wauben, A. Weaver, J.P. Webber, V. Weber, A.M. Wehman, D.J. Weiss, J.A. Welsh, S. Wendt, A.M. Wheelock, Z. Wiener, L. Witte, J. Wolfram, A. Xagorari, P. Xander, J. Xu, X. Yan, M. Yáñez-Mó, H. Yin, Y. Yuana, V. Zappulli, J. Zarubova, V. Žekas, J. ye Zhang, Z. Zhao, L. Zheng, A.R. Zheutlin, A.M. Zickler, P. Zimmermann, A.M. Živkovic, D. Zocco, E.K. Zuba-Surma, Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines, *J. Extracell. Vesicles* 7 (2018), 1535750. <https://doi.org/10.1080/20013078.2018.1535750>.
- [6] M. Tkach, C. Théry, Communication by extracellular vesicles: where we are and where we need to go, *Cell* 164 (2016) 1226–1232. <https://doi.org/10.1016/j.cell.2016.01.043>.
- [7] C. Jiang, Y. Fu, G. Liu, B. Shu, J. Davis, G.K. Tofaris, Multiplexed profiling of extracellular vesicles for biomarker development, *Nano-Micro Lett.* 14 (2022) 3. <https://doi.org/10.1007/s40820-021-00753-w>.
- [8] L. Cheng, A.F. Hill, Therapeutically harnessing extracellular vesicles, *Nat. Rev. Drug Discov.* 21 (2022) 379–399. <https://doi.org/10.1038/s41573-022-00410-w>.
- [9] M. Pirisinu, T.C. Pham, D.X. Zhang, T.N. Hong, L.T. Nguyen, M.T. Le, Extracellular vesicles as natural therapeutic agents and innate drug delivery systems for cancer treatment: recent advances, current obstacles, and challenges for clinical translation, *Semin. Cancer Biol.* 80 (2022) 340–355. <https://doi.org/10.1016/j.semancer.2020.08.007>.
- [10] K. Burbidge, V. Zwikelmaier, B. Cook, M.M. Long, B. Balva, M. Lonigro,

- G. Ispas, D.J. Rademacher, E.M. Campbell, Cargo and cell-specific differences in extracellular vesicle populations identified by multiplexed immunofluorescent analysis, *J. Extracell. Vesicles* 9 (2020), 1789326. <https://doi.org/10.1080/20013078.2020.1789326>.
- [11] E. Willms, C. Cabañas, I. Mäger, M.J.A. Wood, P. Vader, Extracellular vesicle heterogeneity: subpopulations, isolation techniques, and diverse functions in cancer progression, *Front. Immunol.* 9 (2018), 00738. <https://doi.org/10.3389/fimmu.2018.00738>.
- [12] T. Vagner, A. Chin, J. Mariscal, S. Bannykh, D.M. Engman, D. Di Vizio, Protein composition reflects extracellular vesicle heterogeneity, *Proteomics* 19 (2019), 1800167. <https://doi.org/10.1002/pmic.201800167>.
- [13] A. Rai, H. Fang, B. Claridge, R.J. Simpson, D.W. Greening, Proteomic dissection of large extracellular vesicle surfaceome unravels interactive surface platform, *J. Extracell. Vesicles* 10 (2021), 12164. <https://doi.org/10.1002/jev.2.12164>.
- [14] J. Kowal, G. Arras, M. Colombo, M. Jouve, J.P. Morath, B. Primdal-Bengtson, F. Dingli, D. Loew, M. Tkach, C. Théry, Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes, *Proc. Natl. Acad. Sci. U. S. A* 113 (2016) 968–977. <https://doi.org/10.1073/pnas.1521230113>.
- [15] E.S. Martens-Uzunova, G.D. Kusuma, S. Crucitta, H.K. Lim, C. Cooper, J.E. Riches, A. Azad, T. Ochiya, G.M. Boyle, M.C. Southey, M. Del Re, R. Lim, G.A. Ramm, G.W. Jenster, C. Soekmadji, Androgens alter the heterogeneity of small extracellular vesicles and the small RNA cargo in prostate cancer, *J. Extracell. Vesicles* 10 (2021), 12136. <https://doi.org/10.1002/jev.2.12136>.
- [16] P. Luo, K. Mao, J. Xu, F. Wu, X. Wang, S. Wang, M. Zhou, L. Duan, Q. Tan, G. Ma, G. Yang, R. Du, H. Huang, Q. Huang, Y. Li, M. Guo, Y. Jin, Metabolic characteristics of large and small extracellular vesicles from pleural effusion reveal biomarker candidates for the diagnosis of tuberculosis and malignancy, *J. Extracell. Vesicles* 9 (2020), 1790158. <https://doi.org/10.1080/20013078.2020.1790158>.
- [17] F. Kolonics, V. Szefert, C.I. Timár, E. Ligeti, Á.M. Lörincz, The functional heterogeneity of neutrophil-derived extracellular vesicles reflects the status of the parent cell, *Cells* 9 (2020) 2718. <https://doi.org/10.3390/cells9122718>.
- [18] C. Almeria, S. Kreß, V. Weber, D. Egger, C. Kasper, Heterogeneity of mesenchymal stem cell-derived extracellular vesicles is highly impacted by the tissue/cell source and culture conditions, *Cell Biosci.* 12 (2022) 51. <https://doi.org/10.1186/s13578-022-00786-7>.
