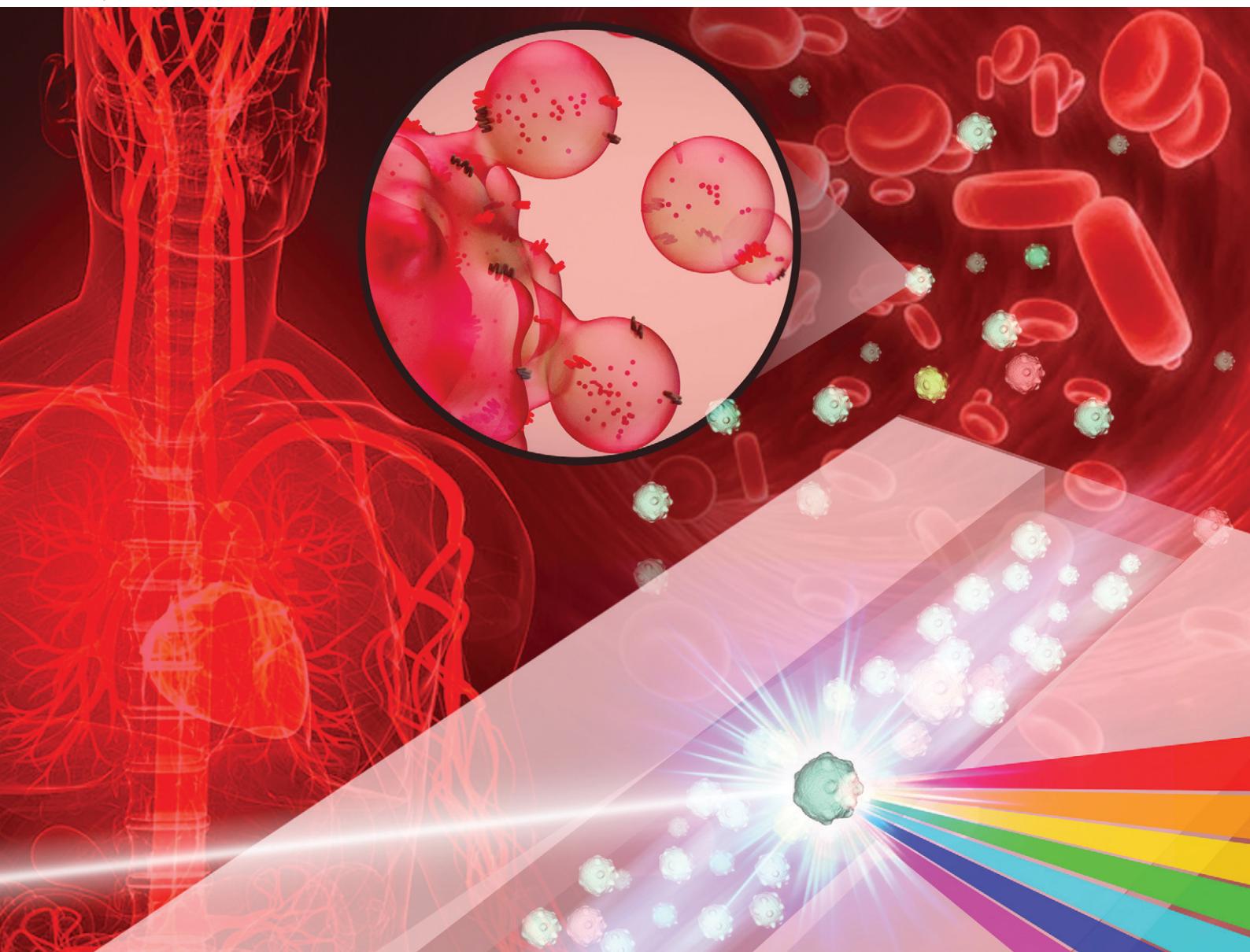


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Emerging technologies for profiling extracellular vesicle heterogeneity

