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Analysis of single extracellular vesicles for biomedical applications with especial emphasis on cancer investigations

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A B S T R A C T

Extracellular vesicles (EVs) are lipid membrane enclosed nano-sized vesicles that are secreted by all known organisms. These vesicles are increasingly recognized as important circulating biomarkers for the diagnosis and prognosis of different diseases including various types of cancer, owing to their essential role in intercellular communication. EVs preserve heterogeneity in both physical properties and cargos, which makes it extraordinarily tough to fully exploit their clinical potential. Therefore, comprehensive characterization of single EVs and their sensitive detection are urgently demanded. In this article, we survey the latest progress in single EVs analysis with innovative discoveries in heterogeneity and highlight the various label-free and labelling approaches of single EVs detection. Furthermore, the state-of-the-art advances in single EV-detection based biomedical applications with especial emphasis on cancer investigations are summarized. To the end, the challenges and prospects for exploiting new system in the field of single EVs study are discussed.

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1. Introduction

EVs are heterogenous, lipid-bilayer-phospholipid membranous vesicles generated by various living cells through active secretion [1,2]. Although initially thought to be cell debris, EVs have been discovered as vital biological species, owing to their physiological and pathological function in organisms. These vesicles carry bioactive molecules such as proteins and nucleic acids that are inherited from parental cells, and thus, affect microenvironment locally and at a distance by transferring cargos to recipient cells [3]. EVs can mediate intercellular communication and have been regarded as potential biomarkers for the diagnosis and treatment of diseases [4,5].

EVs can be released by cells to the extracellular space via different ways. Based on the currently known origin mechanism of EVs, these vesicles can be divided into three categories: exosomes (30–200 nm in diameter), microvesicles (100–1000 nm in diameter) and apoptotic bodies (500–2000 nm in diameter) [2]. In this review, we concern on exosomes and microvesicles and collectively define them as EVs. Exosomes and microvesicles have different modes of biogenesis. In one aspect, exosomes are originated from endocytic pathway. Initially, inward budding of cellular plasma membrane results in the formation of early endosome. Further inward invagination and budding of membrane inside early endosome leads to the formation of multivesicular body (MVB) bearing intraluminal vesicles that carry transmembrane, cytosolic contents, and peripheral proteins. MVBs may then partially fuse with lysosomes and degrades inside cell. Alternatively, MVBs can fuse with plasma membrane and release vesicles to the extracellular environment, which are defined as exosomes. In another aspect, the direct outward budding of the plasma membrane induces the formation of microvesicles [6,7]. Therefore, EVs preserve high heterogeneity in physical characteristics (size, density, morphology) and cargos (protein, lipid content, nucleic acids), mainly owing to their intricate biogenesis processes [8].

Growing evidence has demonstrated the role of EVs in the development of various diseases such as neurodegenerative diseases, acute organ injury and cancer, owing to the bioactive cargos carried and transferred by EVs [5]. In particular, tumour secreted EVs effect critical functions in facilitating intercellular communication in tumour microenvironment and modulate tumour initiation and progression [9]. Additionally, EVs are widely present in

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various bodily fluids and have advantages in high concentration (up to 10^{11}/mL) and stability in the blood circulation. As a result, tumour derived EVs can be used as promising biomarkers for liquid biopsy in cancer patients [4,10]. Notably, many tumour-associated protein biomarkers have been identified in EVs from clinical blood samples, and their types and expression levels are strongly correlated with the presence and progression of certain cancer, which makes the investigation on EVs' heterogeneity important [11]. Since EVs are heterogeneous in sizes and contents, it is of utmost importance to investigate the molecular composition of EVs at single vesicle level in order to completely understand the biological function of various EV subtypes in disease development and exploit their clinical value [11]. Conventional techniques such as western blotting (WB), enzyme linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (RT-PCR) have been utilized to detect EVs contents (protein and mRNA), however, mainly for bulk vesicles, which are unsuitable for single EV analysis [2,5]. Recent years, research progress has been made in analysis of single EV, using various advanced detection technologies [12,13]. In general, detection approaches of single EVs fall into two main categories, label-free and label-based techniques. Label-free methods such as Raman spectroscopy and plasmon resonance are based on the physical properties of EVs, offering non-destructive approaches for single EV detection [14]. Leveraging fluorescently labelled antibody or aptamer, label-based methods can achieve the detection of individual EVs, in conjugation with flow cytometry or fluorescence imaging techniques etc. [15]. Herein, we intend to summarize the recent progress in single EVs analysis and the latest advances in biomedical applications, with especial emphasis on cancer diagnostics based on EV-derived biomarker discovery. In detail, the influence of EVs' heterogeneity on their function and the significance of single EVs analysis are discussed. Additionally, various characterization and detection methods are listed and compared. And the state-of-the-art clinical application in cancer diagnosis based on single EVs analysis are exemplified. Finally, we propose the current challenges and future perspectives in single EV-related research.

2. Significance of single EVs analysis

Cells actively release a large number of EV populations with distinct biomechanical properties and biological functions into the extracellular environment, which exerts diverse biological effects on recipient cells [16]. Exosomes and microvesicles are most concerned EV populations, which are discriminated based primarily on their cellular origin [3]. Nonetheless, increasing evidence has indicated that these EV populations contain various subpopulations with unique function in bioprocesses. For instance, non-membranous nanovesicles and exosome subsets including small and large exosome vesicles from various cancer cell lines have been identified [17]. Additionally, in a recent study, it has been observed that tetraspanins are unevenly distributed across single EVs [18]. The EV subpopulations can reflect the associated biological processes, which enables them to be promising biomarkers for clinical diagnosis. However, these intrinsic diversity and heterogeneity of EVs make it more complex and difficult for investigating their biology and function. Typically, in cancer biology, the various EV subtypes may have unique biological roles in the development of cancer, and the varied distribution of membrane proteins on single EVs may bias sensitivity to multiplexed cancer biomarkers [18,19].

Therefore, one of the major concerns in EV research is to address the heterogeneity within EV populations and investigate the molecular composition of single EVs in detail [12]. Clarifying EVs' diversity will help to better understand the exact function of EV in the physiological and pathophysiological processes, and ultimately accelerate the use of EVs in diagnostics and therapeutics. The significant progress has been made in the field of EVs, owing to the improved characterization and detection techniques, which enable deciphering of the heterogeneity of single EVs for biomedical applications [20].

3. Characterization techniques of single EVs

The characterization of EVs include two parts, one is the physical characterization including morphology, size and distribution, the other is the characterization of the molecular composition [21]. The physical characterization can only be employed to examine the physical characteristics of EVs, while complementary techniques are needed to examine the molecular composition e.g., protein, nucleic acids etc. to ensure the successful isolation of the desired vesicles [22]. There are multiple methods for the physical characterization of EVs including scanning electron microscopy (SEM), transmission electron microscopy (TEM) and dynamic light scattering (DLS) [5]. Different from the above techniques that are commonly employed for bulk EVs examination, as illustrated in Fig. 1, nanoparticle tracking analysis, atomic force microscopy and cryogenic transmission electron microscopy have been proved to be valuable methods for characterizing single EVs [23,24]. Characterization of the molecular composition of single EVs requires optical techniques such as Raman or fluorescence spectroscopy to obtain the molecular features of individual vesicles, either with or without exogenous labels. Commonly, Raman tweezers microspectroscopy and nanoscale flow cytometry have been employed for the molecular characterization of single EVs [25,26]. Mass spectrometry has also been employed to characterize EV proteins specifically expressed by single EV subpopulations [27]. The description and comparison of different characterization techniques are summarized in Table 1.

3.1. Atomic force microscopy

Atomic force microscopy (AFM) is high resolution imaging technique for EV characterization. In AFM, a mechanical cantilever is utilized as a probing tip to pass through the sample surface, with deflection of the tip induced by the interaction forces suggesting the morphology of surface structure. AFM does not require complicated sample preparation, while simple adsorption of EV samples to a mica substrate can be used for imaging upon mild drying. AFM enable accurate morphological and mechanical characterization of EVs with high lateral (1−3 nm) and vertical (<0.1 nm) resolution [28]. Under AFM observation, the isolated EVs are circular, while the phase image shows the bottom structure, indicating that the various constituent elements (such as lipids, proteins) make up their structure. Subgroups of vesicular and non-vesicular objects in the same sample can be distinguished by detecting the mechanical response of individual vesicles deposited on the matrix via AFM [29]. This method offered a tool to distinguish EVs from other nanoparticles and could perform high-throughput quantitative nanomechanical measurement of individual EVs.

3.2. Cryogenic transmission electron microscopy

Cryogenic transmission electron microscopy (Cryo-TEM) has been regarded as one of the most valuable techniques precisely observing the morphology and heterogeneity of EVs [30]. Unlike SEM or TEM, Cryo-TEM does not require complicated sample preparation, heavy metal for fixation or dehydration. This technique can analyse EVs in frozen samples, which shows advantage in avoiding the effects of dehydration and chemical fixation. In this

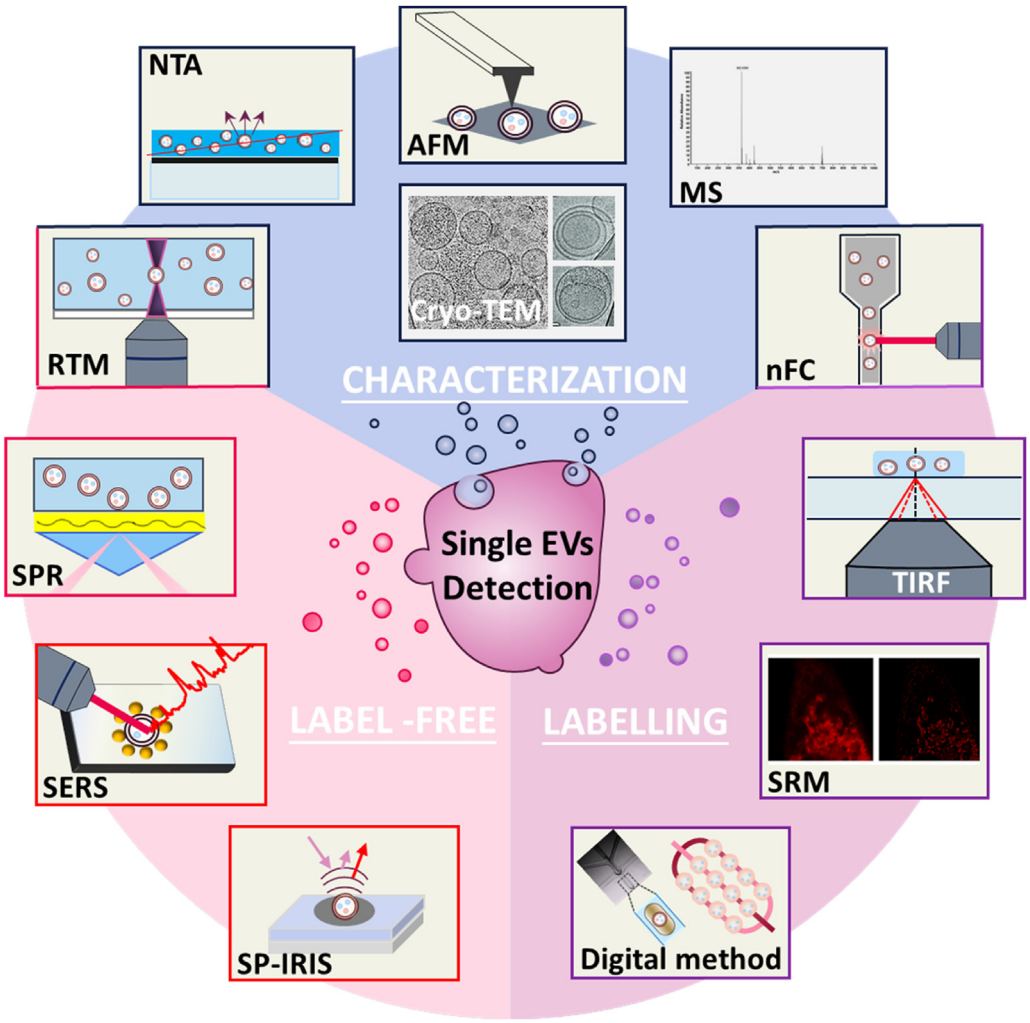

Fig. 1. Various characterization and detection methods of single EVs.

technique, vesicle samples are prepared after rapid freezing with liquid ethane, and then employed for Cryo-TEM observation. Under this high-resolution electron microscope, EVs exhibited round shape, suggesting that the cup-shaped structures observed by other techniques are likely due to the dehydrating. Although limited by the low throughput, Cryo-TEM technique has been utilized to reveal the EV polymorphism by imaging the lipid membranes and internal structures of individual vesicles [31].