- [19] A. Torres Crigna, F. Fricke, K. Nitschke, T. Worst, U. Erb, M. Karremann, D. Buschmann, S. Elvers-Hornung, C. Tucher, M. Schiller, I. Hausser, J. Gebert, K. Bieback, Inter-laboratory comparison of extracellular vesicle isolation based on ultracentrifugation, *Transfus. Med. Hemotherapy* 48 (2021) 48–59. <https://doi.org/10.1159/000508712>.
- [20] C. Peng, J. Wang, Q. Bao, J. Wang, Z. Liu, J. Wen, W. Zhang, Y. Shen, Isolation of extracellular vesicle with different precipitation-based methods exerts a tremendous impact on the biomarker analysis for clinical plasma samples, *Cancer Biomarkers* 29 (2020) 373–385. <https://doi.org/10.3233/CBM-201651>.
- [21] E.J.K. Kowal, D. Ter-Ovanesyan, A. Regev, G.M. Church, Extracellular vesicle isolation and analysis by western blotting, *Methods Mol. Biol.* 1660 (2017) 143–152. https://doi.org/10.1007/978-1-4939-7253-1_12.
- [22] G. Wu, H. Geng, R. Xu, M. Deng, C. Yang, C. Xun, Y. Wang, Q. Cai, P. Chen, Preparation of a CaTiO₃/Al₃₊/Pr₃₊/Sm₃₊ nanocomposite for enrichment of exosomes in human serum, *Talanta* 226 (2021), 122186. <https://doi.org/10.1016/j.talanta.2021.122186>.
- [23] F.A.W. Coumans, E.L. Gool, R. Nieuwland, Bulk immunoassays for analysis of extracellular vesicles, *Platelets* 28 (2017) 242–248. <https://doi.org/10.1080/09537104.2016.1265926>.
- [24] Y. Dai, Y. Cao, J. Köhler, A. Lu, S. Xu, H. Wang, Unbiased RNA-Seq-driven identification and validation of reference genes for quantitative RT-PCR analyses of pooled cancer exosomes, *BMC Genom.* 22 (2021) 27. <https://doi.org/10.1186/s12864-020-07318-y>.
- [25] F. Tian, C. Liu, L. Lin, Q. Chen, J. Sun, Microfluidic analysis of circulating tumor cells and tumor-derived extracellular vesicles, *TrAC, Trends Anal. Chem.* 117 (2019) 128–145. <https://doi.org/10.1016/j.trac.2019.05.013>.
- [26] W. Su, H. Li, W. Chen, J. Qin, Microfluidic strategies for label-free exosomes isolation and analysis, *TrAC, Trends Anal. Chem.* 118 (2019) 686–698. <https://doi.org/10.1016/j.trac.2019.06.037>.
- [27] S. Cheng, Y. Li, H. Yan, Y. Wen, X. Zhou, L. Friedman, Y. Zeng, Advances in microfluidic extracellular vesicle analysis for cancer diagnostics, *Lab Chip* 21 (2021) 3219–3243. <https://doi.org/10.1039/d1lc00443c>.
- [28] B. Lin, Y. Lei, J. Wang, L. Zhu, Y. Wu, H. Zhang, L. Wu, P. Zhang, C. Yang, Microfluidic-based exosome analysis for liquid biopsy, *Small Methods* 5 (2021), 2001131. <https://doi.org/10.1002/smtd.202001131>.
- [29] C.M. Abreu, B. Costa-Silva, R.L. Reis, S.C. Kundu, D. Caballero, Microfluidic platforms for extracellular vesicle isolation, analysis and therapy in cancer, *Lab Chip* 22 (2022) 1093–1125. <https://doi.org/10.1039/d2lc00066g>.
- [30] D. Angraini, N. Ota, Y. Shen, T. Tang, Y. Tanaka, Y. Hosokawa, M. Li, Y. Yalikun, Recent advances in microfluidic devices for single-cell cultivation: methods and applications, *Lab Chip* 22 (2022) 1438–1468. <https://doi.org/10.1039/d1lc01030a>.
- [31] L. Ding, P. Radfar, M. Rezaei, M.E. Warkiani, An easy-to-operate method for single-cell isolation and retrieval using a microfluidic static droplet array, *Microchim. Acta* 188 (2021) 242. <https://doi.org/10.1007/s00604-021-04897-9>.
- [32] H. Nakaoka, Y. Wakamoto, Aging, mortality, and the fast growth trade-off of *Schizosaccharomyces pombe*, *PLoS Biol.* 15 (2017), 2001109. <https://doi.org/10.1371/journal.pbio.2001109>.
- [33] S. Dechantsreiter, A.R. Ambrose, J.D. Worboys, J.M.E. Lim, S. Liu, R. Shah, M.A. Montero, A.M. Quinn, T. Hussell, G.M. Tannahill, D.M. Davis, Heterogeneity in extracellular vesicle secretion by single human macrophages revealed by super-resolution microscopy, *J. Extracell. Vesicles* 11 (2022), 12215. <https://doi.org/10.1002/jev.2.12215>.
- [34] Z. Chen, B. Chen, M. He, B. Hu, Negative magnetophoresis focusing micro-chips online-coupled with ICP-MS for high-throughput single-cell analysis, *Anal. Chem.* 94 (2021) 6649–6656. <https://doi.org/10.1021/acs.analchem.1c04216>.