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Extracellular vesicles (EVs) are membrane-bound vesicles secreted by most cell types and exist in virtually all bodily fluids. They carry on a wealth of proteomic and genetic information including proteins, lipids, miRNAs, mRNA, non-coding RNA and other molecules from parental cells. Increasing evidence shows that within populations of EVs, their biogenesis, physical characteristics (e.g. size, density, morphology) and cargos (e.g. protein, lipid content, nucleic acids) may vary substantially, which accordingly change their biological properties. To fully exploit the potential of EVs, it requires qualified methods to profile EV heterogeneity. In this review, we survey recent approaches for EV isolation with innovative discoveries in heterogeneity. The main challenges in EV heterogeneity research are identified, and the roles of single cell EV profiling and single EV imaging are highlighted. We further discuss promising opportunities for resolving the underlying complexity of EV heterogeneity.

1. Extracellular vesicles

Extracellular vesicles (EVs) are membrane-bound phospholipid vesicles actively secreted by most cells, existing in almost all kinds of body fluids, like blood,^{1,2} ejaculates,^{3,4} urine,^{5,6} cerebrospinal fluid,^{7,8} saliva,⁹ and breast milk.^{10,11} EVs carry specific cargos such as mRNA, miRNA and gDNA fragments, and a myriad of different proteins depending on the cell of origin (Fig. 1). The cargos allow cells to exchange messages, thereby altering the function of the receiving cells and facilitating a range of important cellular functions.



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Based on their biogenesis and biophysical characteristics, EVs have been traditionally classified into three major subtypes (Fig. 1a): (I) exosomes – generated within multivesicular endosomal compartments and secreted when these compartments fuse with the plasma membrane (PM), with sizes in the range of 40–200 nm; (II) ectosomes or shedding microvesicles (MVs) – directly formed and released from the cells' PM, with sizes in the range of 200–2000 nm; (III), apoptotic bodies – formed as a consequence of apoptotic disintegration, with sizes in the range of 500–2000 nm.^{12–15}

The functions of EVs can vary greatly depending on their inherent compositions of each class. Exosomes are known for intercellular communication in both normal and diseased tissue,^{7,8} and its biogenesis starts within the endosomal system, and the release of exosome involves several cellular steps.^{12–14} MVs are involved in the regulation of programmed cell death,¹⁶ modulation of the immune response,¹⁷ inflammation,¹⁸ angiogenesis,¹⁵ and coagulation.¹⁹ Oncosomes are cancer-specific MVs transferring oncogenic messages and protein complexes across cell borders.²⁰ The apoptotic bodies are known for facilitating phagocytosis and are formed only during programmed cell death.²¹ Due to the above specific characteristics, EVs are promising biomarkers in cancer diagnostics and mediators in drug delivery.^{18,19}

2. Heterogeneity – a hidden world beneath population averages

At the current stage, one of the biggest challenges for the EV is to address the heterogeneity within EV populations. For one thing, according to the latest consensus of the International Society for Extracellular Vesicles (ISEV), assigning an EV to its biogenesis pathway is extraordinarily difficult. No isolation methods available can separate EVs based on their biogenesis and the isolation fractions overlap