3.3. Raman tweezers microspectroscopy

Raman microspectroscopy provides fingerprint vibrational spectra of analytes by measuring the scattering effect of laser light upon the interaction of photons and molecular vibrations [37]. When combining optical trapping and Raman probing, Raman tweezers microspectroscopy (RTM) (or defined as "laser tweezers Raman spectroscopy, LTRS") has become an advanced technique to examine the biomolecular content of EVs at single vesicle level [34]. In RTM technique, nanovesicles are trapped by laser mediated tweezing using a microscope objective with a high numerical aperture. Then Raman scattering is excited from the biomolecules, which provides Raman fingerprints of the sample chemical constituents in a time range from seconds to minutes without any exogenous label or sample preparation. For instance, the exosome-to-exosome variability was studied by measuring the Raman spectra of individual exosomes from different cell lines [19]. In another study, the label-free identification and chemical characterization of single extracellular vesicles was achieved by synchronous Rayleigh and Raman scattering [25]. Therefore, RTM could provide information on the chemical composition of individual EVs, providing critical evidence in studying the high diversity of EV subsets.

3.4. Mass spectroscopy

Mass spectroscopy (MS) is a tool to present structural information of the target compound based on the molecular mass and fragmentation patterns [38]. Coupling with other techniques such as chromatography, high resolution mass spectrometry (HRMS) has been extensively applied to the characterization and analysis a variety of compounds due to its advantages in identifying the structure, composition and concentration of compounds in rapid and sensitive manner. For instance, López et al. illustrated the analysis of the natural anti-cancer carotenoids from bacteria using high-performance liquid chromatography-mass spectrometry [38]. In another work, the same group presented the powerful function of various MS techniques in determining the structure, chemical composition and abundance of different anti-inflammatory

Table 1
Summary of characterization techniques for single EVs.

	Techniques	Working principle	Information obtained	Advantages	Disadvantage	Ref
Physical characterization (morphologies, size and distribution)	Nanoparticle tracking analysis (NTA)	Tracking and recording the Brownian motion of nanoparticles in suspension	Size distribution and concentration of particles	Minimum sample preparation, easy to operate, less time-consuming	Low specificity, possible false signal from protein aggregates or other nanoparticles, interference of scattered light from adjacent particles	[5,32]
	Atomic force microscopy (AFM)	Measuring the interaction between the probing tip and sample surface	Morphological and mechanical characteristics of single EVs	No special sample preparation, high resolution, high throughput, integration with other techniques	Limited information obtained; perfectly flat substrate required, time consuming, labour intensive	[28,29]
	Cryogenic transmission electron microscopy (cryo-TEM)	Observing frozen samples under transmission electron microscopic	Morphology and structure	No complicated sample preparation, retain the native structure of EVs, high resolution	Limited information obtained, low throughput, low contrast image	[31,33]
Molecular composition characterization	Raman tweezers microspectroscopy (RTM)	Measuring the Raman spectra of vesicles using laser mediated tweezing of the object	Raman fingerprints of the sample EV's chemical constituents	No exogenous label, non-destructive, timesaving, no sample treatment	Weak signal, low throughput	[25,34]
	Mass spectrometry	Measuring mass-to-charge ratio after ionization and fragmentation of sample molecule in the gas phase	Molecular mass and ion fragmentation pattern of given molecule showing the structural information	No exogenous label, rapid, sensitive, specific, high throughput	Limited information obtained, strict operation condition, coupling with other techniques required	[27,35]
	Nanoscale flow cytometry (nFC)	Detecting the multiparametric scattered light and fluorescence signal emitted by labelled vesicles on a nanoscale flow cytometer	The size distribution and diversity of EV populations, and the protein or nucleic acid content of single EVs	High resolution, high throughput	Interference from unbound dye, highly purified labelled EVs and properly diluted samples required	[26,36]
Multiparametric characterization	AFM-IR, NTA-TIRF	Different techniques are coupled on one setup to measure the same sample simultaneously and measure by two techniques	membrane protein composition and abundance, the size and mechanical properties etc.	Multiparametric analysis of single EVs, detailed information on morphology and structure	Special setup required, different software for processing and analysis	[40,41]

compounds from natural sources [39]. It has also been proved that MS is an effective method to identify EV-derived biomarkers. In a recent work, data-independent acquisition-mass spectrometry (DIA-MS) has been proved to be useful in validating tumour-specific proteins as promising biomarkers. As a result, specific EV subpopulation was determined to be early cancer diagnostic biomarker and DIA-MS was proved to be efficient liquid biopsy for rapid cancer screening [35]. Owing to the combination of MS and other techniques (e.g. WB), general EV markers such as tetraspanins and EV proteins specifically expressed by single EV subtypes (e.g., mitofilin enriched in large EVs, ADAM10 enriched in small low density EVs) have been detected, demonstrating the effectiveness in using MS for characterizing EVs to identify biomarkers [27].

3.5. Multiparametric characterization

Multiparametric characterization could present rich information on single EVs. An AFM-IR method was reported to detect the structural composition of individual EVs with nanoscale resolution [40]. In this protocol, the nano-IR spectra and nano-IR images of single EVs were collected on an AFM-IR instrument. Further data analysis by established software could reveal the composition and structure of single EVs. Recently, a fluorescence based NTA system was proposed to characterize the size and membrane protein expression of single EV by sequentially tracking the scattering and fluorescence signal of single EV [41]. In this experiment, according to a pre-programmed timetable, laser sheets with four different wavelengths were irradiated on the EV in sequence to provide scattering images inserted by three fluorescent images. Moreover, the combination of NTA and total internal reflection fluorescence microscope made it possible for the localization of the tetraspanins on a single vesicle.

4. Detection methods

Various analysis techniques mainly focus on the sensitive detection of single EVs for investigating the heterogeneity, *in vivo* function and disease diagnosis, which can be categorized to two main types: label-free and labelled approaches (as summarized in Table 2). Specifically, label-free techniques generally analyse physical characteristics (e.g., particle size, concentration) and structural components (e.g., lipids, proteins, and nucleic acids). The labelling methods rely on using fluorescently labelled antibodies or aptamers or molecular beacons.

4.1. Label-free approaches

4.1.1. RTM

RTM is such a technique that can not only be employed to characterize the molecular composition of single EVs to study the subpopulations (see section 3.3), but also be utilized as a tool to analyse individual vesicles for further purpose, e.g., cancer diagnosis [42].

Raman spectra of EVs contain unique chemical signature which can be utilized to differentiate between EV populations derived from healthy and cancer cells. Lee et al. presented a classification tool to classify Raman spectra of single EVs that were obtained by RTM for potential prostate cancer biomarker detection [42]. Penders et al. described their studies in using the label-free and nondestructive Single Particle Automated Raman Trapping Analysis

(SPARTA) platform to isolate and analyse individual EVs in their hydrated state in order to identify the compositional differences between EVs of cancerous and noncancerous origin (Fig. 2a). The developed SPARTA system could discriminate between cancer and noncancer EVs with high sensitivity and specificity [39]. In RTM technology, only one of 10^{6-8} photons are scattered inelastically, which is a very inefficient process, so the combination of high laser power, long signal integration time and high sample concentration are required. Although Raman spectroscopy has been utilized for analysing single EVs, it suffers from the drawback of weak signal.

4.1.2. Surface-enhanced Raman Spectroscopy

In order to compensate for the weak Raman signal, Surface-enhanced Raman Spectroscopy (SERS) which is based on using surface enhancement effect of precious metals (e.g., Au or Ag) nanoparticles to amplify Raman signals [70]. Therefore, SERS has been regarded as a highly sensitive technique in single EVs analysis after exposing EVs to signal-enhancing nanoparticles to obtain strengthened Raman signal [71]. Recently, Braeckmans's group showed that single vesicles could be identified by SERS [43,44]. In their work, the cations of AuNP (4-(dimethylamino) pyridine, DMAP) were electrostatic adsorbed to the anion surface of vesicles, forming an irregularly (metal) shaped nanoshell, which generated enhanced Raman signals while maintaining a colloidal suspension of individual vesicles. With the help of data analysis, the proposed method allowed the analysis of the ratio of individual vesicles from different sources in the mixture [43]. In a following study, the same team proposed a method to form Au@Ag core shell nanoparticles by generating an additional silver layer on the surface of the single vesicles, which enabled single EV analysis with higher sensitivity (Fig. 2b) [44]. Although SERS can induce enhanced signal, it can only provide limited information of biomolecules on the outer surface of EVs, owing to the minimum distance required between biomolecule and SERS substrate to generate enhancement effect. Additionally, this label-free technique measures the overall vibrational modes of individual vesicles, which lacks reproducibility and relies on complicated data processing to decode the spectrum [12].

4.1.3. Surface plasmon resonance microscopy

Surface plasmon resonance (SPR) is a technique which detects the changes in refractive index induced by molecular binding to noble metal surface. Combing with plasmonic microscopy which can image samples within 200 nm of the surface and eliminate the noise of the out-of-focus source in the culture medium, SPR microscopy has been developed to an advanced technique for label-free detection of single EVs [45]. Recently, a localized surface plasmon resonance imaging (LSPRi) platform with nanosensing arrays was proposed for single exosome detection (Fig. 2c) [72]. In this study, by adjusting the size of a single sensor to the scale of an exosome, individual exosomes can be captured by the antibodies coated on top of the arrays. The chip with thousands of individual nanosensors can be integrated into a standard optical microscope for multiplexed data collection of single vesicles.

Yang and colleagues presented their research progress in developing interferometric plasmonic microscopy (iPM) which combined surface plasmon stimulation and interferometric scattering effect to image individual exosomes [47]. With this technique, the real-time adsorption of exosomes onto chemically modified Au surface was monitored, which exhibited bright spots of individual exosomes. In successive research, a single EV imaging-integrated plasmonic biosensor was developed. After capturing EVs on sensor surface, SPR images were obtained and processed by deep learning algorithm. In this way, the single EVs derived from human lung cancer A549 cell line was identified [45]. This method could easily and effectively realize the physical and chemical analysis of single EVs, providing a potential approach for clinical application.

4.1.4. Single-particle interferometric reflectance imaging sensor

The single-particle interferometric reflectance imaging sensor (SP-IRIS) is based on a single enhanced scattering signal, which can directly and sensitively perform interference imaging analysis on a single nano-scale particle without a fluorescent label. SP-IRIS could provide the information on the size and multiple surface markers of single vesicles. For example, Daaboul et al. proposed a SP-IRIS-based method that allowed analysis and digital counting of multiple phenotyping of various individual exosomes captured on a microarray-based solid-phase chip (Fig. 2d) [48]. Recently, Mizenko et al. used commercial SP-IRIS platform to check tetraspanins profile of a single EV. In their experiment, individual nanoscale vesicles were captured and measured by the SP-IRIS mode which was based on the interference of two reflected light paths through the bound particles and the substrate [18]. As a result, the authors demonstrated that tetraspanins are evenly distributed across single EVs and bias sensitivity to cancer-related EV surface markers for diagnosis.

