Chapter

High-Throughput Single Extracellular Vesicle Profiling

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Abstract

Extracellular vesicles (EVs) are heterogeneous due to their cell of origins, biogenesis, stimuli in the microenvironment and so on. Single EV analysis is required for the study of EV heterogeneity. Besides the investigation of EV biology, single EV analysis technologies are promising approach for liquid biopsy, which relies on the detection of biomarker EVs readily available in body fluids but in trace amount. However, EVs are nano-scaled structures, which beyond the resolution of conventional technologies like optical microscopes, flow cytometers and so on. In this chapter, we will discuss advanced strategies for studying single EVs, including single EV imaging systems, flow cytometers, nano-sensing technologies and single EV barcoding assay.

Keywords: extracellular vesicles, single EV analysis, imaging, flow cytometer, nano-sensing, Proximity Barcoding Assay

1. Introduction

Extracellular vesicles (EVs) are heterogeneous because of their diverse cell of origins, the process of biogenesis, the specific stimuli in their microenvironment and so on. EVs are produced by cells of bacteria, fungi, plants and animals. Inside human bodies, EVs carry molecular signatures of their parent cells and diffuse freely among blood stream and tissues. EVs can be classified into various subpopulations according to their origin, size, density, biogenesis, compositions etc. The origin of the vesicles gives us terminology of prostasome, oncosome etc. According to the size of EVs, investigators utilized terms of exomere, small EVs, large EVs. Depending on the biogenesis, we defined apoptic bodies, microvesicles and exosomes. Apoptic bodies are large vesicles formed due to apoptosis. Microvesicles are vesicles budding directly from cell membrane. Exosomes are the released intraluminal vesicles (ILVs) from multivesicular bodies (MVBs) through fusion of MVB with cell membrane.

To address the heterogeneity of EVs, scientists dedicated to the development of novel techniques for single EV detection. Besides investigation of EV biology, single EV analysis technologies are promising approach for liquid biopsy, which relies on the detection of biomarker EVs readily available in body fluids. Here in this chapter, we will discuss four main strategies of studying single EVs, including single EV imaging systems, flow cytometers, nano-sensing technologies and single EV barcoding assay.

2. Single EV imaging

Due to the sub-200 nm diameter of most EVs, conventional optical microscopies are precluded for direct observation or imaging of EVs. Nano-scaled imaging methods which are applicable to the scale of EVs include electron microscopies (EM), atomic force microscopy (AFM), total internal reflection fluorescence microscopy (TIRFM) and stochastic optical reconstruction microscopy (STORM).

2.1 Electron microscopies (EM)

Electron microscopies (EM) can be used to study the morphology and size of EVs. Scanning electron microscopy utilizes beam of electrons to bombard the surface of samples and detect the backscattered electrons and secondary electrons to construct the image of the detected surface. EVs under SEM imaging are usually round shaped, but could be collapsed and irregular in shape, possibly due to the deformation of EV structure during sample preparation. Transmission electron microscopy (TEM) and cryo-TEM has become one of the gold standards for EV characterization according to MISEV2018 guideline suggested by International Society of Extracellular Vesicles (ISEV). Under TEM observation, EV are round spheres or cup-shaped with lipid membrane visible as bilayers. Immunogold labelling could be combined with EM to get the composition distribution on EVs (**Figure 1**) [1–3].

2.2 Atomic force microscopy (AFM)

With AFM, an oscillating cantilever scans over a substrate with adhered EVs and simultaneously image the spatial dimension of single EVs. Therefore, the morphology

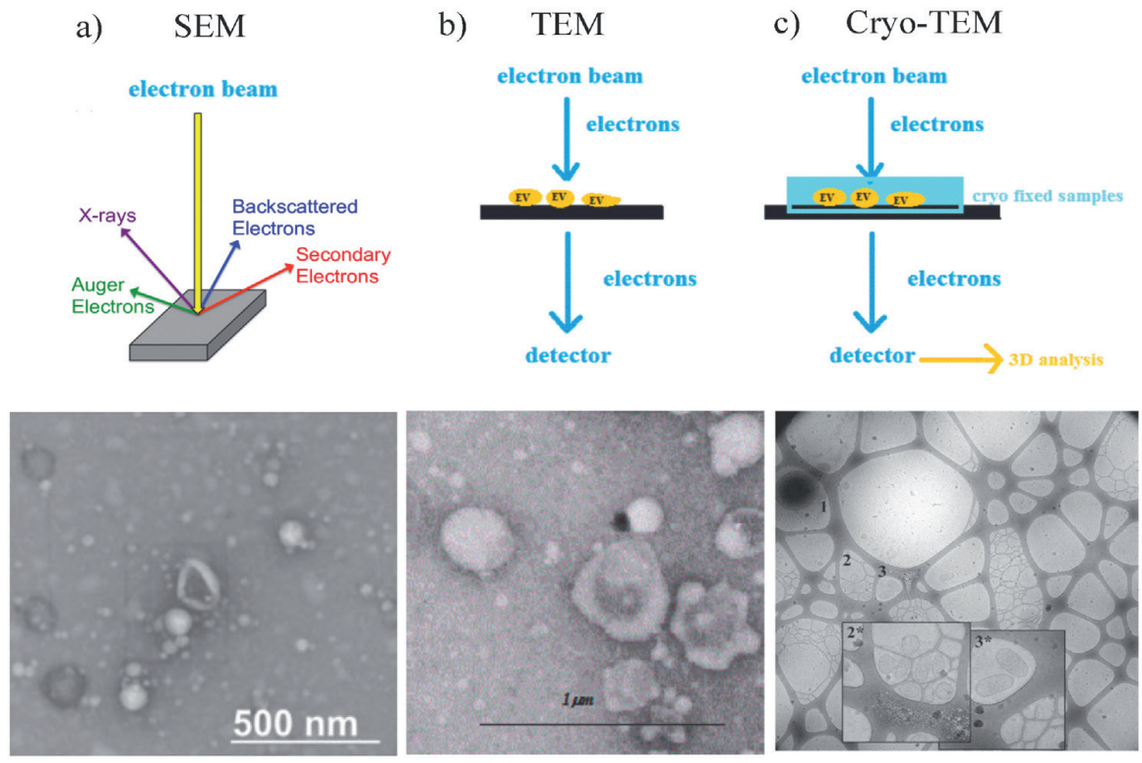

Figure 1.
The principle of a) SEM, b) TEM and c) cryo-TEM with an example of imaged EVs, respectively.