- [35] Z. Bai, Y. Deng, D. Kim, Z. Chen, Y. Xiao, R. Fan, An integrated dielectrophoresis-trapping and nanowell transfer approach to enable double-sub-Poisson single-cell RNA sequencing, *ACS Nano* 14 (2020) 7412–7424. <https://doi.org/10.1021/acsnano.0c02953>.
- [36] J. Lamanna, E.Y. Scott, H.S. Edwards, M.D. Chamberlain, M.D.M. Dryden, J. Peng, B. Mair, A. Lee, C. Chan, A.A. Sklavounos, A. Heffernan, F. Abbas, C. Lam, M.E. Olson, J. Moffat, A.R. Wheeler, Digital microfluidic isolation of single cells for -omics, *Nat. Commun.* 11 (2020) 5632. <https://doi.org/10.1038/s41467-020-19394-5>.
- [37] F. Ahmadi, K. Samlali, P.Q.N. Vo, S.C.C. Shih, An integrated droplet-digital microfluidic system for on-demand droplet creation, mixing, incubation, and sorting, *Lab Chip* 19 (2019) 524–535. <https://doi.org/10.1039/c8lc01170b>.
- [38] K. Hattori, Y. Goda, M. Yamashita, Y. Yoshioka, R. Kojima, S. Ota, Droplet array-based platform for parallel optical analysis of dynamic extracellular vesicle secretion from single cells, *Anal. Chem.* 94 (2022) 11209–11215. <https://doi.org/10.1021/acs.analchem.2c01609>.
- [39] X.D. Zhu, J. Chu, Y.H. Wang, Advances in microfluidics applied to single cell operation, *Biotechnol. J.* 13 (2018), 1700416. <https://doi.org/10.1002/biot.201700416>.
- [40] D. Liu, M. Sun, J. Zhang, R. Hu, W. Fu, T. Xuanyuan, W. Liu, Single-cell droplet microfluidics for biomedical applications, *Analyst* 147 (2022) 2294–2316. <https://doi.org/10.1039/dian02321g>.
- [41] M. Nooranidoost, R. Kumar, Geometry effects of axisymmetric flow-focusing microchambers for single cell encapsulation, *Materials* 12 (2019) 2811. <https://doi.org/10.3390/ma12172811>.
- [42] C.E. Lagerman, S.N. López Acevedo, A.S. Fahad, A.T. Hailemariam, B. Madan, B.J. DeKosky, Ultrasonically-guided flow focusing generates precise emulsion droplets for high-throughput single cell analyses, *J. Biosci. Bioeng.* 128 (2019) 226–233. <https://doi.org/10.1016/j.jbiobioeng.2019.01.020>.
- [43] T. Jiang, Y. Wu, Controlled generation of droplets using an electric field in a flow-focusing paper-based device, *Electrophoresis* 43 (2022) 601–608. <https://doi.org/10.1002/elps.2020100245>.
- [44] M. Navi, N. Abbasi, M. Jeyhani, V. Gnyawali, S.S.H. Tsai, Microfluidic diamagnetic water-in-water droplets: a biocompatible cell encapsulation and manipulation platform, *Lab Chip* 18 (2018) 3361–3370. <https://doi.org/10.1039/C8LC00867A>.
- [45] R. Hu, P. Liu, P. Chen, L. Wu, Y. Wang, X. Feng, B.F. Liu, Encapsulation of single cells into monodisperse droplets by fluorescence-activated droplet formation on a microfluidic chip, *Talanta* 153 (2016) 253–259. <https://doi.org/10.1016/j.talanta.2016.03.013>.
- [46] Z. Yin, Z. Huang, X. Lin, X. Gao, F. Bao, Droplet generation in a flow-focusing microfluidic device with external mechanical vibration, *Micromachines* 11 (2020) 743. <https://doi.org/10.3390/mi11080743>.
- [47] S. Kobel, A. Valero, J. Latt, P. Renaud, M. Lutolf, Optimization of microfluidic single cell trapping for long-term on-chip culture, *Lab Chip* 10 (2010) 857–863. <https://doi.org/10.1039/b918055a>.
- [48] B. Deng, H. Wang, Z. Tan, Y. Quan, Microfluidic cell trapping for single-cell analysis, *Micromachines* 10 (2019) 409. <https://doi.org/10.3390/mi10060409>.
- [49] V. Narayananamurthy, S. Nagarajan, A.Y. Firuz Khan, F. Samsuri, T.M. Sridhar, Microfluidic hydrodynamic trapping for single cell analysis: mechanisms, methods and applications, *Anal. Methods* 9 (2017) 3751–3772. <https://doi.org/10.1039/C7AY00656J>.
- [50] Y. Deng, Y. Guo, B. Xu, Recent development of microfluidic technology for cell trapping in single cell analysis: a review, *Processes* 8 (2020) 1253. <https://doi.org/10.3390/pr8101253>.
- [51] S.L. Faley, M. Copland, D. Włodkowic, W. Kolch, K.T. Seale, J.P. Wikswo, J.M. Cooper, Microfluidic single cell arrays to interrogate signalling dynamics of individual, patient-derived hematopoietic stem cells, *Lab Chip* 9 (2009) 2659–2664. <https://doi.org/10.1039/b902083g>.
- [52] S.L. Angione, N. Oulhen, L.M. Brayboy, A. Tripathi, G.M. Wessel, Simple perfusion apparatus for manipulation, tracking, and study of oocytes and embryos, *Fertil. Steril.* 103 (2015) 281–290. <https://doi.org/10.1016/j.fertnstert.2014.09.039>.