4.2. Labelling approaches

By using different labelling molecules such as fluorophore, antibody or aptamer, the surface signature of single EVs can be sensitively detected. Particularly, when integrating with microfluidics that is advantageous in analysing nanosized particles [73], subtle molecular differences in individual EVs have been discovered, offering new insights into cancer biology and diagnosis.

4.2.1. Flow cytometry

Traditional flow cytometry (FC) faces challenges in detecting particles smaller than 300 nm. Therefore, two main types of protocol have been developed for single EVs detection: one is based on engineered EVs which has enlarged size or enhanced signal to be detected on conventional FC device [74], the other is using customized FC setup with improved settings e.g., IFCM, microFC or nFC to detect fluorescently labelled EVs [75]. In the former case, Shen et al. has employed target-initiation engineering of DNA nanostructures of EVs and enabled the visualization of single EVs using a conventional FC device [74]. In their work, a conformation-convertible DNA probe was bound to EV surface to trigger the engineering design of DNA nanostructures through hybridization chain reaction. Finally, a fluorescent tag was bound to the engineered EV. The proposed strategy could expand the overall size of a single EV to more than 500 nm and combine with multiple fluorophore groups to amplify the signal from a small number of marker molecules located in a limited region on the EV surface. With this technique, the authors revealed differences between molecular markers of EVs derived from breast cancer cell lines and identified EV-originated from cancer cells in heterogeneous EV populations.

In the latter case, Görgens et al. reported their studies in using IFCM which combined FC with imaging for studying single EVs [51]. In this setup, signals were collected through microscope objectives and quantified based on images detected by charge coupled device cameras. In the first work, by using fluorescently labelled (CD63eGFP) single EVs as biological reference materials, the setup has been optimized to detect different antibody-labelled EV subpopulations, even from unpretreated samples. The authors claimed that IFCM could be a robust technique to assess EV heterogeneity and identify specific EV subpopulations as biomarkers for various diseases. In a following work, they demonstrated that the EV subgroups that secreted from malignant gliomas and other cancer

Table 2
A summary of the various detection methods for single EVs.

Detection methods		Working principle	Information detected	Advantages	Disadvantages	Ref.
Label-free approaches	RTM	Refer to Table 1				[39]
	Surface-enhanced Raman spectroscopy (SERS)	Exposing EVs to signal-enhancing nanoparticles to obtain amplified Raman signal	SERS spectra of individual EVs	Enhanced Raman signal, high sensitivity, high throughput	Limited information of EV surface proteins, low reproducibility, complicated data processing	[43,44]
	Surface plasmon resonance imaging (SPRi)	A technique which detects the changes in refractive index induced by molecular binding to noble metal surface	SPR spectra and SPR imaging upon binding of EVs on substrate	High sensitivity, multiplexed data collection, compatibility with microfluidic systems	Low throughput, uniform substrate and modified sensor surface required	[45,46]
	Interferometric plasmonic microscopy (iPM)	Combined surface plasmon stimulation and interferometric scattering effect to detect single EVs	iPM images reflecting the adsorption and binding events of single exosomes	High sensitivity, high spatial resolution, in situ visualization	Low specificity, low throughput	[47]
	Single-particle interferometric reflectance imaging sensor (SP-IRIS)	Detecting individual enhanced scattering signals generated by bound vesicles on layered substrate	Size and multiplexed profiling of membrane biomarkers of EV groups in a single measurement	High sensitivity, high specificity, high throughput, compatibility with microfluidics	Limited lateral resolution, difficult to detect small nanovesicles	[18,48]
	Frequency locking optical whispering evanescent resonator (FLOWER)	Recognizing EVs by tracking changes in resonant frequency of the microtoroid optical resonators	Resonance frequency changes over time indicating the binding of EVs to resonator	High signal-to-noise ratio, high sensitivity	Difficult to identify particle size, limited information obtained	[49]
	Reflection enhanced dark field scattering microscopy (REDFSM)	Recording scattering signal of single EVs after illumination on a reflective surface	The size and scattering intensity of single exosomes	High sensitivity, simplicity, high spatial resolution	Low throughput, low specificity	[50]
Labelling approaches	Imaging flow cytometry (IFCM)	Detection by combined flow cytometry and imaging techniques	Fluorescence images for qualitative and quantitative evaluation of single EVs	Multi-parametric detection, high throughput, stable signal	Dependence on biological reference materials, time consuming	[51,52]
	Micro/Nano-Flow cytometry (MFC/nFC)	Refer to Table 1				[53–55]
	Fluorescence microscopic imaging	Fluorescence from external labels for in vitro and *in vivo* tracking	Fluorescence images of single EVs both in vitro and *in vivo*	Rapid response, multi-colour labelling, high sensitivity, compatibility with microfluidics	Low signal-to-noise ratio, fluctuation of fluorescence induced by low photostability of dyes	[56–61]
	Super-resolution microscopy (SRM)	Fluorescence from external labels detected below the optical diffraction limit	Fluorescence images of single EVs in biological samples	High resolution, in situ visualization, less invasive	Fixed samples required, interference from fixing regent, low throughput	[62]
	Total internal reflection fluorescence (TIRF) microscopy	Imaging of fluorescence signal generated by the evanescent wave induced excitation of external labels at glass-water interface	Fluorescence images of single EVs in aqueous environment	High axial resolution, high signal-to-noise ratio	Limited specimen detected, fluorophore instability and photobleaching	[63,64]
	DNA points accumulation for imaging in nanoscale topography (DNA-PAINT)	Combination of DNA-PAINT and TIRF to measure exosomal biomarkers	Quantitative analysis of exosomal surface biomarkers on individual vesicles	High accuracy, high resolution, multiplexed profiling	Complicated data analysis	[65]
	Droplet digital exosome enzyme-linked immunosorbent assay (ExoELISA)	Detection of the enzymatic fluorescent reporter tagged on sandwich ELISA complexes of exosomes in single droplets	Fluorescence signals from droplets indicating the expression levels of biomarker on single exosomes	High specificity, high sensitivity, high accuracy, high throughput	Magnetic beads dependence, diluted sample required	[66]
	Immune droplet digital polymerase chain reaction (iddPCR)	PCR amplification of the genetic barcode of labelled EVs in single droplets	Absolute quantification of specific targets in individual EVs	Multiplexed analysis, high sensitivity, high throughput	Difficulty in obtaining absolute expression level of biomarker, diluted sample required	[67]
	Proximity barcoding assay (PBA)	Profiling surface proteins of individual EVs using antibody-DNA conjugates and next-generation sequencing	Surface protein patterns of individual EVs	Multiple-recognition assay, high throughput, high specificity	Complicated processing, time consuming	[68]
	Nanoplasmon-enhanced scattering (nPES)	Local plasmon-coupling effect induced by the binding of Au nanostructures to immunocaptured EVs	Dark field images and scattering spectra indicating the concentration of EV and biomarker	High specificity, high sensitivity, no sample pre-treatment, little sample required	Low throughput	[46]
						[69]

Table 2 (*continued*)

Detection methods	Working principle	Information detected	Advantages	Disadvantages	Ref.
Nano-plasmonic EV analysis with enhanced fluorescence detection (nPLEX-FL)	Fluorescence signals of the immunostained EVs are amplified by SPR excited by the Au nanohole structures	Enhanced fluorescence intensity indicating the biomarker profiling of single EVs	Multiplexed analysis, high sensitivity	Complex substrate modification, limited enhancement of fluorescence	

types were detected with high accuracy using IFCM, which was conducive to the identification and separation of tumour-specific EV populations [52].

In order to detect small EVs below 100 nm, Tian et al. developed a home-built high-sensitivity flow cytometry (HSFCM) by applying the strategies for single molecule fluorescence detection in a sheathed flow [55]. The device has three single photon-counting avalanche photodiode (APD) detectors that facilitates the simultaneous detection of side scattering and two-colour fluorescence. The advantages of the developed HSFCM in low probe volume (~25 fL), enhanced laser interacting time and high quantum yield of APD collectively make this system suitable for protein profiling and sizing of individual EVs down to 40 nm with significantly enhanced sensitivity. Kibria and colleagues developed a micro-FC (MFC) platform that could access the expression of surface protein in single circulating exosomes. By combining light scatters of three distinct angle ranges and fluorescent channels [53], the MFC was capable of measuring surface protein profiles of individual EVs derived from cell culture or human blood samples. This technique is rapid, sensitive and high throughput for single EV detection.

Nanoscale flow cytometry (nFC) has been extensively employed for studying the molecular composition of individual EVs that are pre-treated by fluorescent labelling [59]. Taking advantage of the nanoscale flow cytometer, this technique can be employed to determine the size distribution and diversity of EV populations by measuring the multiparametric scattered light and fluorescence signal. Meanwhile, nFC can enable high throughput sorting for the characterization of certain EV features. For instance, Choi and colleagues employed optimized multicolour nFC, structured illumination, and AFM to facilitate the sensitive detection of cancer cell secretomes at single EV level [54]. The authors observed a highly heterogeneous distribution of biologically relevant elements of the EV cargos, and the different uptake rates of various EV subgroups by cancer cells. Consequently, nFC coupled with high-resolution microscopy has great potential to explore the cancer related EVs. However, this method faces the challenges in measuring EV specimens with high concentration, and unbound fluorescent dyes will induce high background fluorescence, therefore affecting the signal-to-noise ratio. Therefore, highly purified labelled EVs are required to avoid the interference from remaining dyes. Also, only EVs with suitable concentration could be well characterized [12].

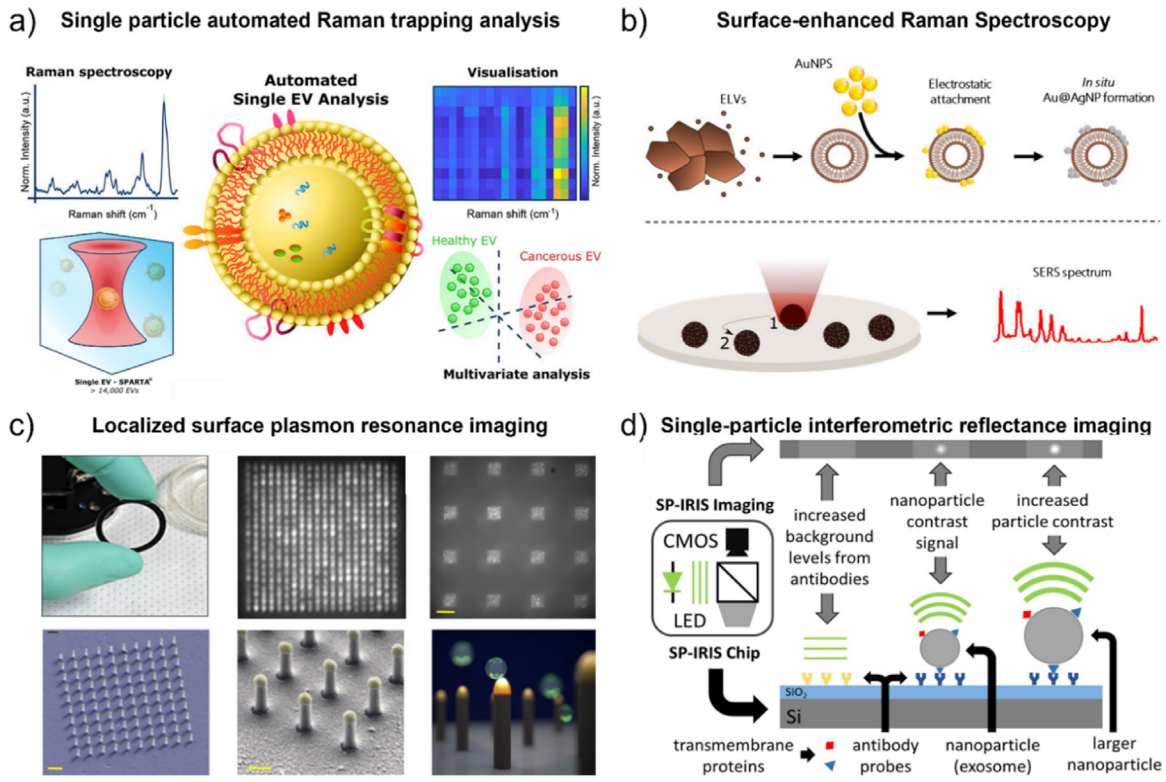

Fig. 2. a) Schematic illustration of the automated single EV analysis using the Single Particle Automated Raman Trapping Analysis (SPARTA). Adapted with permission from Ref. [39] Copyright 2021 American Chemical Society. b) Schematic illustrateion of the label-free identification of individual exosome-like vesicles with Au@Ag nanoparticles as SERS substrate. Adapted with permission from Ref. [44] Copyright 2019 American Chemical Society. c) Schematic diagram of the localized surface plasmon resonance imaging platform. Adapted with permission from Ref. [72]. Copyright 2018 PLoS one. d) Detection principle of single-particle interferometric reflectance imaging (SP-IRIS). Adapted with permission from Ref. [48]. Copyright 2016 Springer Nature.