and particle size distribution are analyzed in AFM [2]. AFM tip indents the EVs and records the force-distance curve to provide insight into the mechanical properties, including mechanical stiffness, Young's modulus and bending modulus [4]. Tumor-exosomes of bladder cancer cell lines were proven to show differential mechanical properties dependent on the malignant state [5]. Although constrained in the throughput of EV analysis, the precise analysis of structural and biophysical properties via AFM provides unique measures in EV engineering for targeted delivery and therapeutic (**Figure 2**) [4].

2.3 Microfluidic device for single EV imaging

Combining with fluorescent microscopies, microfluidic devices are widely designed for single EV analysis. The EVs were biotinylated and captured on the streptavidin coated glass surface of a microfluidic device. The repeated cycles of immune staining-imaging-quenching steps allowed the investigators to analyze up to 11 biomarkers on immobilized individual EVs with an inverted microscope (Nikon Eclipse TE2000S) equipped with a sCMOS camera. After image processing, about 600 individual EVs were mapped onto a 2D plot via t-distributed stochastic neighbor embedding (tSNE) [6]. However, Biomarkers expressed on single EVs are at very

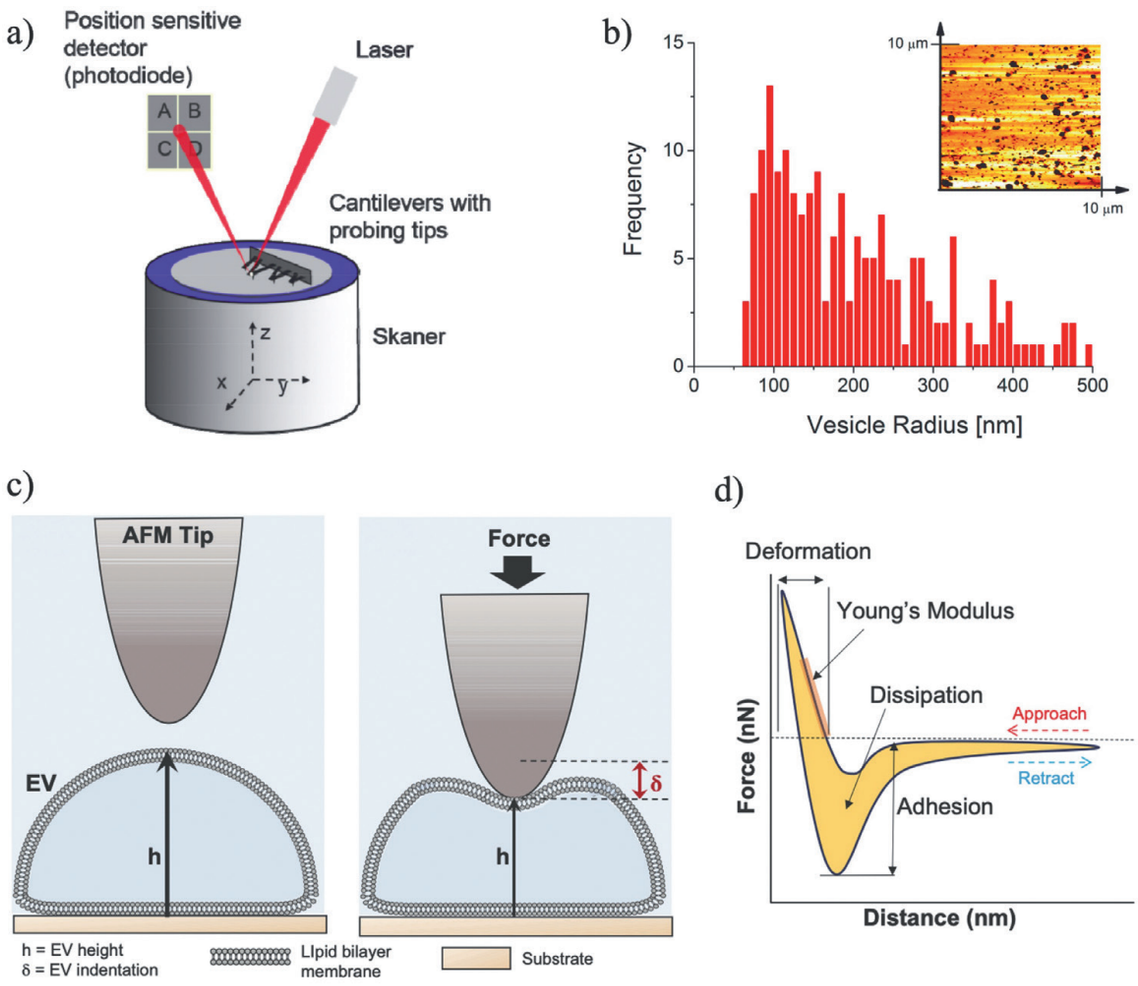

Figure 2.
AFM for analysis of single EVs. a) Principles of AFM detection. Panel b) shows an typical AFM readout of EV morphology detection and particle size distribution plot. c) and d) AFM measurement of biomechanical properties of EVs.

low abundant or has limited number of epitopes for immunostaining. Therefore, EV biomarker could be undetected due to weak signals. Molecular profiling of single EVs often requires high resolution optical microscopies or signal amplification strategies, such as rolling circle amplification, branched DNA probes or enzymatic reactions.