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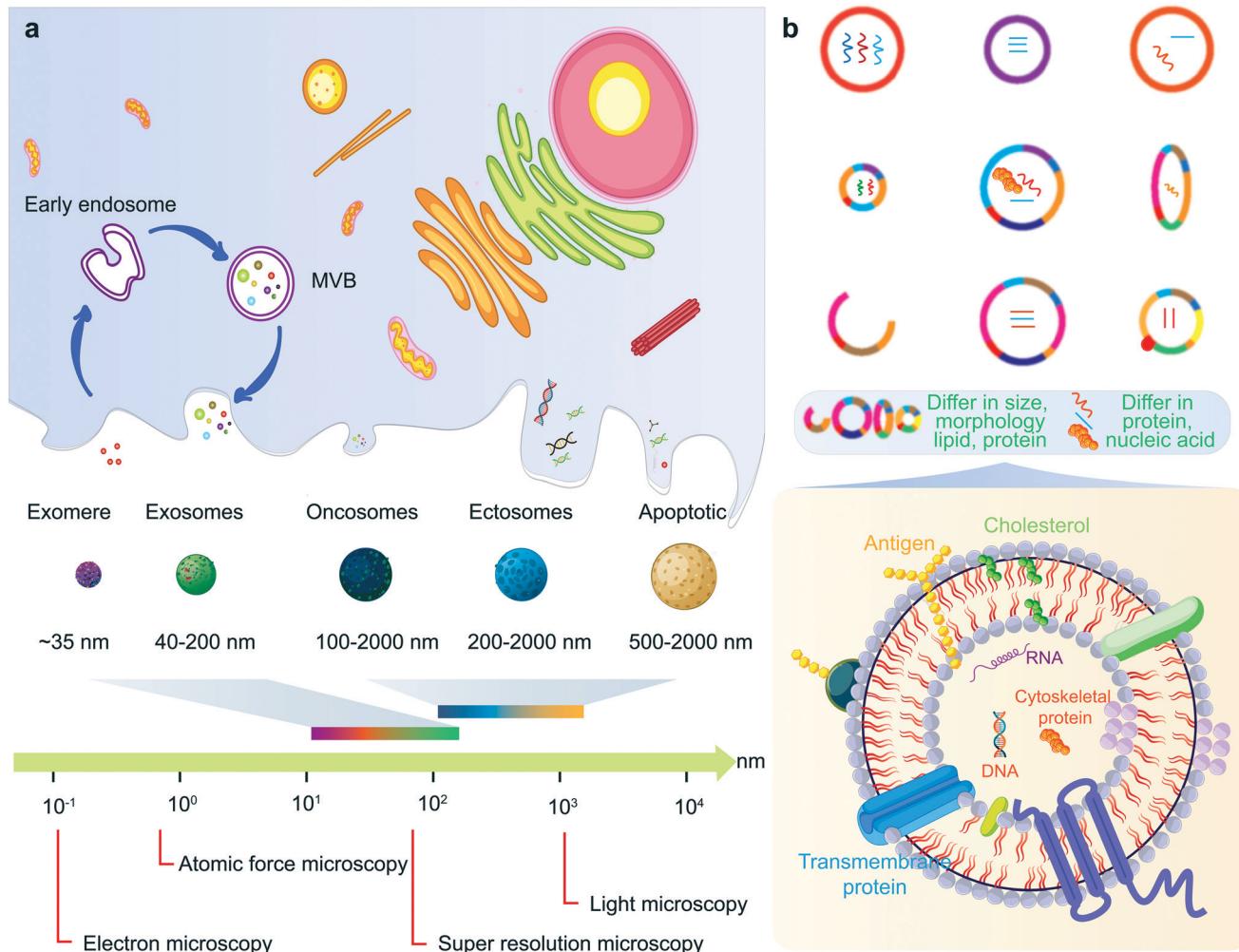
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when using current isolation methods.²² Growing evidence shows that within the biogenesis classifications of EVs, various subpopulations may exist, even with EVs originating from the same parental cells.^{23–28} Their physical characteristics (size, density, morphology), cargos (protein, lipid content, nucleic acids) vary substantially, and biological properties may be changed accordingly. In this way, all above contribute to the heterogeneity of EVs (Fig. 1b). A recent guideline established in ISEV has suggested the use of (a) physical characteristics of EVs, including the size (small EVs (sEVs) and medium/large EVs (m/IEVs) and density; (b) biochemical composition (CD63⁺/CD81⁺ EVs, annexin A5-stained EVs, etc.); or (c) descriptions of conditions or cell of origin (e.g., oncosomes, hypoxic EVs) for describing EV subtypes unless their biogenesis pathway can be clearly defined.²²

EVs have a diversity of physical characteristics. It is well known that EVs have diverse sizes and diverse density, and even EVs with similar sizes could have different densities,²⁹ as discussed in more detail in many previous reviews.^{26,30,31} To be emphasized here is that the size and density of different EV subpopulations, even from the same parental cells, may significantly overlap due to the limitation of current isolation technologies. Furthermore, various morphologies of EVs have been confirmed by electron microscopy techniques. It has been shown that non-spherical EVs exist, and EVs can be featured with different shapes (such as long tubule-like, round, protrusions on surface, a round incomplete structure and so on) even if they are produced by a single cell type.^{32–35} The protrusions could be made up of proteins with specific functions, e.g. facilitating membrane fusion, which could allow cargo delivery into the cytoplasm of a potential target cell.³⁶ The variations in the morphologies of the EVs are indicative of the existence of different subpopulations that may have different functions.