- [53] K. Zhang, X. Han, Y. Li, S.Y. Li, Y. Zu, Z. Wang, L. Qin, Hand-held and integrated single-cell pipettes, *J. Am. Chem. Soc.* 136 (2014) 10858–10861. <https://doi.org/10.1021/ja5053279>.
- [54] H. Zhang, M. Lu, Z. Xiong, J. Yang, M. Tan, L. Huang, X. Zhu, Z. Lu, Z. Liang, H. Liu, Rapid trapping and tagging of microparticles in controlled flow by in situ digital projection lithography, *Lab Chip* 22 (2022) 1951–1961. <https://doi.org/10.1039/d2lc00186a>.
- [55] J.M. Nikoloff, M.A. Saucedo-Espinosa, A. Kling, P.S. Dittrich, Identifying

- extracellular vesicle populations from single cells, Proc. Natl. Acad. Sci. U. S. A 118 (2021), 2106630118. <https://doi.org/10.1073/pnas.2106630118>.
- [56] Y. Sun, B. Cai, X. Wei, Z. Wang, L. Rao, Q.F. Meng, Q. Liao, W. Liu, S. Guo, X. Zhao, A valve-based microfluidic device for on-chip single cell treatments, *Electrophoresis* 40 (2019) 961–968. <https://doi.org/10.1002/ELPS.201800213>.
- [57] J. Briones, W. Espulgar, S. Koyama, H. Takamatsu, E. Tamiya, M. Saito, A design and optimization of a high throughput valve based microfluidic device for single cell compartmentalization and analysis, *Sci. Rep.* 11 (2021), 12995. <https://doi.org/10.1038/s41598-021-92472-w>.
- [58] Y. Moradi, M. Ibrahim, K. Chakrabarty, U. Schlichtmann, An efficient fault-tolerant valve-based microfluidic routing fabric for droplet barcoding in single-cell analysis, *IEEE Trans. Comput. Des. Integr. Circ. Syst.* 39 (2020) 359–372. <https://doi.org/10.1109/TCAD.2018.2889765>.
- [59] P. Li, M. Kaslan, S.H. Lee, J. Yao, Z. Gao, Progress in exosome isolation techniques, *Theranostics* 7 (2017) 789–804. <https://doi.org/10.7150/thno.18133>.
- [60] K.J. Son, A. Rahimian, D.S. Shin, C. Siltanen, T. Patel, A. Revzin, Microfluidic compartments with sensing microbeads for dynamic monitoring of cytokine and exosome release from single cells, *Analyst* 141 (2016) 679–688. <https://doi.org/10.1039/c5an01648g>.
- [61] Y.J. Chiu, W. Cai, Y.R.V. Shih, I. Lian, Y.H. Lo, A single-cell assay for time lapse studies of exosome secretion and cell behaviors, *Small* 12 (2016) 3658–3666. <https://doi.org/10.1002/smll.201600725>.
- [62] W. Cai, Y.J. Chiu, V. Ramakrishnan, Y. Tsai, C. Chen, Y.H. Lo, A single-cell translocation and secretion assay (TransSeA), *Lab Chip* 18 (2018) 3154–3162. <https://doi.org/10.1039/c8lc00821c>.
- [63] Y. Ji, D. Qi, L. Li, H. Su, X. Li, Y. Luo, B. Sun, F. Zhang, B. Lin, T. Liu, Y. Lu, Multiplexed profiling of single-cell extracellular vesicles secretion, *Proc. Natl. Acad. Sci. U. S. A* 116 (2019) 5979–5984. <https://doi.org/10.1073/pnas.1814348116>.
- [64] C. Liu, J. Zhao, F. Tian, J. Chang, W. Zhang, J. Sun, λ -DNA- and aptamer-mediated sorting and analysis of extracellular vesicles, *J. Am. Chem. Soc.* 141 (2019) 3817–3821. <https://doi.org/10.1021/jacs.9b00007>.
- [65] G. Ströhle, J. Gan, H. Li, Affinity-based isolation of extracellular vesicles and the effects on downstream molecular analysis, *Anal. Bioanal. Chem.* 414 (2022) 7051–7067. <https://doi.org/10.1007/s00216-022-04178-1>.
- [66] Z. Zhou, Y. Chen, X. Qian, Target-specific exosome isolation through aptamer-based microfluidics, *Biosensors* 12 (2022) 257. <https://doi.org/10.3390/bios12040257>.
- [67] C. Liu, J. Zhao, F. Tian, L. Cai, W. Zhang, Q. Feng, J. Chang, F. Wan, Y. Yang, B. Dai, Y. Cong, B. Ding, J. Sun, W. Tan, Low-cost thermophoretic profiling of extracellular-vesicle surface proteins for the early detection and classification of cancers, *Nat. Biomed. Eng.* 3 (2019) 183–193. <https://doi.org/10.1038/s41551-018-0343-6>.
- [68] Y. Ren, K. Ge, D. Sun, Z. Hong, C. Jia, H. Hu, F. Shao, B. Yao, Rapid enrichment and sensitive detection of extracellular vesicles through measuring the phospholipids and transmembrane protein in a microfluidic chip, *Biosens. Bioelectron.* 199 (2022), 113870. <https://doi.org/10.1016/j.bios.2021.113870>.