2.4 Total internal reflection fluorescence microscopy (TIRFM)

Total internal reflection fluorescence microscopy (TIRFM) is an optical technique utilized to observe single molecule fluorescence at surfaces and interfaces. It provides a high axial resolution below 100 nm. Single-vesicle imaging analysis could be done via TIRFM. The investigators can visualize multiple marker expressions of individual EVs by using fluorescent probes, and can also classify EV subpopulations by analyzing co-localization of markers [7]. With TIRFM system, targeted miRNA detection at single EVs could be realized with the co-delivery of inactive split DNAzymes and fluorescence-quenched substrates into EVs and a miRNA-activated catalytic cleavage reaction that amplifies fluorescence signal (**Figures 3** and **4**) [8].

2.5 Stochastic optical reconstruction microscopy (STORM)

Stochastic optical reconstruction microscopy (STORM) and direct-STORM (d-STORM) are single-molecule super-resolution imaging techniques with a practical resolution limit of 20 nm. STORM utilized the photoswitchable fluorescent probes to precisely localize detected molecules at a high spatial resolution. AlexaFluor 647-conjugated anti-CD63 antibodies were employed to detect cancer cell-derived EVs [9]. Using photo-switchable lipid dyes such as Dil, d-STORM imaging enabled rapid detection of EVs down to 20–30 nm in size and imaging of EV uptake by live cells in culture [10]. With STORM, droplet-based single-exosome-counting enzyme-linked immunoassay (droplet digital ExoELISA) approach enables absolute counting of EVs with cancer-specific biomarkers (**Figure 5**) [11].

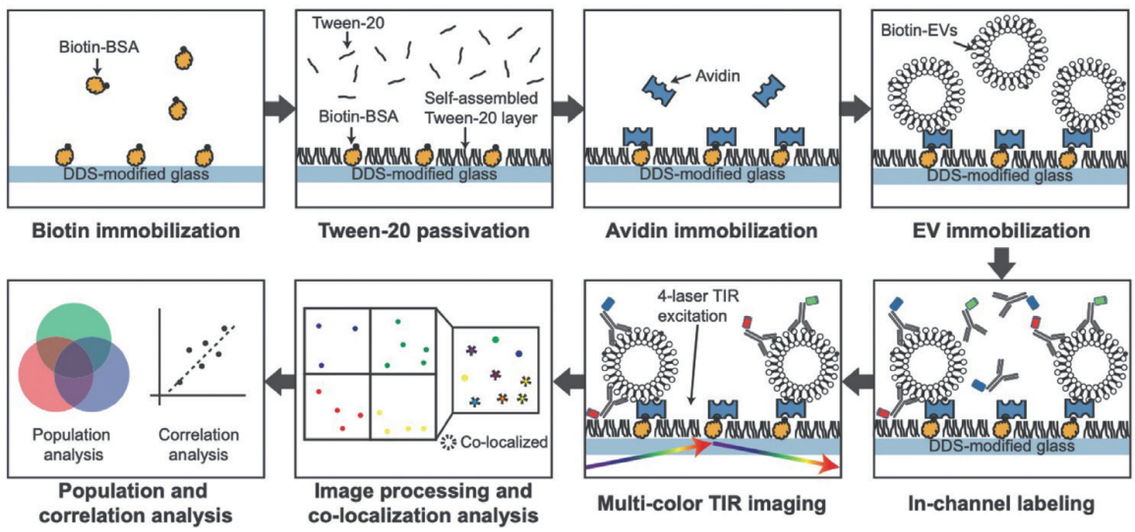

Figure 3.
TIRFM for single EV 3-plexed proteomic biomarker analysis. The colocalization of multiple biomarkers could be simultaneously recorded via multi-color TIR imaging.

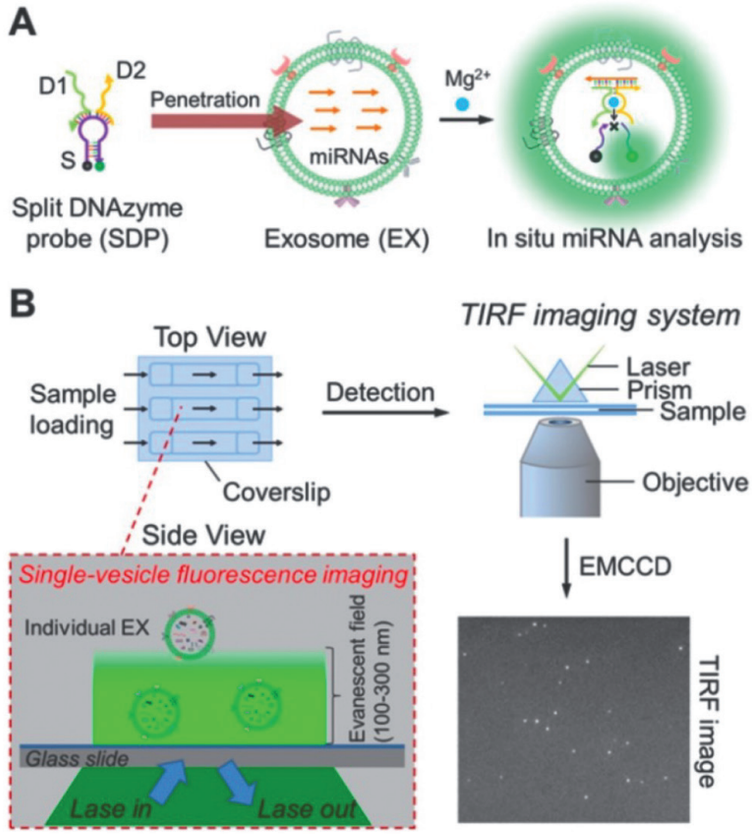

Figure 4.
TIRFM for single EV miRNA biomarker analysis.