4.2.2. Fluorescence-based imaging techniques

a. Fluorescence microscopy. Fluorescence imaging on microscope has been demonstrated to be an advanced tool for bioimaging of intracellular molecules [76,77], which could facilitate the visualized monitoring of individual nanovesicles both *ex vivo* and *in vivo* [78].

For *ex vivo* detection, microfluidic platforms have been integrated with fluorescence imaging for single EV analysis, mainly owing to the miniaturization of the device, multiple analysis and high-level integration. For instance, Lee et al. described a single EVs analysis technique on a microfluidic chip that could achieve the measurement of the diverse protein biomarkers on a single vesicle [56]. Immobilized single EVs were firstly stained with fluorescent antibodies and then imaged by fluorescence microscopy. A repeated stain and imaging cycle was employed for detecting different markers on the immobilized vesicle. Afterwards, multidimensional data analysis was utilized to identify EV populations. This technology enabled molecular identification of tumour-derived EV and had the potential to solve fundamental issues in vesicle biology and clinical applications. Liu et al. reported a λ-DNA-mediated sorting of EV subpopulations and aptamer-based analysis of individual EVs on microchip [58]. In this approach, cell derived EVs including exosomes, microvesicles and apoptotic bodies were prelabelled with fluorescent HER2 and EpCAM aptamers and injected into the microchip. Afterwards, size-selective separation of EV subpopulations by λ-DNA mediated viscoelastic microfluidics was achieved, which was observed under fluorescent microscopy at single EV resolution (Fig. 3a). In this manner, target proteins on the EV surface were identified by aptamer-based single EV analysis with high signal-to-noise ratios. Therefore, this DNA- and aptamer-mediated microfluidic platform would contribute to unveiling of the heterogeneity of single EVs. Recently, Bally's group has proposed a fluorescence-based nanofluidic devices that functions as nano flow cytometers for the detection and characterization of small lipid vesicles on a single particle basis. In this technology, when a single vesicle passed through parallel nanochannels in a pressure-driven flow, it could be observed and measured by fluorescence microscope [59]. In another study, Zhang et al. developed a localized fluorescent imaging method (namely Digital Profiling of Proteins on Individual EV, DPPIE) to analyse multiple proteins on single EVs (Fig. 3b) [57]. After isolating an EV from clinical plasma samples on an anti-CD9 antibody engineered biochip, the EVs were specifically detected by multiple DNA aptamers (CD63/EpCAM/MUC1), which were then amplified by rolling loop to generate localized fluorescence signals. The local amplification method of fluorescence signal could light up multiple proteins on the individual EVs, which greatly improved the sensitivity.

For *in vivo* measurement, the monitoring of EVs in living organisms will help to understand their vital function during physiological and pathological processes, especially in tumour progression and metastasis. To investigate the circulating tumour single EV in living organisms, Hyenne and colleagues reported their work on tracing tumour EVs in zebrafish embryo models at high spatiotemporal resolution (Fig. 3c) [60]. In their study, tumour EVs were labelled with MemBright (a cyanine-based membrane probe) and introduced to zebrafish embryo for *in vivo* monitoring. They observed the uptake of circulating tumour EVs by endothelial cells and patrolling macrophages at single EV sensitivity, which provided valuable information on studying the function of circulating tumour EVs *in vivo*. In a parallel study, Niel et al. reported their progress in live-visualizing single endogenous EVs in the zebrafish embryo after labelling the EVs with CD63-pHluorin [61]. The authors observed the release of exosomes from the yolk syncytial layer into the blood stream and the similar uptake event by macrophages and endothelial cells in the tail of the embryo. Therefore, fluorescent imaging offers a facile tool for observing single vesicles both in vitro and *in vivo*. However, this method demands fluorescent dyes with high photostability to generate stable signal with high signal-to-noise ratio [12].

b. Super-resolution microscopy (SRM). Recently, many investigators have utilized high-resolution optical microscopy and fluorescent signal tracers to allow single-vesicle analysis of EVs. In this technique, stable fluorescent labelling is required for imaging. For instance, using single molecule localization-based super-resolution microscopy, Cui's group has successfully imaged and tracked cancer-derived exosomes in living cells (Fig. 3d, i) [62]. In this work, cancer-derived exosomes membrane receptors were labelled with photo-switchable probes (Alexa Fluor 488 and Alexa Fluor 647), and these membrane receptors could be imaged using photo-activated localization microscopy (PALM) or stochastic optical reconstruction microscopy (STORM). They demonstrated simultaneous dual-colour PALM/STORM imaging of two membrane receptors on the exosome membrane and the localization of cancer-derived exosomes and lysosomes in recipient cells.

Quantitative single molecule localization microscopy (qSMLM) is a highly sensitive fluorescence imaging technique which can detect nanoparticles with single molecule sensitivity. Lennon et al. applied qSMLM to comprehensively analysing single EVs from pancreatic cancer cell culture and clinical samples (Fig. 3d, ii) [79]. In their protocol, EVs were firstly immunocaptured by surface glycoproteins (EGFR and CA19-9) enriched in pancreatic cancer. Afterwards, fluorescently labelled antibodies were used to bind EVs isolated from pancreatic cancer cells and normal pancreatic ductal epithelial cells, which was then applied to qSMLM imaging for evaluating the size, quantity and content of the isolated single EVs. The results showed that there was a greater number of EVs with higher EGFR and CA19-9 content from cancer cells than normal cells, indicating the effectiveness of qSMLM for analysis of individual EVs.

c. *Total internal reflection fluorescence microscopy.* Total internal reflection fluorescence (TIRF) microscopy has been applied to study single EVs due to the enhanced contrast and high sensitivity. Recently, He et al. have demonstrated the imaging and detection of single EVs using TIRF microscopy [63]. In their work, an activatable aptamer probe (AAP) consisted of a targeted aptamer sequence (A-strand), a poly-T linker (L-strand) and a DNA trigger (T-strand) was prepared to specifically bind to tyrosine-protein kinase-like 7 exosomes (PTK7-Exo) that was captured on the surface of TIRF platform, resulting in readable fluorescence. Further applying fluorescent molecular hairpins (H1 and H2) to the system would induce enhanced fluorescence response due to the triggered self-assembly of this DNA nanodevice. Based on the developed approach, the circulating PTK7-Exo in plasma could be directly imaged and quantified without pre-treatment. Afterwards, the same group reported their work on a new TIRF imaging platform. In this assay, the authors introduced DNAzymes to construct fluorescent probes. When the reasonably designed DNAzyme probe penetrated the entire exosomes, it could specifically bind to target miRNAs to generate amplified fluorescent signals [64]. Mg^{2+}-dependent DNAzyme was utilized as a model to amplify the label. A split DNAzyme probe (SDP) composed of two isolated DNAzyme fragments (D1 and D2) was constructed, which had an inactive structure and self-quenching fluorescence. Using the hsa-miR-21 (miR-21) to target miRNA, which was derived from multiple types of cancer, was supposed to mediate tumour growth in invasion and metastasis. After mixing the target miR-21 with SDP in the presence of Mg^{2+} ions, the catalytic activity of SDP on miR-21 (SDP21) was monitored by activated fluorescence. And the results show that TIRF imaging technology can not only quantify the target miRNA content of the entire exosomes with a high signal-to-noise ratio at