- [69] X. Dong, J. Chi, L. Zheng, B. Ma, Z. Li, S. Wang, C. Zhao, H. Liu, Efficient isolation and sensitive quantification of extracellular vesicles based on an integrated ExoID-Chip using photonic crystals, *Lab Chip* 19 (2019) 2897–2904. <https://doi.org/10.1039/c9lc00445a>.
- [70] J.T. Smith, B.H. Wunsch, N. Dogra, M.E. Ahsen, K. Lee, K.K. Yadav, R. Weil, M.A. Pereira, J.V. Patel, E.A. Duch, J.M. Papalia, M.F. Lofaro, M. Gupta, A.K. Tewari, C. Cordon-Cardo, G. Stolovitzky, S.M. Gifford, Integrated nanoscale deterministic lateral displacement arrays for separation of extracellular vesicles from clinically-relevant volumes of biological samples, *Lab Chip* 18 (2018) 3913–3925. <https://doi.org/10.1039/C8LC01017j>.
- [71] M. Wu, Y. Ouyang, Z. Wang, R. Zhang, P.H. Huang, C. Chen, H. Li, P. Li, D. Quinn, M. Dao, S. Suresh, Y. Sadovsky, T.J. Huang, Isolation of exosomes from whole blood by integrating acoustics and microfluidics, *Proc. Natl. Acad. Sci. U. S. A* 114 (2017) 10584–10589. <https://doi.org/10.1073/pnas.1709210114>.
- [72] S. Ayala-Mar, V.H. Perez-Gonzalez, M.A. Mata-Gómez, R.C. Gallo-Villanueva, J. González-Valdez, Electrokinetically driven exosome separation and concentration using dielectrophoretic-enhanced PDMS-based microfluidics, *Anal. Chem.* 91 (2019) 14975–14982. <https://doi.org/10.1021/acs.analchem.9b03448>.
- [73] C. Paganini, B. Hettich, M.R.G. Kopp, A. Eördögh, U. Capasso Palmiero, G. Adamo, N. Touzet, M. Manno, A. Bongiovanni, P. Rivera-Fuentes, J.C. Leroux, P. Arosio, Rapid characterization and quantification of extracellular vesicles by fluorescence-based microfluidic diffusion sizing, *Adv. Healthc. Mater.* 11 (2022), 2100021. <https://doi.org/10.1002/adhm.202100021>.
- [74] S.H. Hilton, I.M. White, Advances in the analysis of single extracellular vesicles: a critical review, *Sens. Actuators Rep.* 3 (2021), 100052. <https://doi.org/10.1016/j.snr.2021.100052>.
- [75] S. Qiu, Y. Weng, Y. Li, Y. Chen, Y. Pan, J. Liu, W. Lin, X. Chen, M. Li, T. Lin, W. Liu, L. Zhang, D. Lin, Raman profile alterations of irradiated human nasopharyngeal cancer cells detected with laser tweezer Raman spectroscopy, *RSC Adv.* 10 (2020) 14368–14373. <https://doi.org/10.1039/d0ra01173h>.
- [76] S.G. Kruglik, F. Royo, J.M. Guignier, L. Palomo, O. Seksek, P.Y. Turpin, I. Tatischeff, J.M. Falcón-Pérez, Raman tweezers microspectroscopy of: circa 100 nm extracellular vesicles, *Nanoscale* 11 (2019) 1661–1679. <https://doi.org/10.1039/c8nr04677h>.
- [77] S. Stremersch, M. Marro, B. El Pinchasik, P. Baatsen, A. Hendrix, S.C. De Smedt, P. Loza-Alvarez, A.G. Skirtach, K. Raemdonck, K. Braeckmans, Identification of individual exosome-like vesicles by surface enhanced Raman spectroscopy, *Small* 12 (2016) 3292–3301. <https://doi.org/10.1002/smll.201600393>.
- [78] D. Sun, F. Cao, Y. Tian, A. Li, W. Xu, Q. Chen, W. Shi, S. Xu, Label-free detection of multiplexed metabolites at single-cell level via a SERS-microfluidic droplet platform, *Anal. Chem.* 91 (2019) 15484–15490. <https://doi.org/10.1021/acs.analchem.9b03294>.
- [79] Y. Zhang, L. Wu, K. Yang, S. Zong, Z. Wang, Y. Cui, 2D profiling of tumor chemotactic and molecular phenotype at single cell resolution using a SERS-microfluidic chip, *Nano Res.* 15 (2022) 4357–4365. <https://doi.org/10.1007/s12274-022-4100-5>.
- [80] S. Kreimer, A.M. Belov, I. Ghiran, S.K. Murthy, D.A. Frank, A.R. Ivanov, Mass-spectrometry-based molecular characterization of extracellular vesicles: lipidomics and proteomics, *J. Proteome Res.* 14 (2015) 2367–2384. https://doi.org/10.1021/PR501279T/ASSET/IMAGES/MEDIUM/PR-2014-01279T_0002.GIF.
- [81] A. Ali, Y. Abouleila, Y. Shimizu, E. Hiyama, S. Emara, A. Mashaghi, T. Hankemeier, Single-cell metabolomics by mass spectrometry: advances, challenges, and future applications, *TrAC, Trends Anal. Chem.* 120 (2019), 115436. <https://doi.org/10.1016/j.trac.2019.02.033>.