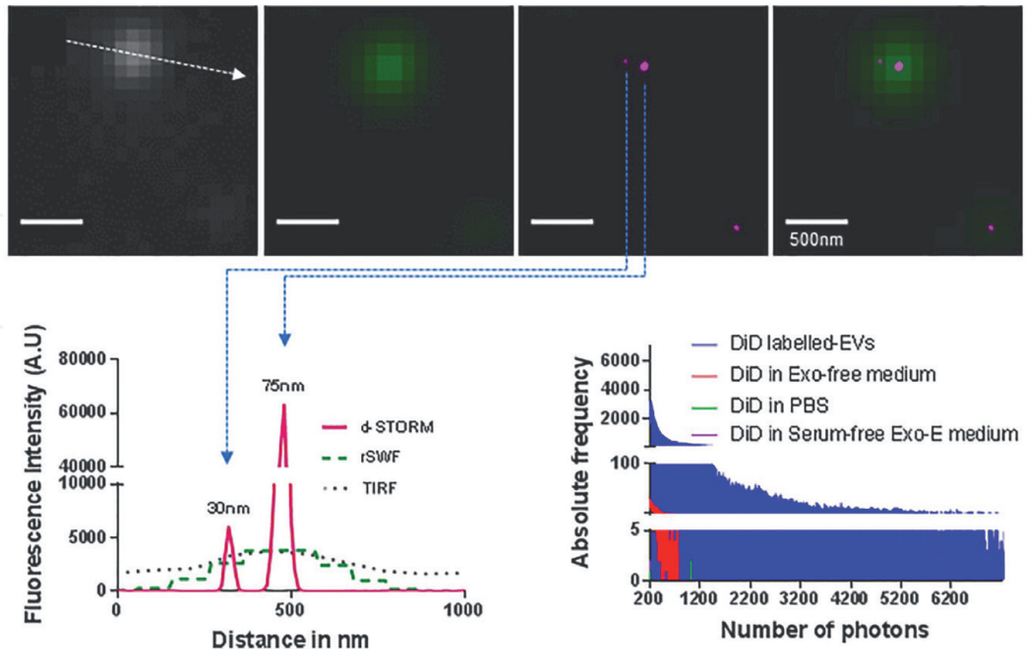

Figure 5.
Imaging of two adjacent exosomes with conventional TIRFM image, PALM/STORM image. Cross-sectional profiles of the two adjacent exosomes shown.

3. Flow cytometer (FCM)

Despite of the advanced technologies superior to the conventional microscopic systems, imaging methods are still not suitable for high-throughput or rapid detection. Therefore, scientists are committed to developing simple, sensitive and high-throughput exosome detection methods. Inspired by single cell technologies, flow cytometer attracted interests of scientists for multiplex large-scale single EV characterization. However, it is difficult to detect particles below 500 nm in size with the conventional FCM. Two approaches could be applied to overcome the problem. First, recognition of proteins on the single EVs was followed by a signal amplification step that could produce detectable structures through FCM. Second, the FCM were customized and optimized for detection in nanoscale.

3.1 Conventional FCM combined with amplified single EV signals

In the first approach, several methods are feasible for signal amplification to produce detectable structures via FCM. The proteins on single EVs could be detected

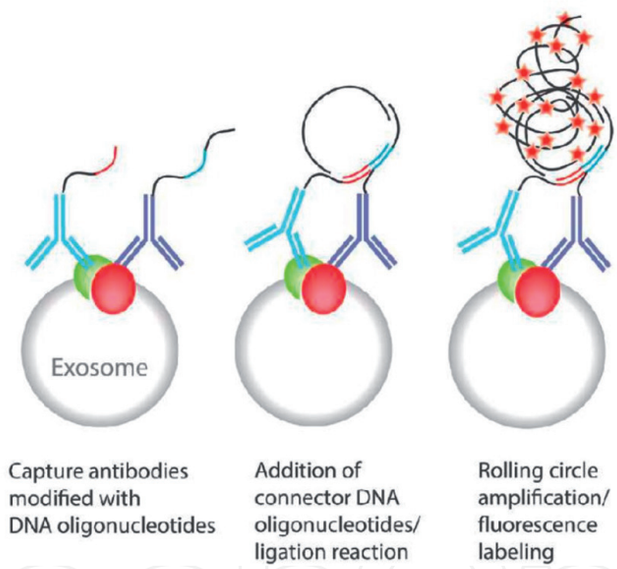

Figure 6.
Colocalization of two proteins on the same individual EV were detected via proximity ligation assay (PLA) and the signal was amplified via rolling circle amplification (RCA) to form a detectable structure under FCM.

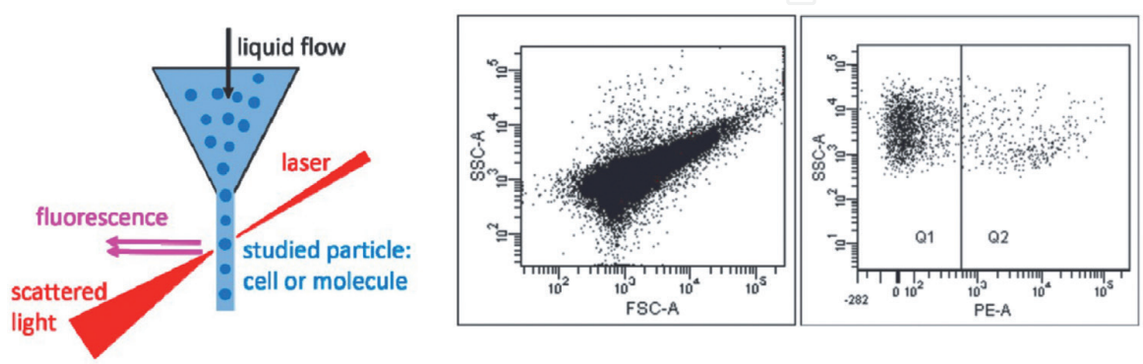

Figure 7.
The principle of flow cytometer (FCM) and an example of detected EV samples with scattered signals and fluorescently labeled Her-2 antigen.

with aptamers with a trigger sequence at the end, which then trigger hybridization chain reaction via DNA hairpins with biotin labels. Addition of avidin-fluorescent labels lead to the formation of an 500 nm fluorescently labeled EV-centered complex to be detect in FCM. Signal amplification via hybridization chain reaction occurred after single EV biomarker recognition by a conformation switchable aptamer. Two biomarkers could be targeted and analyzed simultaneously [12]. In another study, investigators utilized DNA oligonucleotides labeled antibodies to detect two proteins on the same EV. Proximity ligation assay occurs and rolling circle amplification (RCA) reaction produce thousands of copies of DNA sequences for fluorescent labels to bind and consequently forms a structure detectable in FCM (**Figure 6**) [13].