Moreover, EVs also have a diversity of cargos. Too much overlap in the size of isolated EVs prohibits clear-cut differentiation by proteomic quantitation and complicates the study of EV heterogeneity in biological properties.^{37–39} Different EV subpopulations secreted by neuroblastoma cells were reported to differ in the mutually exclusive presence of tetraspanin CD63 and amyloid precursor protein, which result in their different capabilities to target different cells.¹⁷ Compared to proteomics, studies on lipid contents in EVs are under inadequate representation.⁴⁰ The lipid bilayer consists of two chains of the hydrophilic phosphate head and the hydrophobic tail, with a variety of possible modifications and configurations, which could lead to heterogeneity in lipid contents.⁴¹ The main limitation in EV proteomics and lipidomics largely depends on the isolation techniques.⁴² Similarly, profiling the nucleic acid content of EVs faces the same challenges.^{28,43}

The EV content was generally thought of as stochastically packaged. Evidence has shown that the uptake of cytoplasmic components during classical exosome biogenesis is not random, and the exosome loading can be a highly regulated



process.⁴³ RNA sorting into exosomes is unlikely to be random as well.^{44–46} The EVs' cargo is a reflection of what the cell is experiencing in response to different physiological and pathological conditions. EVs are a promising disease biomarker and could serve as a potential therapeutic tool. The main obstacle to understanding “disease-specific” EVs is the lack of sufficient tools to reveal the mysteries of EV heterogeneity among overlapped populations.

3. Current approaches for EV heterogeneity analysis

The limited capabilities of current sEV isolation and analysis techniques can only reveal a tip of the iceberg of EV heterogeneity.⁴⁷ Most isolation methods, including ultracentrifugation, commercial precipitation kits or size-exclusion chromatography, can only yield overlapped

populations of EVs based on the underlying physical mechanisms such as size, density and solubility. In addition, the isolated EVs are easily contaminated by protein aggregates as well as lipoprotein particles, which could lead to artefacts and complicate downstream analysis.^{16,48} In this part, we will specifically discuss a few representative physical separation approaches that can fractionate different populations with innovative findings contributing to the EV heterogeneity study.

Ultracentrifugation & density gradients

Ultracentrifugation is the most popular method to separate EVs with a specific size range. Recently, a novel population of EVs (P200 fraction) smaller than exosomes which can promote cell proliferation was identified, distinguishing them from exosomes in size, protein, and biogenesis

pathway.²⁷ However, ultracentrifugation is not a good choice for separating heterogeneous EV subpopulations, but density gradients can move one step further.

Density gradients, based on either sucrose or iodixanol are used in ultracentrifugation to separate EVs according to their flotation speed and equilibrium density. Protein aggregates sediment into a sucrose or iodixanol gradient, whereas lipid-containing vesicles with different densities float upward to the different positions of equilibrium buoyant density fractions (Fig. 2a). Such an approach, when combined with ultracentrifugation, has allowed for the first time the identification of mutant proteins that can be secreted with exosomes and ectosomes.⁵⁵ Parallel quantitative proteomics analysis on the EV subtypes has further revealed that CD81 and MMP2 can be used as exosomal and ectosomal markers, respectively. Using the same method, different types of EVs, specific exosomal (CD63, CD81, or CD9) and non-exosomal subpopulations within small EVs from a single cell type can be isolated (Fig. 2b).²³ This approach represents one of the earliest attempts to address EV heterogeneity. Recently, a high-resolution iodixanol density gradient method was utilized to isolate small EVs from a non-vesicular gradient. The study found that Argonaute 1–4 and major vault proteins are released free of small EVs and annexin A1 is a specific marker of MVs shed from the PM.⁴¹ The progress would benefit the proper evaluation of the molecular mechanisms of biogenesis and the respective functions of EV subtypes.