- [82] S. Wang, I.A. Blair, C. Mesaros, Analytical methods for mass spectrometry-based metabolomics studies, *Adv. Exp. Med. Biol.* 1140 (2019) 635–647. https://doi.org/10.1007/978-3-030-15950-4_38.
- [83] R.T. Kelly, Single-cell proteomics: progress and prospects, *Mol. Cell. Proteomics* 19 (2020) 1739–1748. <https://doi.org/10.1074/mcp.R120.002234>.
- [84] Á. Végvári, J.E. Rodriguez, R.A. Zubarev, Single cell proteomics using multiplexed isobaric labeling for mass spectrometric analysis, *Methods Mol. Biol.* 2386 (2022) 113–127. https://doi.org/10.1007/978-1-0716-1771-7_8.
- [85] R. Liu, Z. Yang, Single cell metabolomics using mass spectrometry: techniques and data analysis, *Anal. Chim. Acta* 1143 (2021) 124–134. <https://doi.org/10.1016/j.aca.2020.11.020>.
- [86] D. Feng, H. Li, T. Xu, F. Zheng, C. Hu, X. Shi, G. Xu, High-throughput single cell metabolomics and cellular heterogeneity exploration by inertial microfluidics coupled with pulsed electric field-induced electrospray ionization-high resolution mass spectrometry, *Anal. Chim. Acta* 1221 (2022), 340116. <https://doi.org/10.1016/j.aca.2022.340116>.
- [87] X.C. Zhang, Z.W. Wei, X.Y. Gong, X.Y. Si, Y.Y. Zhao, C.D. Yang, S.C. Zhang, X.R. Zhang, Integrated droplet-based microextraction with ESI-MS for removal of matrix interference in single-cell analysis, *Sci. Rep.* 6 (2016), 24730. <https://doi.org/10.1038/srep24730>.
- [88] D.S. Verkhoturov, B.P. Crulhas, M.J. Eller, Y.D. Han, S.V. Verkhoturov, Y. Bisrat, A. Revzin, E.A. Schweikert, Nanoprojectile secondary ion mass spectrometry for analysis of extracellular vesicles, *Anal. Chem.* 93 (2021) 7481–7490. https://doi.org/10.1021/ACS.ANALCHEM.1C00689/ASSET/IMAGES/LARGE/AC1C00689_0007.JPG.
- [89] W. Shen, K. Guo, G.B. Adkins, Q. Jiang, Y. Liu, S. Sedano, Y. Duan, W. Yan, S.E. Wang, K. Bergersen, D. Worth, E.H. Wilson, W. Zhong, A single extracellular vesicle (EV) flow cytometry approach to reveal EV heterogeneity, *Angew. Chem. Int. Ed.* 57 (2018) 15675–15680. <https://doi.org/10.1002anie.201806901>.
- [90] L. de Rond, E. van der Pol, P.R. Bloemen, T. Van Den Broeck, L. Monheim, R. Nieuwland, T.G. van Leeuwen, F.A.W. Coumans, A systematic approach to improve scatter sensitivity of a flow cytometer for detection of extracellular vesicles, *Cytom. Part A* 97 (2020) 582–591. <https://doi.org/10.1002/cyto.a.23974>.
- [91] G.J.A. Arkesteijn, E. Lozano-Andrés, S.F.W.M. Libregts, M.H.M. Wauben, Improved flow cytometric light scatter detection of submicron-sized particles by reduction of optical background signals, *Cytom. Part A* 97 (2020) 610–619. <https://doi.org/10.1002/cyto.a.24036>.
- [92] Y. Tian, L. Ma, M. Gong, G. Su, S. Zhu, W. Zhang, S. Wang, Z. Li, C. Chen, L. Li, L. Wu, X. Yan, Protein profiling and sizing of extracellular vesicles from colorectal cancer patients via flow cytometry, *ACS Nano* 12 (2018) 671–680. <https://doi.org/10.1021/acsnano.7b07782>.
- [93] D. Choi, L. Montermini, H. Jeong, S. Sharma, B. Meehan, J. Rak, Mapping subpopulations of cancer cell-derived extracellular vesicles and particles by nano-flow cytometry, *ACS Nano* 13 (2019) 10499–10511. <https://doi.org/10.1021/ACSNANO.9B04480>.
- [94] V. Ponath, N. Hoffmann, L. Bergmann, C. Mäder, B.A. Alhamwe, C. Preußler, E.P. von Strandmann, Secreted ligands of the nk cell receptor nkp30: B7-h6 is in contrast to bag6 only marginally released via extracellular vesicles, *Int. J. Mol. Sci.* 22 (2021) 2189. <https://doi.org/10.3390/ijms22042189>.
- [95] A. Morales-Kastresana, J.A. Welsh, J.C. Jones, Detection and sorting of extracellular vesicles and viruses using nanoFACS, *Curr. Protoc. Cytom.* 95 (2020) 81. <https://doi.org/10.1002/cpcy.81>.