3.2 High resolution FCM and nano FCM

In the second approach, FCM were configured to overcome the limitations of detectable particle size and adapt to the analysis of nanoscale EVs. The improved sensitivity for EV detection includes higher laser power, slower flow and longer signal integration times and so on [14, 15]. Fluorescence based triggering could be superior over light scatter based triggering [16, 17]. Short wavelength laser can detect smaller particle size, for example 405 nm violet side scatter (VSSC) in Beckman Coulter CytoFLEX S [18]. As particle size decreases, the scattered light decreases rapidly. Data acquisition always needs to be optimized for better SSC resolution and efficacy of the detection of dim fluorescent single EVs. Therefore, novel noise reduction algorithm is applied for the high sensitivity detection of small particles. Calibrating light scatter detection for EV analysis have been proposed [19]. Nowadays, Apogee flow cytometry with micro-PLUS mode announced a detectable size of as low as 80 nm [20]. Yan's lab developed nano flow cytometer (nFCM) employing single molecule fluorescence detection in a sheathed flow. Two single-photon counting avalanche photodiodes (APDs) were used to detect side scatter and fluorescence of individual EVs, respectively, which enables phenotyping of single EVs as small as 40 nm (**Figure 7**) [21].

4. Nano-sensing technology

4.1 Nanoparticle tracking analysis (NTA)

Nanoparticle tracking analysis (NTA) is well acknowledged and commercially available technique for EV characterization. By tracking the Brownian motion of each particle, the hydrodynamic diameter of each particle is calculated with Stockes-Einstein equation. Thereafter, size distribution and concentration of EVs in a fluid sample could be obtained. Combined with fluorescent labeled antibodies or molecular beacons, EV subpopulation with expression of certain protein or miRNAs, respectively, could be quantified (**Figure 8**).

As a modified tracking analysis of EVs, an on-chip microcapillary electrophoresis system was built with a laser dark-field microscope. The tracking analysis of the electrophoretic migration of individual exosomes were performed and the zeta potential distribution of exosomes were able to be analyzed. The system consists of a chip, a pair of platinum electrodes, a DC power supply, a laser source, an inverted microscope, and an EMCCD camera (**Figure 9**) [22].

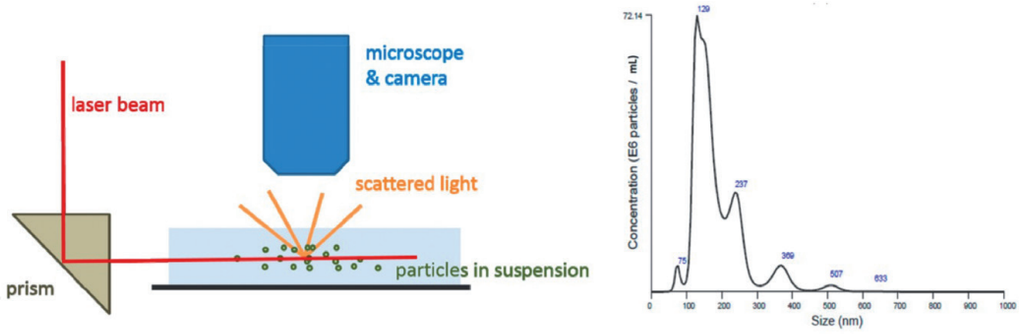

Figure 8.
The principle of nanoparticle tracking analysis (NTA) and its obtained EV size distribution.

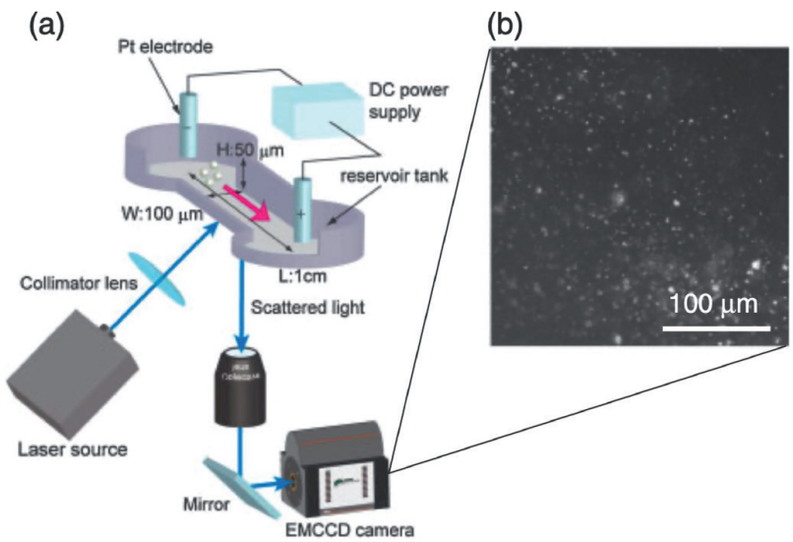

Figure 9.
Tracking analysis combined with zeta potential measurement of single EVs.

4.2 Fluorescence correlation spectroscopy (FCS)

Fluorescence Correlation Spectroscopy (FCS) were set up for characterize the GFP labeled vesicles at the single molecule – single vesicle level. FCS detects temporal fluorescence fluctuations in a defined confocal volume at a single photon sensitivity [23].