Sequential centrifugal ultrafiltration

Sequential centrifugal ultrafiltration (SCUF) allows for the separation of EV subtypes by utilizing the centrifugal force that allows substances of specific relative molecular mass to pass through or to be intercepted on the ultrafiltration membrane.^{56,57} It typically requires centrifugation at 100 000g for 1–2 h to obtain nanometer-sized EVs from the filtrate. Solvent and small molecules can be filtered through the membrane, while the molecules with higher relative molecular masses can be trapped in the ultrafiltration membrane, thereby achieving the separation. SCUF is usually used in combination with size-exclusion chromatography to achieve a higher yield and purity.⁵⁸ A series of different pore-sized polyvinylidene fluoride ultrafilters has been applied for isolating both small EVs and large EVs from LIM1863 colon cancer cells.⁵⁹ Notedly, it was found that the large EVs promote invasion to recipient NIH3T3 cells more significantly (3-fold more) than small EVs. However, a critical limitation of ultrafiltration is that it cannot separate dissolved low molecular weight species. In addition, efficient fractionation by ultrafiltration is only possible if the species differ in molecular weight by a factor of 10 or more.

Asymmetric flow-field flow fractionation

Asymmetric flow-field flow fractionation (AF4) can be used to separate different sizes of EVs based on their density and hydrodynamic properties (Fig. 2e). By using two

perpendicular flows, forward laminar channel flow and variable crossflow in AF4, two exosome subpopulations have been identified recently. Importantly, the technique has led to the discovery of an abundant subpopulation of even smaller non-membranous nanoparticles (~35 nm, named exomeres) (Fig. 2f).⁵⁰ Different populations show unique N-glycosylation, protein, lipid, DNA and RNA profiles and organ biodistribution. Compared with traditional centrifugation methods, AF4 relies on more specialized fractionation equipment and requires highly trained personnel to operate.

Immunoaffinity capture

Most of the above isolation approaches rely on specific physical properties of EVs to passively select subpopulations of EVs. They don't allow the purification of EVs with desired biochemical properties. In comparison, the use of capture antibodies attached to different matrices such as microbeads, nanoparticles, membranes or chip surface allows the analysis of a specific subgroup of EVs expressing the surface markers recognized by the capture antibodies. Using a combination of capture and detection antibodies, the immunoaffinity capture method enables easy screening of surface markers on specific populations of EVs. Among them, antibody coated microbeads are especially popular, and several relevant products have come into the market (Fig. 2c). In recent years, several interesting discoveries have been found using the immunoaffinity capture approach. A novel multiplexed bead-based platform has been demonstrated to investigate up to 39 different surface markers in one sample (Fig. 2d). It has been found that NK cell-derived EVs and platelet-derived EVs are devoid of CD9 or CD81, and that EVs isolated from activated B cells comprise different EV subpopulations.⁴⁹ Furthermore, the contents of EVs can be analyzed by a subsequent lysing of EVs captured on the matrices, but correlating the inner contents to individual isolated EVs remains elusive. More recently, it was found that small EVs do not contain DNA and active secretion of cytosolic DNA occurs through an amphisome-dependent mechanism.⁴¹ This progress takes one step forward to understand how nucleic acid contributes to EV heterogeneity. However, compared with label-free methods, the immunoaffinity capture method detects only EVs with known surface markers; thus, EVs not expressing known markers or with unknown markers will be lost. The EVs captured by the microbeads can be further analyzed by conventional flow cytometry, but the approach cannot profile individual EVs because many EVs are captured on a bead.