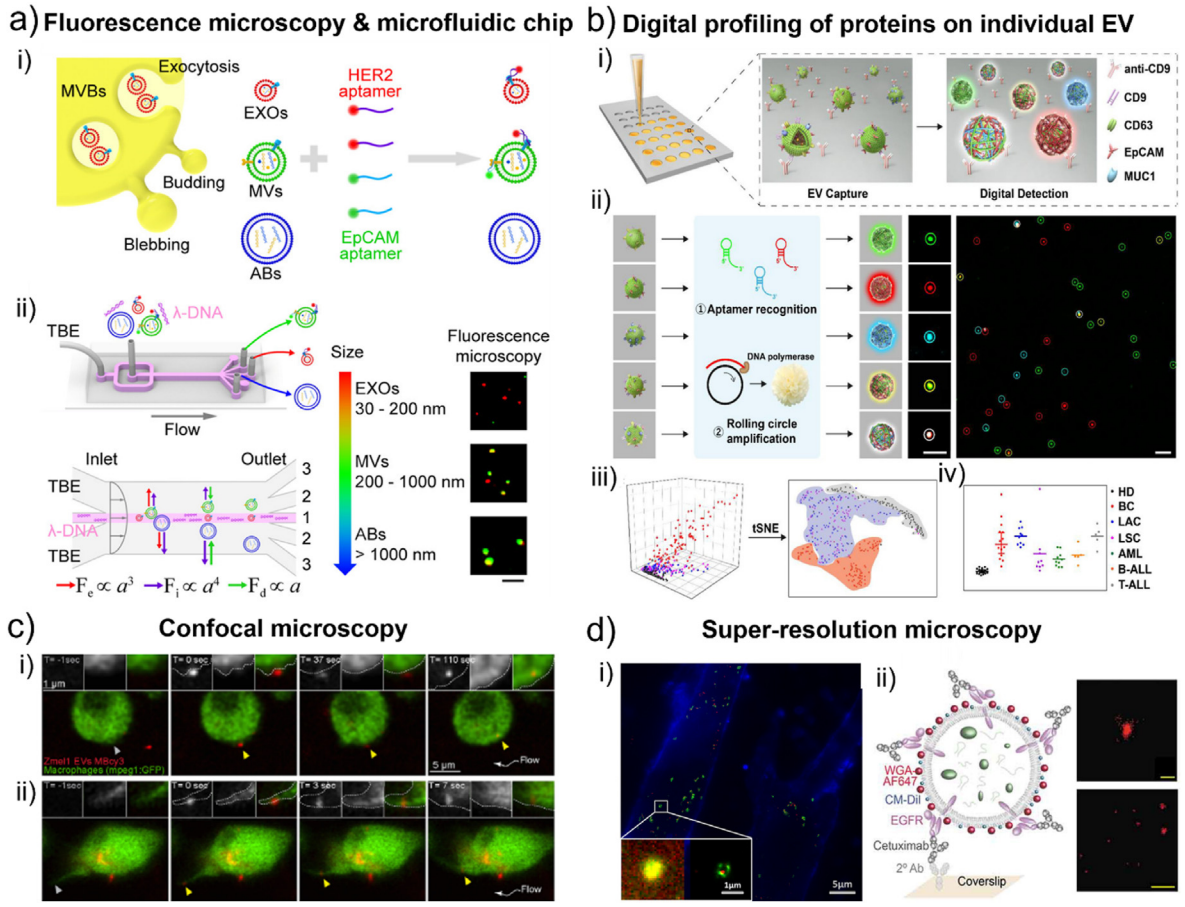

Fig. 3. a) Schematic of λ-DNA-mediated sorting of EV subpopulations and aptamer-based analysis of individual EVs. i) Labeling of cell originating EVs including exosomes (EXOs, red), microvesicles (MVs, green), and apoptotic bodies (ABs, blue) with fluorescent HER2 and EpCAM aptamers. ii) Size-selective separation of EV subpopulations. Fluorescence microscopy images showed HER2 (red) and EpCAM (green) expression of isolated individual EVs. Scale bar, 5 μm. Adapted with permission from Ref. [58]. Copyright 2018 American Chemical Society. b) Schematic diagram of digital profiling of proteins on individual EV (DPPIE) for high-dimensional individual EV analysis and multi-cancer classification. i) Overview of the steps in DPPIE assay. ii) The captured EV is labelled with DNA aptamers, followed by RCA to generate localized amplified fluorescent signals that can be imaged by confocal microscopy. Fluorescence images showed CD63 (green), EpCAM (red) and MUC1 (blue) expression on individual EV. Scale bar: 3 μm iii) Individual EV analysis data from different cells are classified using tSNE algorithm. iv) Multi-cancer diagnosis and classification. Adapted with permission from Ref. [57]. Copyright 2020 John Wiley and Sons. c) Single-plane confocal images of Tg (mpeg1: GFP) embryos injected with Zmel1 MemBright-Cy3 (MBCy3) EVs extracted from time-lapses generated immediately after injection and showing: i) the attachment and uptake of EVs by endocytosis and ii) the sliding of EVs on the macrophage protrusion and its fast internalization. Adapted with permission from Ref. [60]. Copyright 2019 Elsevier. d) i) Colocalization of MRC-5 lysosomes (green) and internalized SKBR3 exosomes (red). Figure inset showed the enlarged dual-colour PALM/STORM image that exosomes were clearly visualized in the interior of lysosomes. Adapted with permission from Ref. [62]. Copyright 2016 American Chemical Society. ii) Left, scheme of an EV affinity isolated with cetuximab and labelled with WGA-AF647 as a reporter. Right, filtered dSTORM images of WGA-AF647 with localizations in red. A single EV (top; scale bar, 100 nm) and a larger field of view (bottom; scale bar, 1 μm) are shown. Adapted with permission from Ref. [79]. Copyright 2019 Taylor & Francis Group.

the single vesicle level, but also obtain the accurate stoichiometry of miRNA and exosomes.

In a most recent work, Zhou et al. introduced a High-throughput Nano-bioChip Integrated System for Liquid Biopsy (HNCIB) system by combining nano-biochips for EV capture and TIRF for high-resolution imaging to simultaneously detect proteins and mRNA/miRNA in a single EV (Fig. 4a) [80]. Using the HNCIB system, EVs were firstly captured on the nano-biochip. Then, RNA was detected with molecular beacons (MBs), and antibodies were then applied to visualize the protein. Furthermore, deep learning algorithms were utilized to automate the analysis to obtain the distribution of mRNA/miRNA and membrane proteins. This technology could enable rapid single EV analysis. Taking advantage of the single vesicle imaging analysis method of TIRF microscopy, Han and colleagues could visualize the expression of multiple EV-related markers in a single EV [81]. In this experiment, simple fluid channels made of DDS functionalized cover plates and glass slides were incubated with biotinylated BSA and then passivated with Tween-20. Avidin was introduced to the surface to immobilize biotinylated EV. Unbound molecules were washed away after each step. The immobilized EVs were labelled with probes, and the EVs were imaged with multiple excitation lasers and a multi-colour synchro-fluorescence imaging device equipped with EMCCD cameras. Signal counting and co-localization analysis were performed on the acquired signals to study EV heterogeneity and subpopulations.

d. DNA points accumulation for imaging in nanoscale topography. DNA points accumulation for imaging in nanoscale topography (DNA-PAINT) is a localization-based super-resolution microscopy method. The combination of DNA-PAINT and TIRF can reduce the interference of impure extraction, further improve theaccuracy of sequential quantitative analysis of multiple exosomal surface biomarkers. Cui's group recently used the DNA-PAINT method to achieve quantitative analysis of exosomal surface biomarkers (e.g., HER2,GPC-1,EpCAM, EGFR) on individual vesicles [65]. In their experiment, the imaging chain stained with the immuno-stained exosomes of a DNA-coupled primary antibody was combined with the target protein docking chain. The transient hybridization of the two chains represented the fluorescence on

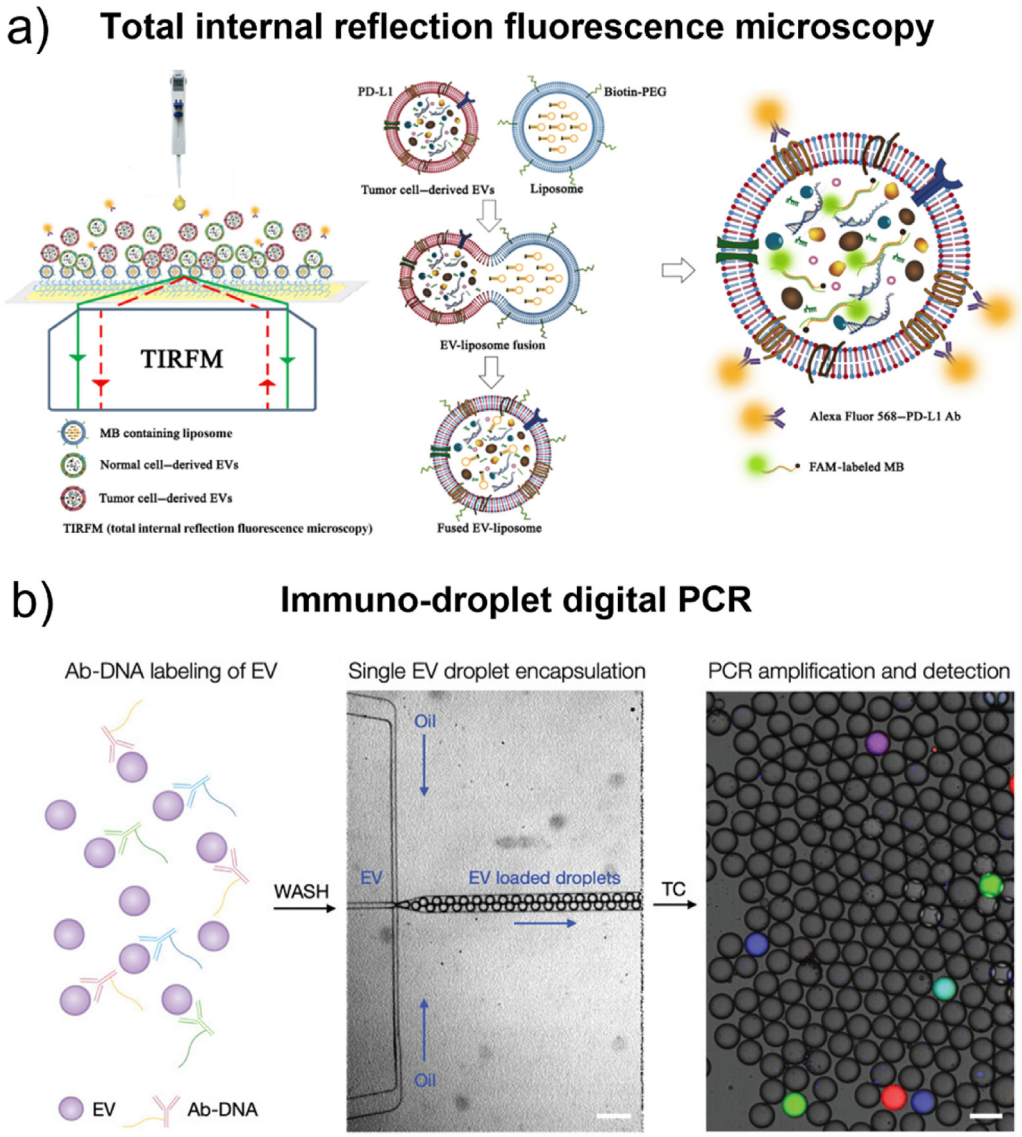

Fig. 4. a) Illustration of the HNCIB system for simultaneous detection of PD-L1 membrane protein and mRNA in a single EV. Adapted with permission from Ref. [80]. Copyright 2020 American Association for the Advancement of Science. b) Schematic of droplet-based single EV detection. Adapted with permission from Ref. [67]. Copyright 2020 John Wiley and Sons.

and off states, respectively. These two states could be detected continuously by TIRF microscopy and analysed with computer algorithms. In a proof-of-concept experiment, the proposed technology was applied to profile four exosomal surface biomarkers (HER2, GPC-1, EpCAM, EGFR) to identify exosomes from cancer-derived blood samples.

4.2.3. Digital method

Digital methods such as digital ELISA and digital PCR have been applied to the accurate quantification of proteins and nucleic acids. In this method, by using diluted samples and combining sample introduction system, single EVs could be individually encapsulated or trapped in microdroplet or microcavity, then applied for the detection of single EVs [13]. A digital quantitative immunosorbent assay for single target exosomes based on droplet microfluidic technology has been reported in a recent study [66]. Single exosomes with biomarkers of target membrane proteins were identified by exosome enzyme-linked immunosorbent assay (ExoELISA). Firstly, single exosome was immobilized to the magnetic microbeads by sandwich ELISA complexes labelled with enzymatic fluorescent reporters. Then the constructed beads were further separated and encapsulated into a sufficient number of droplets by the microfluidic chip. Finally, cancer-specific exosomes were counted by droplet-based single exosome counting ELISA, with the limit of detection down to 10 enzyme-labelled exosome complexes per microliter. As a result, ExoELISA could be used to quantify individual exosomes in a small number of samples, which was conducive to identification of exosomal biomarkers.

Recently, Ko et al. described their work in using antibody-based immune droplet digital polymerase chain reaction (iddPCR) for multiplexed single EV protein profiling (Fig. 4b) [67]. In this experiment, barcode antibodies were firstly incubated with EV for labelling. Then the labelled EV was added to the two-channel droplet microfluidic device, which was transformed by PCR and amplified by barcode signal. Afterwards, the individual droplet was imaged through a fluorescence microscope. Due to the digital nature of iddPCR, the system was able to exponentially amplify the signal from very low EV proteins to produce bright fluorescent

drops of 70 μm. And it had unique advantages over other digital methods, including 1) high detection sensitivity and signal-to-noise ratios; 2) easy multiplexing with different DNA barcodes; 3) independent of the microscopic resolution required to identify a single EV. This method was applied to profiling the PD-L1 contents of cancer cell derived single EVs. Then, the same group proposed a new antibody-based immuno-sequencing method to perform multiplexed protein profiling of a single EV [82]. In their experiment, droplet microfluidics was utilized to separate and barcode individual EVs, and then the barcode/antibody-DNA were sequenced to determine the protein composition. This method used different barcodes to define protein types and single vesicles, enabling the detection of specific proteins at the level of a single EV.

In another work, Wu and his colleagues developed a technique for the analysis of single exosome surface proteins using proximity-dependent barcoding assay (PBA), which allowed the simultaneous analysis of the presence of 38 surface proteins on a single exosome [68]. The prepared PBA probe was used to capture barcoding individual exosomes with single-stranded DNA clusters in microtiter wells. The combined antibody-DNA conjugates converted the protein composition on the surface of a single exosome into DNA sequence information. After PCR amplification, DNA sequencing was performed to record the numbers of molecules with a specific combination of markers, thus identifying the surface protein composition of individual exosomes.

4.2.4. Nanoplasmonic sensing

Using SPR imaging technology, the binding kinetics of EV at different sensing points can be monitored in real time. However, the shortcomings of precise optical alignment, miniaturization, and limited adjustment of the sensing adjustment range have promoted the development of nanoplasma platforms [83]. Currently, metallic nanostructures and/or nanoparticles are commonly employed to generate localized SPR and used to analyse single EVs. For instance, Liang et al. described a nanoplasmon-enhanced scattering (nPES) method that directly quantifies tumour-derived single EV from plasma [46]. In this experiment, EVs were firstly captured by EV-specific antibody on a silicon-dioxide sensor chip. Then another two antibody-conjugated gold nanospheres (AuS) and gold nanorods (AuR) were added to the chip and bound to the immobilized EVs to form AuS-EV-AuR complexes. AuS and AuR could be detected by dark-field microscopy, while the formed AuS-EV-AuR complexes could produce nanoplasmons that obviously shift the spectra and increase the scattering intensity, allowing the detection of single EVs. By using tumour EV-specific antibody, the developed nPES assay could be applied to sensitively quantitate tumour-derived EVs directly from clinical samples.