4.3 Surface plasmon resonance (SPR)

Surface plasmon resonance (SPR) detects real-time interactions between the receptors and the targeted biomolecules. SPR sensors show high sensitivity for label-free exosome quantification, which could be attributed to a nanoscale sensing range closing to exosome size. The receptors were immobilized on the surface of the biosensor. When a solution of biomolecules flows across, the targeted biomolecules interact with the receptors to change the refractive index of the sensor surface. The response of the sensor is measured in resonance units (RU) and is proportional to the mass of molecules interacting with the immobilized receptor. (**Figure 10**) Nano-plasmonic exosome (nPLEX) system was developed based on SPR principles and utilized 200 nm plasmonic nanoholes for EV capture and detection. nPLEX enables quantitative detection and proteomic profiling of EVs at the throughput of 12 biomarkers

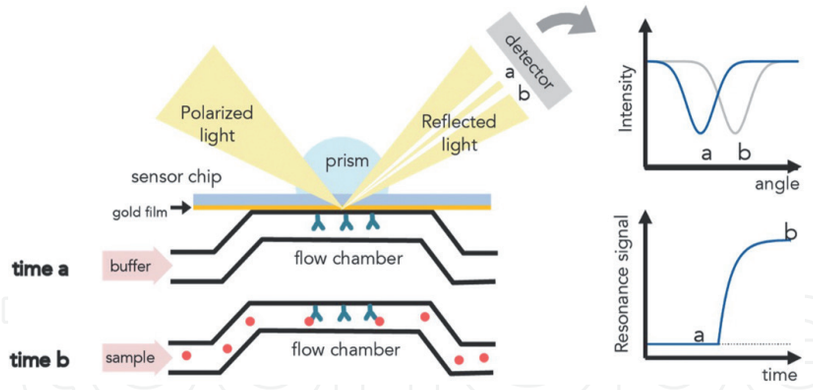

Figure 10.
The priciple of surface plasmon resonance (SPR).

detection with antibodies immobilized at 12 channels of a microfluidic chip [24]. The nPLEX-FL assay enables multiplexed single EV analysis of targeted markers with improved sensitivities. In nPLEX-FL assay, EVs are biotinylated and then captured on the nanohole surface with avidin coating. EVs are immunostained by fluorescent labeled antibodies. Plasmon-enhanced fluorescence detection can amplify fluorescence signals using plasmonic metallic nanostructures. The labeled EVs are imaged, and their fluorescent intensities are analyzed. Therefore, biomarker distribution analysis could be performed on a single-EV level [25]. A localized surface plasmon resonance imaging (LSPRi) platform improves the limit of detection down to the single exosome limit. With a 400 nanopillar array sensor chip, single EVs are captured by nanopillar for both imaging and spectrometer measurement (**Figure 11**) [26].

4.4 Raman spectroscopy

Raman spectroscopy is a spectral analysis method that determines the chemical properties of samples by measuring the vibration mode of covalent bonds caused by a radiation laser. Raman spectroscopy is non-destructive label-free analysis and therefore is suitable for analysis of biological samples. Laser tweezers Raman spectroscopy (LTRS)explore the chemical content of individual EVs, in which a tightly focused laser beam traps small particles at the laser's focal point. A confocal detection of Raman scattering from the precise focal volume allows EVs to be studied individually. Based on the exosomal chemical differences, EVs could be classified into subpopulations (**Figure 12**) [27–29].

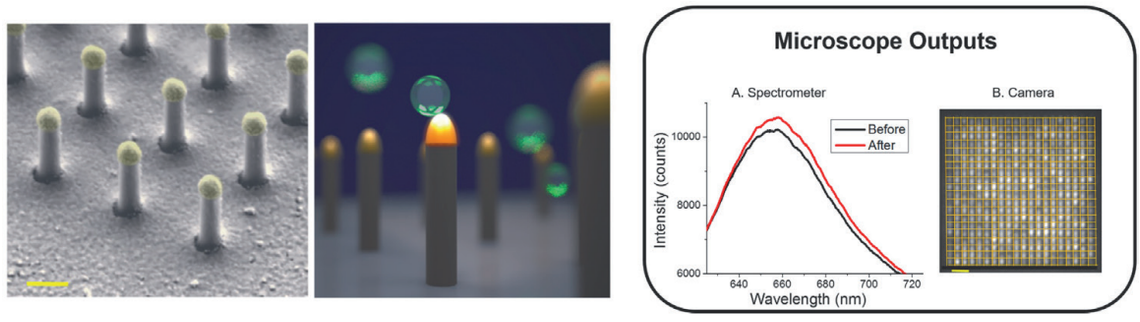

Figure 11.
A localized surface plasmon resonance imaging (LSPRi) nanopillar platform enables both spectrum and image readout.

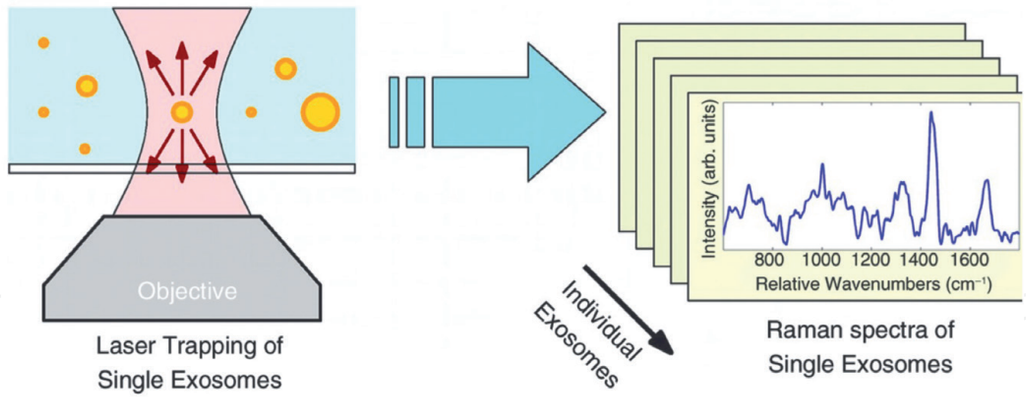

Figure 12.
The principle of laser tweezers Raman spectroscopy (LTRS).