- [96] A. Morales-Kastresana, T.A. Musich, J.A. Welsh, W. Telford, T. Demberg, J.C.S. Wood, M. Bigos, C.D. Ross, A. Kachynski, A. Dean, E.J. Felton, J. Van Dyke, J. Tigges, V. Toxavidis, D.R. Parks, W.R. Overton, A.H. Kesarwala, G.J. Freeman, A. Rosner, S.P. Perfetto, L. Pasquet, M. Terabe, K. McKinnon, V. Kapoor, J.B. Trepel, A. Puri, H. Kobayashi, B. Yung, X. Chen, P. Guion, P. Choyke, S.J. Knox, I. Ghiran, M. Robert-Guroff, J.A. Berzofsky, J.C. Jones, High-fidelity detection and sorting of nanoscale vesicles in viral disease and cancer, *J. Extracell. Vesicles* 8 (2019), 1597603. <https://doi.org/10.1080/10.1080/1597603>

- 20013078.2019.1597603.**
- [97] A. Görgens, M. Bremer, R. Ferrer-Tur, F. Murke, T. Tertel, P.A. Horn, S. Thalmann, J.A. Welsh, C. Probst, C. Guerin, C.M. Boulanger, J.C. Jones, H. Hanenberg, U. Erdbrügger, J. Lannigan, F.L. Ricklefs, S. El-Andaloussi, B. Giebel, Optimisation of imaging flow cytometry for the analysis of single extracellular vesicles by using fluorescence-tagged vesicles as biological reference material, *J. Extracell. Vesicles* 8 (2019). 1587567. <https://doi.org/10.1080/20013078.2019.1587567>.
- [98] F.L. Ricklefs, C.L. Maire, R. Reimer, L. Dührsen, K. Kolbe, M. Holz, E. Schneider, A. Rissiek, A. Babayan, C. Hille, K. Pantel, S. Krasemann, M. Glatzel, D.H. Heiland, J. Flitsch, T. Martens, N.O. Schmidt, S. Peine, X.O. Breakefield, S. Lawler, E.A. Chiocca, B. Fehse, B. Giebel, A. Görgens, M. Westphal, K. Lamszus, Imaging flow cytometry facilitates multiparametric characterization of extracellular vesicles in malignant brain tumours, *J. Extracell. Vesicles* 8 (2019). 1588555. <https://doi.org/10.1080/20013078.2019.1588555>.
- [99] M. Yamanaka, N.I. Smith, K. Fujita, Introduction to super-resolution microscopy, *Microscopy* 63 (2014) 177–192. <https://doi.org/10.1093/jmicro/dfu007>.
- [100] E. Betzig, G.H. Patterson, R. Sougrat, O.W. Lindwasser, S. Olenych, J.S. Bonifacino, M.W. Davidson, J. Lippincott-Schwartz, H.F. Hess, Imaging intracellular fluorescent proteins at nanometer resolution, *Science* 313 (2006) 1642–1645. <https://doi.org/10.1126/science.1127344>.
- [101] M.J. Rust, M. Bates, X. Zhuang, Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM), *Nat. Methods* 3 (2006) 793–795. <https://doi.org/10.1038/nmeth929>.
- [102] C. Chen, S. Zong, Z. Wang, J. Lu, D. Zhu, Y. Zhang, Y. Cui, Imaging and intracellular tracking of cancer-derived exosomes using single-molecule localization-based super-resolution microscope, *ACS Appl. Mater. Interfaces* 8 (2016) 25825–25833. <https://doi.org/10.1021/acsami.6b09442>.
- [103] Z. Nizamudeen, R. Markus, R. Lodge, C. Parmenter, M. Platt, L. Chakrabarti, V. Sottile, Rapid and accurate analysis of stem cell-derived extracellular vesicles with super resolution microscopy and live imaging, *Biochim. Biophys. Acta Mol. Cell Res.* 1865 (2018) 1891–1900. <https://doi.org/10.1016/j.bbamcr.2018.09.008>.
- [104] R.P. McNamara, Y. Zhou, A.B. Eason, J.T. Landis, M.G. Chambers, S. Willcox, T.A. Peterson, B. Schouest, N.J. Maness, A.G. MacLean, L.M. Costantini, J.D. Griffith, D.P. Dittmer, Imaging of surface microdomains on individual extracellular vesicles in 3-D, *J. Extracell. Vesicles* 11 (2022). 12191. <https://doi.org/10.1002/jev.212191>.
- [105] R. Skovronova, C. Grange, V. Dimuccio, M.C. Deregibus, G. Camussi, B. Bussolati, Surface marker expression in small and medium/large mesenchymal stromal cell-derived extracellular vesicles in naïve or apoptotic condition using orthogonal techniques, *Cells* 10 (2021) 2948. <https://doi.org/10.3390/cells10112948>.
- [106] M.G. Chambers, R.P. McNamara, D.P. Dittmer, Direct stochastic optical reconstruction microscopy of extracellular vesicles in three dimensions, *J. Vis. Exp.* 2021 (2021). 62845. <https://doi.org/10.3791/62845>.
- [107] M. Wolf, R.W. Poupartdin, P. Ebner-Peking, A.C. Andrade, C. Blöchl, A. Obermayer, F.G. Comes, B. Vari, N. Maeding, E. Eminger, H.M. Binder, A.M. Raninger, S. Hochmann, G. Bracht, A. Spittler, T. Heuser, R. Ofir, C.G. Huber, Z. Aberman, K. Schallmoser, H.D. Volk, D. Strunk, A functional corona around extracellular vesicles enhances angiogenesis, skin regeneration and immunomodulation, *J. Extracell. Vesicles* 11 (2022). 12207. <https://doi.org/10.1002/jev.212207>.
- [108] G. Binnig, C.F. Quate, C. Gerber, Atomic force microscope, *Phys. Rev. Lett.* 56 (1986) 930–933. <https://doi.org/10.1103/PhysRevLett.56.930>.