Moreover, based on the next generation nanoplasmonic exosome (nPLEX) detection, Min et al. performed multiplex single EV analysis on the target membrane and intravesicular markers [69]. The EVs were captured on the surface of the nanopore and immunostained by fluorescent detection probes. The labelled EVs were imaged in different fluorescence channels, and a single EV was detected by plasmon-enhanced fluorescence that amplified the fluorescence signals through surface plasmon resonances excited by the periodic Au nanopore structures. Subsequently, they succeeded in using the platform to verify the biomarkers (e.g., EGFR and EGFRvIII) and tetraspanins (e.g., CD9, CD63, and CD81) of glioblastoma.

5. Single EVs analysis-based biomedical applications

Growing evidence has demonstrated that EVs have potential in clinical application due to the diverse biomolecules they carry and the vital role of EVs in intercellular communication. Additionally, cell secreted EVs bear distinct biophysical properties and biological functions. Therefore, analysis of EVs at single vesicle level will help to fully exploit the potential function of EVs as noninvasive tools for disease diagnosis and treatment [84,85]. With the development of single EV analysis, studies have shown improved biomedical applications (mainly disease diagnosis) based on detection of single EVs (Table 3). Furthermore, the heterogeneity of EVs in drug delivery has also attracted significant interest. Herein, the translation of single EV analysis to clinics is discussed, with especial emphasis on cancer investigations.

Tumour-derived EVs mediate cancer development and influence the metastasis of primary tumors by transferring bioactive contents [86]. Studies have revealed that tumour cells release heterogeneous EV populations that carry various tumour-related EV biomarkers, with their types and expression levels tightly related to the stages of certain cancers [58,87]. Thus, investigation on the EVs at single vesicle level will be beneficial to unveil specific molecular biomarkers for various cancers to advance disease detection, treatment and prognosis monitoring. In one aspect, generic cancer biomarkers have been identified for cancer diagnostics. For instance, exosomal miRNA-21 has been reported to be a useful biomarker for discriminating between cancer patients (melanoma, breast and cervical cancer) and healthy controls based on analysis of single EVs using TIRF. However, this research lack specificity in discriminating different cancers [64]. In another aspect, with the development of single EV analysis, specific biomolecules on individual EVs have been identified for diagnosing clinical cancers using a variety of techniques.

5.1. Breast cancer

According to the Global Cancer Statistics 2020, female breast cancer has surpassed lung cancer to be the most commonly diagnosed cancer type, having approximate new cases of 2.3 million. And breast cancer is the leading cause of cancer death among females worldwide [88]. Thus, it is urgently demanded for identifying specific biomarker for early and noninvasive detection of breast cancer. Kibria et al. has applied the MFC platform for profiling of the surface proteins of individual breast cancer EVs. A significant difference of CD47 expression level was recorded between EVs derived from blood samples of cancer patients ($n = 60$) and EVs from healthy controls ($n = 60$, $p = 0.037$). This result suggested that the expression level of CD47 in blood-derived individual circulating exosomes could be related to breast cancer status [53]. Droplet digital ExoELISA was applied to analyse single EVs isolated from breast cancer cell culture, showing that the expression of GPC-1 on a single EV was indicative of breast cancer. Further validation experiment was performed by detecting individual GPC-1 (+) EVs in clinical samples from serum of healthy sample (HS, $n = 5$), different patients with benign breast disease (BBD, $n = 5$), breast cancer (BC, $n = 12$), and after surgery (BC-AS, $n = 2$), respectively. The results proved that the expression of GPC-1 significantly increased in tumour EVs than those from normal and benign breast disease samples. Additionally, the levels of GPC-1 (+) EVs in BC-AS samples were obvious lower than those of BC. Thus, GPC-1 can be deemed as a prominent exosomal biomarker for breast cancer diagnosis and Droplet digital ExoELISA is useful for GPC-1 detection on single EVs for pre- and postsurgical monitoring [66].

Taking advantage of the DNA-mediated microfluidic platform approach for single EVs detection, Liu and colleagues indicated that HER2 overexpression correlated with breast cancer happened in the EV subgroups from HER2-positive cell lines. The single EV analysis approach was then applied to differentiate the Stage II breast cancer patients with varied immunohistochemical expression of HER2 ($n = 7{:}5$ for HER2 (3+/2+) and 2 for HER2 (1+/0)

Table 3
Summary of the main application based on single EV detection for disease diagnosis.

Disease type	Sample	Biomarkers	Patient cohort/healthy control (HC)	Sample volume	Isolation technique	Detection method	Analysis time	Diagnostic accuracy (%)	Ref.
Breast cancer	Serum	CD47	Cancer patients n = 60/HC n = 60	500 μL	Ultracentrifugation	Micro flow cytometry (MFC)	~55 min	N/A	[53]
	Serum	GPC-1	Cancer patients n = 12/ patients with benign disease n = 5/patients after surgery n = 2/HC n = 5	50 μL	Ultracentrifugation	Droplet digital ExoELISA	~2 h	~100	[66]
	Serum	HER2, EpCAM	Cancer patients n = 7/HC n = 4	2.5 μL	Differential centrifugation	Fluorescence microscopy	>40 min	97.42	[58]
	Plasma	CD63, EpCAM, MUC1	Cancer patients n = 14/HC n = 15	1 μL	Immunocapture on biochip	Digital Profiling of Proteins on Individual EV (DPPIE) assay	>1h15min	91	[57]
Lung cancer	Plasma	miR-21, PD-L1	Cancer patients n = 34/HC n = 35	~90 μL	Precipitation & centrifugation	High-throughput Nano-bioChip Integrated System for Liquid Biopsy (HNCIB) assay	~6 h	N/A	[80]
	Serum	PD-1/PD-L1 mRNAs	Cancer patients n = 27/HC n = 27	3 μL	Filtration & centrifugation	High-resolution TIRF microscopy on ^{Au}SERP biochip	~2 h	93.2	[89]
	Plasma	CD63, EpCAM, MUC1	Cancer patients of subtype I n = 10/Cancer patients of subtype II n = 9/HC n = 15	30 μL	Immunocapture on biochip	DPPIE assay	>1h15min	N/A	[57]
Pancreatic cancer	Plasma	EphA2	Cancer patients n = 27/HC n = 27	1 μL	Immunocapture on nanoplasmon-enhanced scattering (nPES) platform	Dark-field microscopy on nPES platform	5 h	94	[46]
	Plasma	EGFR, CA19-9	Cancer patients n = 5/HC n = 6	~200 μL	Size exclusion chromatography	Quantitative single molecule localization microscopy (qSMLM) assay	~2.5 h	N/A	[79]
	Serum	glypican 1	Cancer patients n = 56/ patients with benign disease n = 6/HC n = 20	250 μL	Ultracentrifugation	FC	>2.5 h	100	[90]
	Serum	HULC lncRNA	Cancer patients n = 20/ patients with benign disease n = 22/HC n = 21	500 μL	Ultracentrifugation	Digital PCR	>30 min	92	[91]
Colorectal cancer	Plasma	CD147	Cancer patients n = 37/HC n = 32	50 μL	Ultracentrifugation	High-sensitivity flow cytometry (HSFCM)	~7 h	93.2	[55]
	Plasma	Somatic BRAF and KRAS mutations	Cancer patients n = 21/HC n = 46	1 mL	Nickel-based isolation	Droplet digital PCR	N/A	93	[92]
Prostate cancer	Plasma	Circulating prostate microparticles (PMPs)	Cancer patients n = 256/ patients with metastatic cancer n = 67/patients with benign disease n = 156/HC n = 22	20 μL	Fluorescence-activated cell sorting (FACS) isolation	nFC	N/A	N/A	[93]
	Serum and plasma	Beta-sheet-rich proteins	Cancer patients n = 4/ patients with benign disease n = 4/HC n = 1	2 mL	Differential centrifugation	Infrared & Raman spectroscopy	>3 h	N/A	[94]
	Plasma	AR-V7 mRNA	Cancer patients n = 16/ patients with metastatic cancer n = 35/HC n = 10	500 μL	Ultracentrifugation	Droplet digital PCR	>2 h	N/A	[95]
	Serum	PSA mRNA	Cancer patients n = 27/ patients with benign prostatic hyperplasia n = 15/HC n = 16	2 μL	FRET-based DNA tetrahedron (FDT) incubation	DNA tetrahedron-based thermophoretic assay & qRT-PCR	~2h45min	0.93	[96]
Glioma cancer	Cerebrospinal fluid	Mutant IDH1 transcript	Cancer patients n = 14/HC n = 4	500 μL	Ultracentrifugation	BEAMing RT-PCR & droplet digital PCR	~2 h	N/A	[97]

Leukaemia	Plasma	CD63, EpCAM, MUC1	Cancer patients with subtype I n = 10/patients with subtype II n = 5/ patients with subtype II n = 5/HC n = 15	30 μL	Immunocapture on biochip	DPPIE assay	>1h15 min	N/A	[57]
	Plasma	Tyrosine protein kinase-like 7 (PTK7)	Cancer patients n = 5/HC n = 5	50 μL	Immunocapture on coverslip	TIRF assay	>7 h	N/A	[63]

expressions) from healthy controls (n = 4). They also proved that EpCAM was also a reliable biomarker for breast cancer diagnostics (Fig. 5a). Based on a LDA algorithm using the concentrations of HER2-and EpCAM-positive EVs as the input, an obvious discrimination by microvesicles among cancer patients was verified [58].

5.2. Lung cancer

Lung cancer is the leading cause of cancer death, with an estimated 1.8 million deaths worldwide in 2020 [88]. Early diagnosis using prominent biomarkers could enable significant clinical benefits and improve the survival rates of patients. Since exosomal miRNAs, mRNA, and proteins could be used as potential cancer biomarkers, the concurrent detection of multiple biomarkers at a single-EV level could result in tremendously improved diagnosis and prognosis. Zhou et al. applied a developed HNCIB system to analyse individual EVs derived from plasma samples of patients with lung adenocarcinoma (LUAD) (n = 34) and healthy controls (n = 35) (Fig. 5b). The result showed that the expression levels of miR-21, programmed death-ligand 1 (PD-L1) mRNA and protein of EV from LUAD patient were much higher than the healthy donors. Hence, the simultaneous detection of up-regulated expression of these biomarkers in clinical samples by the as-built HNCIB system could facilitate improved diagnosis of LUAD with high reliability and sensitivity [80].

Tumour PD-L1 expression has been regarded as a predictive biomarker for anti-PD-1/PD-L1 immunotherapy for non-small cell lung cancer (NSCLC). Based on an immunogold biochip, Nguyen et al. sensitively quantified the level of PD-1/PD-L1 surface proteins and mRNAs cargos in single EVs isolated from the serum samples of NSCLC patients. By testing a cohort of 27 patients and 27 health donors, the authors proved that single-EV mRNA cargo exhibited advantageous performance in diagnosing NSCLC (accuracy 93.2%) and predicting patient responses to immunotherapy (accuracy 72.2%), compared to single-EV protein biomarkers. Therefore, the developed biochip could be applied to sensitively detect the single-EV PD-1/PDL1 mRNA and serve as an effective tool for NSCLC diagnosis and immunotherapy prediction [89].

5.3. Pancreatic cancer

Pancreatic cancer accounts for 93.9% death rate with almost identical mortality and incidence cases, mainly due to the dire prognosis. It is characterized by aggressive local invasion, early metastasis and highly treatment resistance [88]. Conventional biomarker CA19-9 could be applied to diagnose pancreatic cancer, however, with low specificity. Thus, novel circulating biomarkers and sensitive systems need to be identified and developed for early detection of pancreatic cancer and differentiating from other diseases e.g. chronic pancreatitis.