Flow cytometry

Flow cytometry is one of the most popular techniques for detecting heterogeneous mixtures of single particles (Fig. 2g). Conventional flow cytometry uses light scattering as a trigger parameter and is limited to analyzing particles ≥ 300 nm.⁵² The small size of EVs makes it difficult to measure EVs

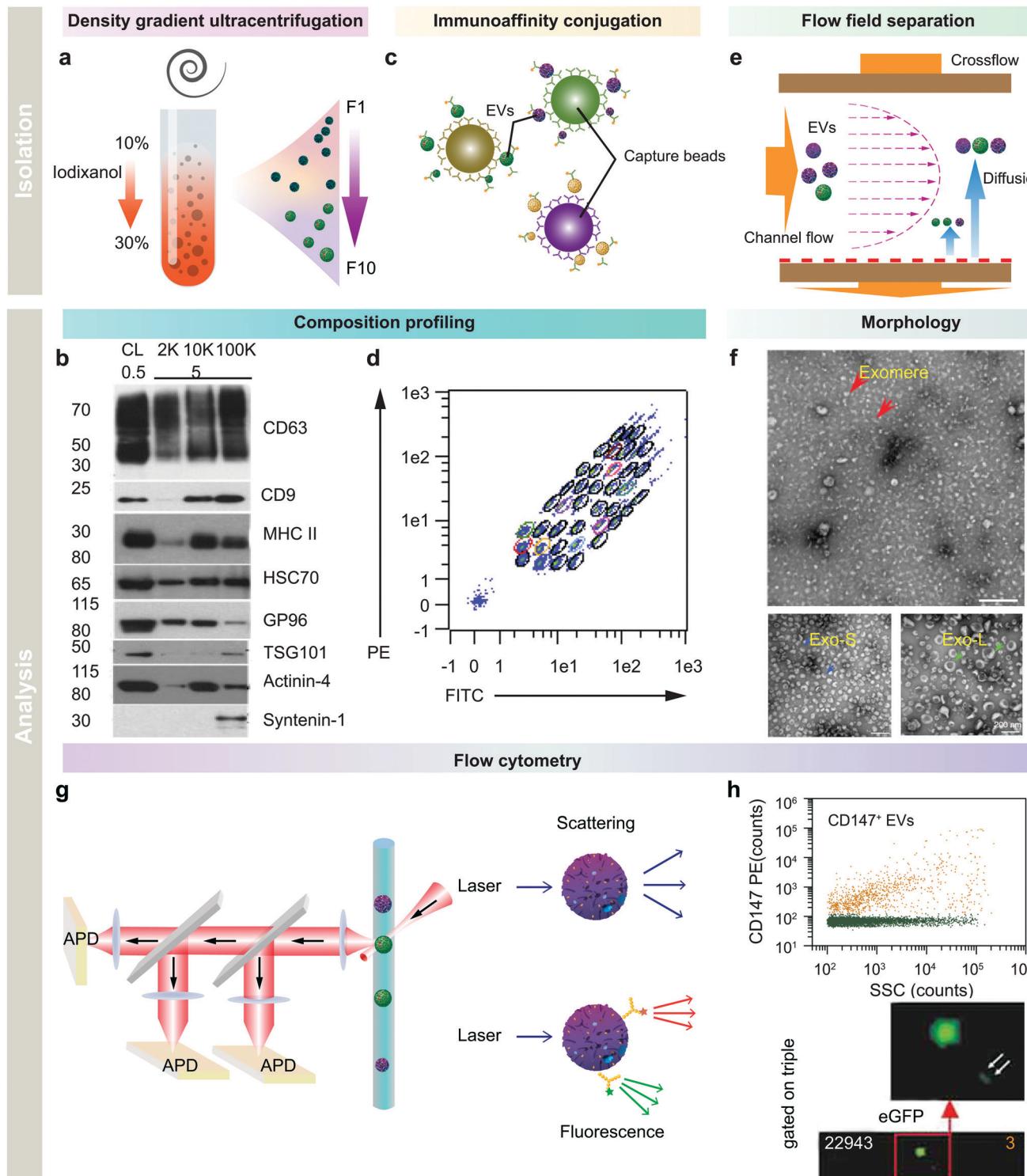


Fig. 2 Representatives of current isolation approaches for EV heterogeneity research. (a) Overview of iodixanol density gradient ultracentrifugation. (b) Western blot analysis of different proteins in EVs isolated from mouse bone marrow-derived dendritic cells.²³ Syntenin-1 and TSG101 are present only in the 100K pellet. GP96 are mainly present in 2K and 10K pellets. HSC70, actinin-4 and MHC II are present in all EVs. Small EVs (100K pellet) are co-enriched in CD63 and CD9 tetraspanins. (c) Schematic of immunoaffinity capture method. (d) Isolated EVs are incubated with 39 different bead populations.⁴⁹ Each bead population is coupled with a different capture antibody which is distinguishable by flow cytometry. Positive bead populations are highlighted in colors. (e) Principle of EV isolation by asymmetric flow field fractionation. (f) TEM imaging analysis of fractionated exomere, small exosome and large exosome subpopulations.⁵⁰ (g) Flow cytometry for EV heterogeneity analysis. Conventional flow cytometry is enabled by scattered light signals, fluorescence staining, signal amplification,⁵¹ higher laser power and slower flow^{52,53} that can be used for EV heterogeneity analysis. (h) High-sensitivity nanoparticle flow cytometry analysis of EVs at the single-particle level.^{53–55} Top: Bivariate dot plot of the PE orange fluorescence versus side scattered signal for EVs isolated from a patient sample upon immunofluorescence staining with PE-conjugated MAb against CD147.⁵³ Bottom: enlarged image demonstrates that even faint spots very close to each other are counted correctly as distinct spots/EVs.⁵⁴

directly using conventional flow cytometry as the scatter intensity from EVs drops off rapidly with the decrease of the particle size. Single EV analysis in conventional flow cytometers can be enabled by applying signal amplification methods. For instance, target-initiated engineering of DNA nanostructures on individual EVs allows an increase of the fluorescence signals from single EVs.