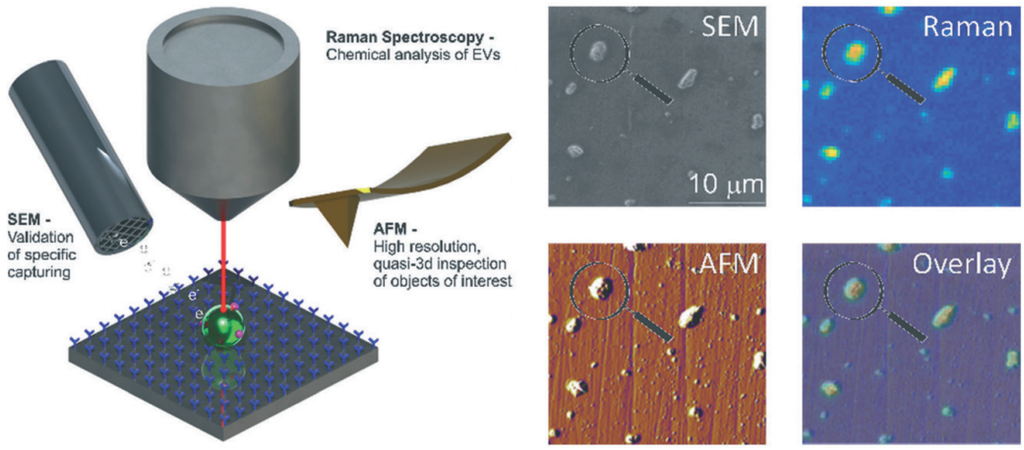

Figure 13.
Multi modal characterization of antibody captured individual EVs via SEM, AFM and Raman spectroscopy.

Surface-enhanced Raman spectroscopy (SERS) is a type of molecular vibration spectrum with enhanced Raman scattering signals from molecules adsorbed on noble metal (Ag, Au) nanostructures. SERS has been applied for the detection of cancer exosomes due to its high sensitivity, specificity, and multiplexing capability. Single EV analysis was performed in several studies. An graphene-coated periodic gold-pyramid were used as SERS substrate to detect single EVs and EV samples from different biological sources were distinguished in their Raman signature [30]. In another study, gold nanoparticles (AuNP) coated single EVs were formed due to electrostatic interaction between cationic AuNP and anionic EV membrane. SERS spectra of individual ELVs with a high density of AuNP were then acquired and variations in the SERS spectra of individual ELVs could be used for EV heterogeneity analysis [31]. Multi modal characterization of individual EVs could be achieved after EV capture on antibody functionalized stainless steel substrate followed by characterization via SEM, AFM and Raman spectroscopy (**Figure 13**) [32].

5. Barcoding based single EV analysis

In the methods described above, the number of simultaneously analyzed biomarkers is limited to types of distinguishable readout signals like fluorescent dyes etc.

To achieve multiplexed high-throughput single EV analysis, researchers unitized molecular tools named Proximity Barcoding Assay (PBA) to detect colocalized proteins on the same EV [33]. The principle is that only proteins on the same individual EV are in proximity and therefore labeled with the same EV tags in PBA.

PBA probes were prepared by labelling of selected antibodies with DNA oligonucleotides containing unique 8-nt protein tag (**Figure 14a**). Rolling circle amplification