Since its expression is significantly higher in human pancreatic cancer samples than chronic pancreatitis or normal pancreatic samples, ephrin type-A receptor 2 (EphA2) has been regarded as a pancreatic cancer biomarker. Based on a nPES analysis system, Liang and colleagues quantified single pancreatic-cancer-derived EVs isolated from clinical blood samples by detecting the CD9 (+)/EphA2 (+) EVs using dark field microscopy. It was indicated that there was a strong correlation between the level of circulating EphA2-EV and pancreatic cancer. The authors further investigated whether nPES EphA2-EV could recognise early pancreatic cancer cases by analysing the plasma samples of a larger cohort including pancreatic cancer patients with early-stage disease (pancreatic cancer stages I and II). The results clearly demonstrated that EphA2-EV signal was obviously higher in plasma samples of early

pancreatic cancer than those from pancreatitis patients or normal controls. As a result, nPES EphA2-EV blood test may provide significant value for early diagnosis of pancreatic cancer and thus improve patient treatment outcomes [46].

Using the single molecule characterization technique qSMLM, Lennon et al. quantified the size and biomarker content of individual EVs from pancreatic cancer (Fig. 5c). Equivalent EVs were obtained from plasma samples of both Pancreatic Ductal Adenocarcinoma (PDAC) patients and healthy subjects, which were applied to further detection. By immuno-isolating and staining target EVs using either cetuximab-AF647 or anti CA19-9 Ab-AF647, qSMLM enabled quantification of size and content of single EVs. The authors observed a much higher number of EVs from cancer samples than those from health controls and an average of 5-and 15-fold increases in EV quantities for EGFR and CA19-9, respectively. Meanwhile, the abundant presence of EGFR- and CA19-9-enriched microvesicles represents typical characteristic of PDAC. These clinical data evidently proved that analysis of EGFR and CA19-9 using qSMLM has diagnostic potential for effective PDAC detection [79].

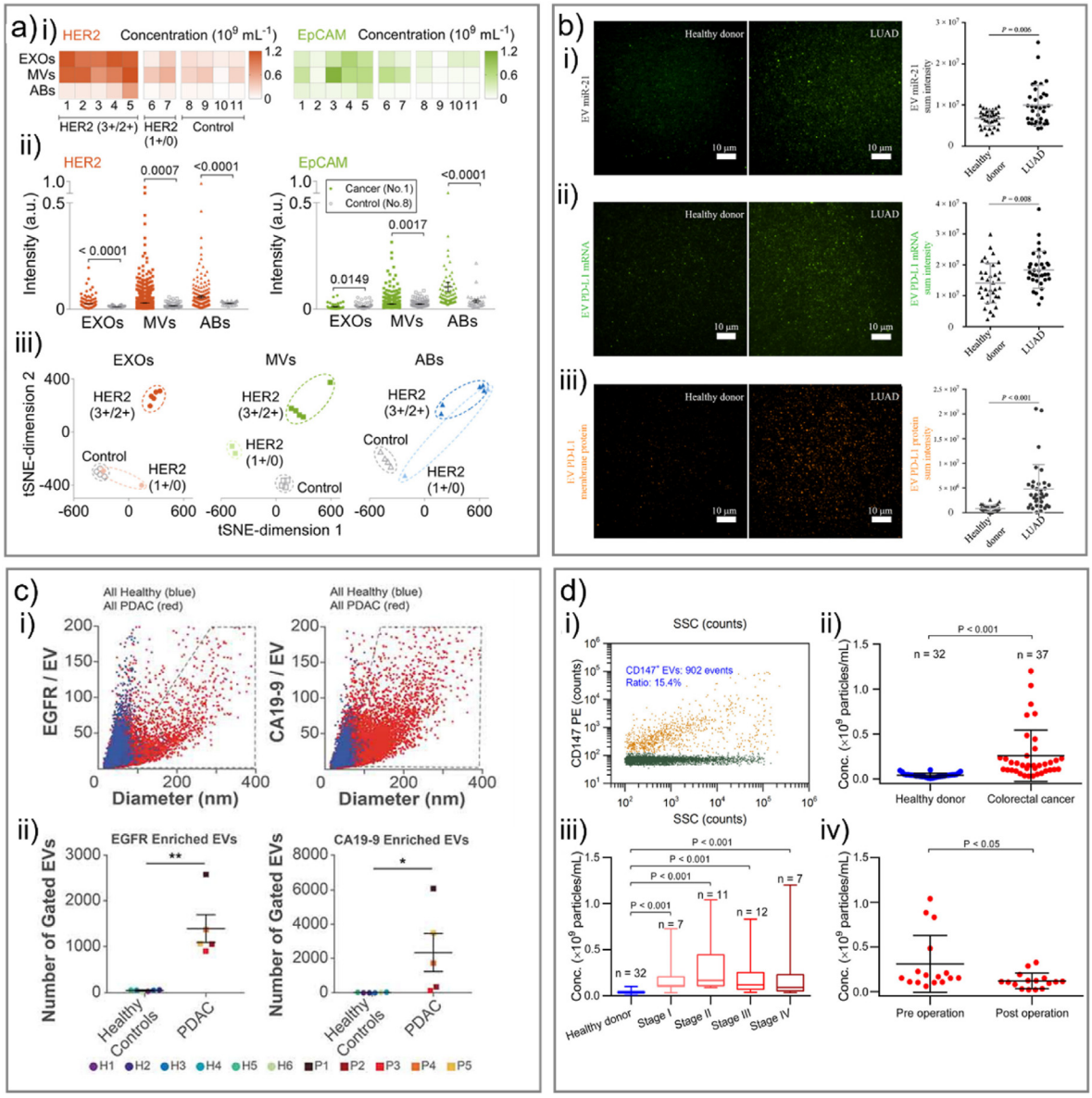

Fig. 5. Representative studies in cancer diagnostics using single EV analysis. a) Discrimination of the Stage II breast cancer patients and healthy controls. i) The concentrations of HER2-positive EXOs, MVs, and ABs in the clinical cohort. ii) Comparison of expression levels of HER2 and EpCAM on individual EXOs, MVs, and ABs between a cancer patient and a control. iii) t-SNE visualization of discrimination among cancer patients with varied HER2 expressions and controls by different EV subpopulations. Adapted with permission from Ref. [58]. Copyright 2019 American Chemical Society. b) Measurement of miRNA, mRNA, and protein expression of EVs isolated from human plasma. Samples were from 35 healthy donors and 34 patients with LUAD. Representative images and statistical analysis of i) EV miR-21, ii) EV PD-L1 mRNA and iii) EV PD-L1 membrane protein. Adapted with permission from Ref. [80]. Copyright 2020 American Association for the Advancement of Science. c) Quantification of EVs from patient plasma. i) Combined EGFR-enriched (left) and CA19-9-enriched (right) EVs from healthy subjects and PDAC patients. ii) Number of EVs in gated area (grey polygons, panel i). Adapted with permission from Ref. [79]. Copyright 2019 Taylor & Francis Group. d) Analysis of CD147-positive EVs in plasma samples of colorectal cancer patients and heathy donors by HSFCM. i) Bivariate dot-plot of the PE orange fluorescence versus SSC for EVs from patient sample upon immunofluorescent staining with PE-conjugated MAb against CD147. Comparison of plasma particle concentrations of CD147-positive EVs in ii) healthy donors and colorectal cancer patients, iii) healthy donors and cancer patients at different stages and iv) cancer patients (n = 16) before (pre-operation) and after (7–10 days postoperation) surgical removal of the tumour (mean ± s.d.). Adapted with permission from Ref. [55]. Copyright 2018 American Chemical Society.

Intriguingly, Melo et al. identified a promising biomarker glypican-1 (GPC1) which was enriched on pancreatic cancer cell derived EVs. Using flow cytometry, single GPC1+ circulating EVs was detected in the serum of cancer patients, which showed absolute specificity and sensitivity in discriminating patients with pancreas cancer from patients with benign pancreas disease. Therefore, GPC1+ EVs may offer a potential non-invasive tool for diagnosis of early stages of pancreas cancer [90]. In another study, the long noncoding RNA highly upregulated in liver cancer (HULC) was found to highly expressed in PDAC cells and secreted EVs. Analysis of serum EV HULC expression of a cohort of subjects including patients with PDAC and intraductal papillary mucinous neoplasm (IPMN), and 21 healthy controls showed that EV HULC expression was significantly upregulated in PDAC patients compared to IPMN patients and health subjects. In addition, HULC exhibited predictive potential for distinguishing PDAC, indicating EV-encapsulated HULC could behave as a chemotaxis biomarker for the timely diagnosis of human PDAC at an early stage [91].

5.4. Colorectal cancer

According to the newest statistics, there are more than 1.9 million new colorectal cancer cases and 935,000 deaths in 2020 worldwide. And colorectal cancer ranks third in terms of incidence (after breast and lung cancer) but second (after lung cancer) in terms of mortality among all kinds of cancer types [88]. Hence, the early diagnosis and intervention of colorectal cancer is critical to facilitate possible curative surgical therapy. As a high expression level of CD147 was confirmed from EVs isolated from colorectal cancer cell lines, the potential role of CD147 as biomarker for colorectal cancer diagnosis was investigated. Based on home built HSFCM, Yan group analysed the plasma concentrations of CD147 (+) EVs derived from blood samples of colorectal cancer patients and controls (Fig. 5d). HSFCM could not only reveal the percentage of EVs that express CD147, but also contribute to correlating the protein abundance with the EV size at the single vesicle level. The results indicated that the concentration of CD147 (+) EVs exhibited high classification with an AUC value of 0.932. Additionally, the elevated level of CD147 (+) EVs of patients at all cancer stages suggested the utility for diagnosing colorectal cancer in the at onset [55]. Another study has identified somatic BRAF and KRAS mutations in the EVs derived from plasma of metastatic colorectal cancer patients. The detection of oil encapsulated single EVs was based on digital PCR, which matched the tissue biopsy results of colorectal cancer [92].

5.5. Prostate cancer

For males, prostate cancer is the most frequently diagnosed cancer type and accounts for the second leading cause of cancer death worldwide [88]. Although prostate-specific antigen (PSA) is the prevalently applied biomarker for detecting prostate cancer, this biomolecule suffers from the low specificity in differentiating cancer and benign diseases. Apart from this issue, prostate cancer is such a cancer type whose survival rate could not be improved by early detection, mainly owing to the side effects induced by overdiagnosis and overtreatment of low-grade tumors.

In order to overcome these challenges, a number of research has been devoted to the discovery of intrinsic biomarkers [98]. For instance, Biggs et al. have detected the levels of circulating prostate microparticles (PMPs, a type of EV) in plasma from patients with castration resistant prostate cancer (CRPC), localized prostate cancer and benign prostatic hyperplasia, as well as healthy donors. By measuring the concentration of PMPs using single vesicle analysis technique nanoscale flow cytometry, it was observed that the PMP levels were significantly higher in the plasma from patients with Gleason Score $\geq$8. Thus, prostate patients with aggressive tumour (Gleason Score of 8 or higher) could be differentiated by the PMP level in plasma, regardless of PSA. Meanwhile, due to the decreased level after surgical resection of the prostate, PMP exhibited prognostic monitoring potential after treatment [93]. Other EV related characteristics have also been observed for screening prostate cancer. In Krafft et al.'s study, the reduction of alpha-helix-rich proteins, accompanied by the enhancement of beta-sheet-rich proteins offered great confidence as a cancer-specific EV signature. Subsequent experiment in measuring single EV samples from serum and plasma samples of patients proved the effectiveness in using this shift feature in identifying high grade prostate cancer patients from those with benign hypoplasia [94].