- [109] S. Sharma, M. Leclaire, J.K. Gimzewski, Ascent of atomic force microscopy as a nanoanalytical tool for exosomes and other extracellular vesicles, *Nanotechnology* 29 (2018). 132001. <https://doi.org/10.1088/1361-6528/aaab06>.
- [110] R. Szatanek, M. Baj-Krzyworzeka, J. Zimoch, M. Lekka, M. Siedlar, J. Baran, The methods of choice for extracellular vesicles (EVs) characterization, *Int. J. Mol. Sci.* 18 (2017) 1153. <https://doi.org/10.3390/ijms18061153>.
- [111] P. Parisse, I. Rago, L. Ulloa Severino, F. Perissinotto, E. Ambrosetti, P. Paoletti, M. Ricci, A.P. Beltram, D. Cesselli, L. Casalis, Atomic force microscopy analysis of extracellular vesicles, *Eur. Biophys. J.* 46 (2017) 813–820. <https://doi.org/10.1007/s00249-017-1252-4>.
- [112] M. Skliar, V.S. Chernyshev, Imaging of extracellular vesicles by atomic force microscopy, *J. Vis. Exp.* 2019 (2019). 59254. <https://doi.org/10.3791/59254>.
- [113] M. LeClaire, J.A. Wohlschlegel, H. Chang, M. Wadehra, W. Yu, J.Y. Rao, D. Elashoff, J.K. Gimzewski, S. Sharma, Nanoscale extracellular vesicles carry the mechanobiology signatures of breast cancer cells, *ACS Appl. Nano Mater.* 4 (2021) 9876–9885. <https://doi.org/10.1021/acsnano.1c02299>.
- [114] S. Ye, W. Li, H. Wang, L. Zhu, C. Wang, Y. Yang, Quantitative nanomechanical analysis of small extracellular vesicles for tumor malignancy indication, *Adv. Sci.* 8 (2021). 2100825. <https://doi.org/10.1002/advs.202100825>.
- [115] V. Bairamukov, A. Bukatin, S. Landa, V. Burdakov, T. Shtam, I. Chelnokova, N. Fedorova, M. Filatov, M. Starodubtseva, Biomechanical properties of blood plasma extracellular vesicles revealed by atomic force microscopy, *Biology* 10 (2021) 4. <https://doi.org/10.3390/biology10010004>.
- [116] S.A. Melo, L.B. Luecke, C. Kahler, A.F. Fernandez, S.T. Gammon, J. Kaye, V.S. LeBleu, E.A. Mittendorf, J. Weitz, N. Rahbari, C. Reissfelder, C. Pilarsky, M.F. Fraga, D. Piwnica-Worms, R. Kalluri, Glycan-1 identifies cancer exosomes and detects early pancreatic cancer, *Nature* 523 (2015) 177–182. <https://doi.org/10.1038/nature14581>.
- [117] P. Beekman, A. Enciso-Martinez, H.S. Rho, S.P. Pujari, A. Lenferink, H. Zuilhof, L.W.M.M. Terstappen, C. Otto, S. Le Gac, Immuno-capture of extracellular vesicles for individual multi-modal characterization using AFM, SEM and Raman spectroscopy, *Lab Chip* 19 (2019) 2526–2536. <https://doi.org/10.1039/c9lc00081j>.
- [118] X. Luo, Y. Guo, Y. Huang, M. Cheng, X. Wu, Y. Gong, Characterization and proteomics of chicken seminal plasma extracellular vesicles, *Reprod. Domest. Anim.* 57 (2022) 98–110. <https://doi.org/10.1111/rda.14033>.
- [119] H. Shao, H. Im, C.M. Castro, X. Breakefield, R. Weissleder, H. Lee, New technologies for analysis of extracellular vesicles, *Chem. Rev.* 118 (2018) 1917–1950. <https://doi.org/10.1021/cracs.7b00534>.
- [120] C. Gardiner, Y.J. Ferreira, R.A. Dragovic, C.W.G. Redman, I.L. Sargent, Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis, *J. Extracell. Vesicles* 2 (2013). 19671. <https://doi.org/10.3402/jev.v2i0.19671>.
- [121] S. Cho, J. Yi, Y. Kwon, H. Kang, C. Han, J. Park, Multifluorescence single extracellular vesicle analysis by time-sequential illumination and tracking, *ACS Nano* 15 (2021) 11753–11761. <https://doi.org/10.1021/acsnano.1c02556>.
- [122] R.A. Haraszti, M.C. Didiot, E. Sapp, J. Leszyk, S.A. Shaffer, H.E. Rockwell, F. Gao, N.R. Narain, M. DiFiglia, M.A. Kiebisch, N. Aronin, A. Khvorova, High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources, *J. Extracell. Vesicles* 5 (2016). 32570. <https://doi.org/10.3402/jev.v5.32570>.
- [123] G. Huang, G. Lin, Y. Zhu, W. Duan, D. Jin, Emerging technologies for profiling extracellular vesicle heterogeneity, *Lab Chip* 20 (2020) 2423–2437. <https://doi.org/10.1039/d0lc00431f>.
- [124] F. Song, C. Wang, C. Wang, J. Wang, Y. Wu, Y. Wang, H. Liu, Y. Zhang, L. Han, Multi-phenotypic exosome secretion profiling microfluidic platform for exploring single-cell heterogeneity, *Small Methods* 6 (2022). 2200717. <https://doi.org/10.1002/smtd.202200717>.