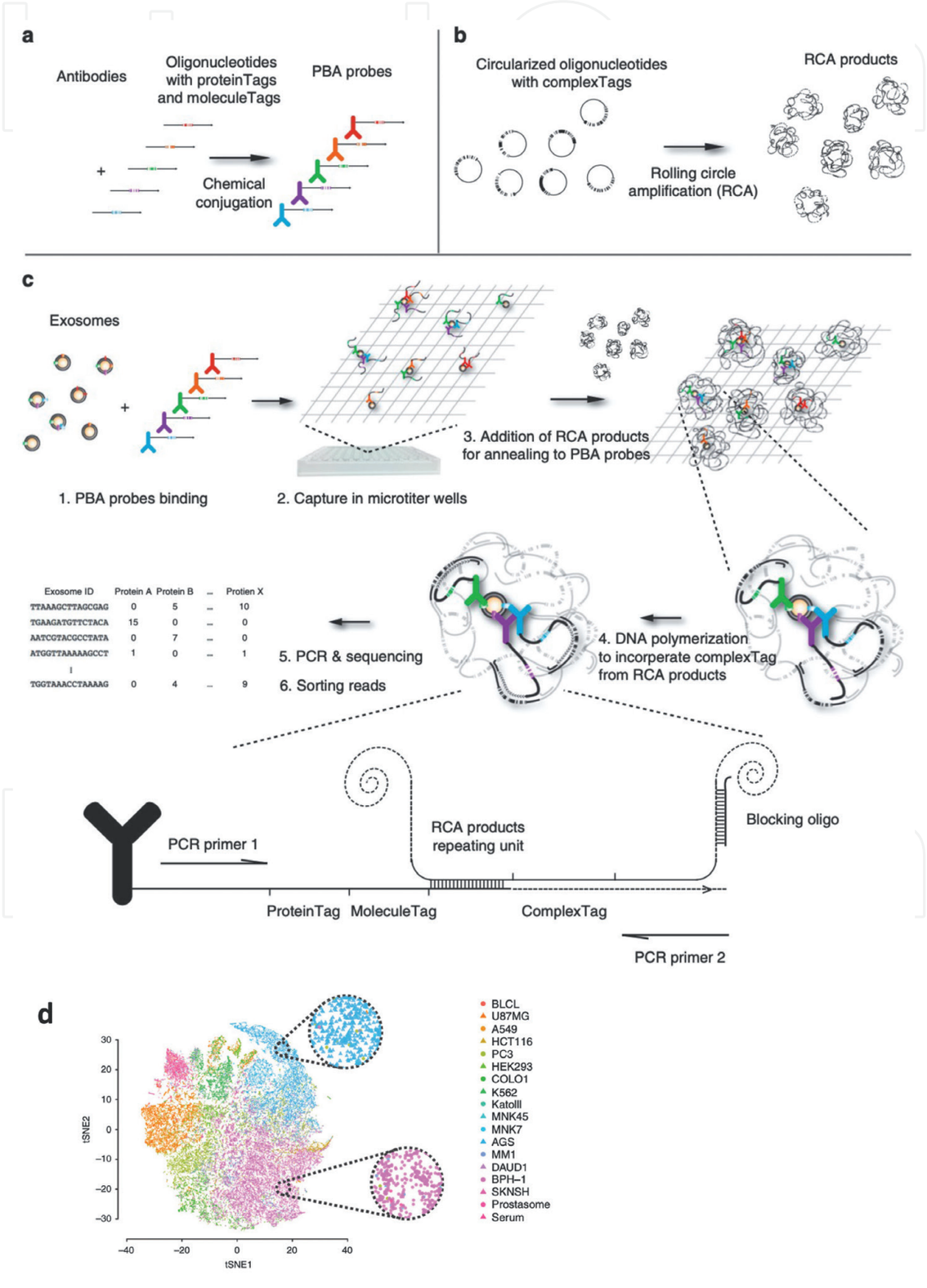

Figure 14.
Schematic illustration of proximity barcoding assay (PBA) and 38-plex analysis of 18 EV samples.

	Analytical methods	capabilities	Throughput	Advantages	Disadvantages	Sample requirement
Imaging	AFM	Dimensional and mechanical properties	1–100 EVs per image	High resolution Unique mechanical property	Device dependent No composition information	Purified EV sample
	SEM	Morphology and size	1–100 EVs per image	High resolution	Device dependent No composition information Collapse of EV during imaging	Purified EV sample
	TEM	Morphology and size	1–100 EVs per image	High resolution	Device dependent No composition information	Purified EV sample 300 μl
	TIRFM	Fluorescently labeled EVs Protein on single EVs	1–100 EVs per image	Biomarker analysis	Resolution of about 100 nm Limited number of biomarkers	Purified EV sample 300 μl
	STORM	Fluorescently labeled EVs Protein on single EVs	1–100 EVs per image	High resolution of about 20 nm Biomarker analysis	Limited number of biomarkers	Purified EV sample
Flow cytometer	Signal amplification for conventional FCM	Proteins on single EVs	10^3–10^4 EVs per minute	Biomarker analysis	Device needed Dependent on antibody affinity Limited number of biomarkers	Purified EV sample, 500 μl 10^8–10^{10} particle/ml
	Nano FCM or high resolution FCM	Biomarkers on single EVs	10^3–10^4 EVs per minute	Biomarker analysis Direct analysis of EVs	Not for small EVs Limited by antibody affinity Limited number of biomarkers	Purified EV sample, 500 μl 10^8–10^{10} particle/ml

	Analytical methods	capabilities	Throughput	Advantages	Disadvantages	Sample requirement
Nano-sensing	NTA	Size distribution Concentration of EV samples	10^6 EVs	Precise size distribution and quantification	Limited possibility in detection of biomarker	Purified EV sample 100 µl 10^8–10^{10} particle/ml
	SPR	Amount of EVs interacting with specific receptor	NA	Label-free Real time High sensitivity	Device dependent, Limited possibility in detection of biomarkers	Purified EV sample
	Raman spectroscopy	Chemical composition	10–100 EVs per analysis	Label-free, real time,	Device dependent, Limited possibility in detection of biomarkers	Purified EV sample
Barcoding	Proximity Barcoding Assay	Proteomic profiling (38-plex protein count for each EV)	$>10^6$ EVs per test	Highly multiplexed High- throughput Small sample volume No EV enrichment needed	Dependent on antibody affinity	Body fluid (cell-free) or purified EVs 2–20 µl

Table 1.
Comparison of single EV analysis methods.

(RCA) products were prepared from circularized oligonucleotides comprising a 15-nt random sequence. Because15-nt random sequence are capable of encoding 4^{15} (about one billion) unique sequences to work as EV tags, RCA products are utilized as templates to barcode antibody conjugated oligonucleotides on single EVs (**Figure 14b**). EVs were incubated with PBA probes and then captured via immobilized cholera toxin subunit B (CTB). Oligonucleotides on PBA probes brought together by binding the same EV are allowed to hybridize to a unique RCA product and therefore obtain the same EV tag via enzymatic extension. Successfully extended DNA molecules on PBA probes are amplified by PCR. The PCR product were subjected to DNA sequencing to record the combinations of EV tag - protein tag thus revealing the proteomic profiles of individual EVs.

In the study of 38-plex PBA analysis, individual EVs of 16 types of cell lines, prostasome and serum could be profiled. The information researchers could obtain includes protein expression and the pattern of protein combinations on individual EVs. The differences of samples were visualized by t-SNE according to their protein compositions.

6. Conclusion

Based on the technology, we discussed the general strategies of single EVs analysis including imaging, flow cytometer, nano-sensing and single EV barcoding. EVs could be classified into subsets according to their chemical composition, size, expression of specific biomarkers etc. The advancing of technologies to reveal EV heterogeneity are the pursuit of scientists from multiple disciplines.

Each EV has a unique story, the start and the end of their journey, the cargo they carried and the mission they accomplished. Revealing the heterogeneity of EVs and getting closer to the detailed facts of the abundant populations of EV will endow us the knowledge of how cells in our bodies talks to each other and what they say about our health (**Table 1**).

Acknowledgements

This work was supported by the National Natural Science Foundation of China under Grant 81802052; China Postdoctoral Science Foundation under Grant 2018 M643318; the Guangdong Key Laboratory funds of Systems Biology and Synthetic Biology for Urogenital Tumors under Grant 2017B030301015.

Conflict of interest

D.W. has filed a patent application (PCT/SE2014/051133) describing the PBA technique. D.W. is a shareholder of Vesicode and Secretech.

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