EV-derived mRNA has been regarded to provide unprecedented opportunities for non-invasive and rapid diagnosis of cancers. In a recent work, Joncas et al. identified AR-V7 (androgen receptor splice variant 7) mRNA in blood EVs to be reliable prognostic biomarker for patients with metastatic CRPC. Furthermore, they highlighted that detectable level of AR-V7 mRNA was associated with a shorter time to progression (median, 16.0 vs 28.0 months; $P = 0.049$) [95]. In Han et al.'s research, they measured the PSA mRNA in serum EVs using the developed FRET-based DNA tetrahedron. With the help of RNA extraction and enzyme amplification, the DNA tetrahedron-based thermophoretic assay (DTTA) achieved a limit of detection (LoD) of 14 aM, which exhibited better diagnostic accuracy over conventional serum PSA protein in differentiating patients with prostate cancer and benign prostatic hyperplasia (area under the curve: 0.93 versus 0.74; 42 patients) [96].

5.6. Glioma cancer

Glioma is the most common primary intracranial tumors, which accounts for 24.7% of primary and 74.6% of all malignant brain tumors globally [99]. The demand for novel biomarkers has promoted research in searching for valuable strategies for cancer diagnostics. For instance, The RNA mutation profiling of individual EVs derived from the cerebrospinal fluid of patients with glioma tumors were measured by BEAMing (beads, emulsion, amplification, magnetics) RT-PCR (EV-BEAMing) and droplet digital PCR. The results indicated that these EVs contain mutant IDH1 transcripts, which could be applied to detecting wild-type IDH1 RNA transcripts in the cerebrospinal fluid of patients with gliomas. Therefore, IDH1 RNA transcripts could behave as a reliable biomarker, and EV-BEAMing and EV-ddPCR could aid the development of advanced diagnostic and therapeutic strategies [97].

5.7. Leukaemia

Leukaemia is a heterogeneous group of haemopoietic cancers that represent the 10th and 11th most frequent cause of cancer occurrence and death globally. Acute lymphoblastic leukaemia is the main subtype of leukaemia occurred in children [100]. Currently, the diagnoses of leukaemia mostly rely on laboratory investigations and the diagnostic validity can affect the treatment and clinical follow-up. Thus, effective biomarkers and analysis methods may provide great opportunity for early detection of hematological malignancies and is beneficial to designing potential curative treatment options.

Based on the fluorescence imaging of single EVs, followed by a rolling circle amplification, multiple proteins on individual EVs were analysed. The detection of EVs from plasma samples of leukaemia patients including acute myeloid leukaemia patients (AML, $n = 10$), B-cell acute lymphoblastic leukaemia (B-ALL, $n = 5$)

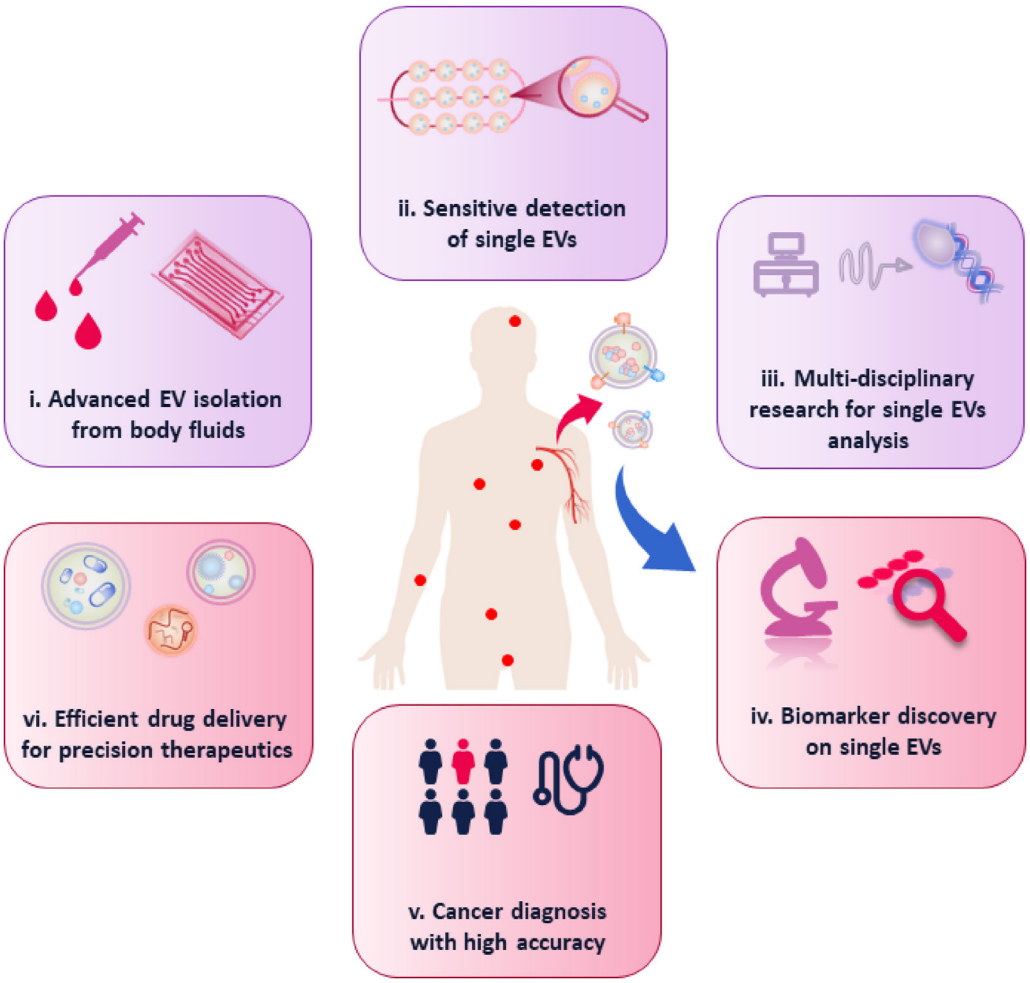

Fig. 6. Overview of critical future perspectives in single EVs analysis and related biomedical applications.

and T-cell acute lymphoblastic leukaemia (T-ALL, n = 5) revealed the existence of CD63/EpCAM/MUC1-triple-positive EV in the plasma samples of patients with B-ALL and T-ALL. Therefore, CD63/EpCAM/MUC1-triple-positive EV could serve as potential biomarker for diagnosing hematologic malignancies [57]. In another study, owing to the different contents of PTK7, the single vesicle-based TIRF analysis of PTK7-exosomes could be applied to differentiate target tumour models from controls. Further TIRF imaging analysis of PTK7-exosomes were conducted using the plasma samples of five human acute-lymphoblastic-leukaemia patients and five healthy individuals. The results illustrated that the level of PTK7-exosomes in plasma samples of cancer patients was significantly higher than that of healthy donors, which proved the efficacy of using the developed approach to identify cancer patients in clinical practice [63].

6. Conclusions and future perspectives

EVs are present in nearly all types of biofluids and contain membrane and cytoplasmic components representing the parental cells, including proteins, nucleic acids, and lipids. Findings on the role of EVs have indicated their diverse biological functions, which makes EVs be reliable biomarkers for diseases diagnosis. Increasing evidence has demonstrated that EVs are heterogeneous in size and molecular contents, which accordingly change their biological properties. This intrinsic heterogeneity introduces extra complexity in studying EV biology and function. However, reported methods focus mainly on the bulk analysis of vesicles, which makes it challengeable to study the various EV types and subpopulations, as well as understanding the distinct biomarker expression. Large efforts have been devoted to overcoming these challenges and producing single EV-optimized analytical platforms. Most of novel analytical technologies are superior to traditional methods, effectively measuring the specific properties from single vesicle. In this article, as summarized in Table 2, current and emerging approaches for analysis of single EVs are categorized into label-free and labelling groups. Label-free methods including RTM, SERS and SPR etc. are based on the analysis of EVs' physical characteristics and overall spectral properties. The labelling methods are mainly based on modifying surface protein with fluorescent dyes, in combination with FC, TIRF or high-resolution imaging techniques to reveal the detection of individual vesicles. Technologies like microfluidics has been successfully integrated with various labelling approaches, achieving the detection of EVs at single vesicle level.

The development of single EVs analysis accelerates not only the understanding of EVs' biology, but also the improved biomedical application with prominent disease diagnosis potential. With an emphasis for cancer investigations, we conclude several critical points including low volume of body fluid to analyse EVs, rapid isolation and analysis of single EVs, reliable biomarkers for cancer diagnosis, high throughput EV analysis and high diagnostic

accuracy *etc.* These technological parameters are crucial for the evaluation of analysis techniques, with the aim to promote the translation to clinics.

Despite the increasing knowledge of EV subpopulations after decades, EV-based research is still in its infancy. Limitations in isolation and detection of single EVs, as well as the in-depth understanding of the roles of EV subpopulations in physiology remain hurdles in the development of EVs research. Based on the advances and the remaining challenges in single EV-related research, we describe the critical issues that we consider for future development in this field (Fig. 6). First, advanced single EVs isolation technologies are still demanded to investigate the exosome-to-exosome phenotypic heterogeneity and their accurate quantification. Owing to the nanometer sizes, isolation of single EVs is still challengeable. In this regard, microfluidic and nanofluidic devices show their advantages due to the comparable dimensions. Combined with nanopatterns and immune-affinity technology, the microfluidic systems are expected to isolate single EVs with high sensitivity and specificity. Therefore, more innovative microfluidic chips with elaborate design are highly desired to achieve highly efficient isolation of single EVs, not only from cell culture medium, but also from complicated clinical samples.

Second, single EVs research requires highly sensitive detection to address the heterogeneity of biophysical characteristics and composition of EVs. As a result, advanced techniques such as fluorescent imaging-based high-resolution microscopy is under focus. Super resolution microscopy has been utilized for imaging of single EVs, due to the ultrahigh resolution down to a few tens of nanometers. Research efforts has been devoted to the development of instrumentation and labelling methods to realize highly sensitive single EV detection. Therefore, in one aspect, the advancement of instrument, for instance three-dimensional super-resolution microscopy will be of utmost importance for investigating the unique characteristics including sizing and biomarker expression of single nanovesicles. In another aspect, promising labelling strategies such as immunoaffinity combined with DNA assisted extension will help to amplify the signals, which enables sensitive quantification of the surface proteins of single EVs. Furthermore, microfluidic systems provide an advanced platform for single EVs detection. Taking advantage of the nanochannels and precise fluid control, individual vesicles can be visualized after proper fluorescence labelling.

Third, the development of single EV research will be based on the joint efforts of associated technologies. And thus, multidisciplinary research including nanotechnology, fluorescence imaging, micro-fabrication and machine learning etc. will contribute to the advancing of single EV-related field. For instance, further advancement in micro- and nanofabrication will offer powerful platforms for studying the single EV transportation by mimicking biosystems (e.g., tumour microenvironment).

After combining with functional labelling linkers and advanced imaging techniques, precise investigation on the roles of EV subpopulation in disease development would accelerate the single EV-based research.

Finally, biomedical application of single EV analysis still relies on the advancement of powerful detection approaches and more clinical practice to generate translational effect. In the past few years, precise medicine, such as personalized pharmacotherapy and tumour screening, has gained great attention in clinical oncology. The increasing development of EV-related research provides tremendous prospects for biomedicine. Biomedical applications based on single EV strategies require the merits of low sample consumption, rapid response, early detection, and capability of differentiation between different stages of certain cancer types. Therefore, the various emerging systems for detecting individual EVs will be beneficial to understanding the role of EV subpopulations in cancer biology and facilitating the development of new strategies for clinical diagnosis and therapeutics. For cancer diagnosis, since different EV subpopulations exert unique roles in tumour development, the screen of reliable biomarkers via single EV analysis has great potential to facilitate accurate cancer diagnosis. Additionally, cell derived EVs have been regarded to be promising drug carriers, due to their nanoscale structure, biocompatibility, and their capability in crossing blood-brain barrier. Therefore, EV heterogeneity must be considered in terms of biological distribution and intracellular delivery, and specific EV subtypes could be emerging efficient drug delivery tools for precision therapeutics.

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.

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