⁵¹ This technique employed a conformation-switchable DNA probe to bind to the EV surface marker, which triggers a hybridization chain reaction (HCR) of DNA nanostructures. The HCR method can enlarge the overall size of single EVs to beyond 500 nm, where multiple fluorophores can be bound to amplify the signal. This enabled the visualization of single EVs in a conventional flow cytometer and greatly simplified the measurement of multiple markers on the same EV. Compared with light scattering, fluorescence has some advantages as a trigger parameter, including an improved resolution against the background and well-established fluorescence calibration protocols.

In addition to the chemical methods for signal amplification, advances in optical engineering have enabled the detection of single EVs in flow cytometry. Recently, a two-color high-sensitivity flow cytometer (HSFCM) has been demonstrated by using higher laser power and slower flow rates than what are typically used in conventional instruments.⁵² The higher laser power can excite more fluorophores into higher states, and the slow flow rates allow more emitted photons to be collected by using a high-sensitivity single photon counting avalanche photodiode with a high quantum yield. HSFCM allows the enumeration and estimation of the size of individual EVs down to 40 nm, as well as the measurement of the presence of surface markers to identify phenotypic subsets of EVs (Fig. 2h).^{53,55} Moreover, HSFCM could offer a rapid approach for quantitative multi-parameter analysis of single EVs with an analysis rate of up to 10 000 particles per minute. Imaging flow cytometry is a technique complementary to conventional flow cytometry based on intensity measurement. It allows imaging of samples in a flow, and all HSFCM signals are collected through the microscope objectives and quantified based on images detected by a charge-coupled-device camera. More acquisition and analysis parameters including the morphology and size of EVs could be defined and optimized for the analysis of single EVs (Fig. 2h).⁵⁴ When analyzing the function of a single type of EV (*e.g.*, sEVs or m/LEVs) exclusively, one may overlook a potentially important function of an EV subtype due to the presence of the other subpopulations if they are studied in a mixture. The methods above are usually combined together to isolate heterogeneous EVs.⁶⁰

Microfluidic-based EV isolation approaches

Microfluidics-based EV isolation approaches have become a research focus in the field of EV research. In general, microfluidic techniques have been used in the separation of different types of particles with sizes ranging from a few

nanometers to a few hundreds of micrometres.⁶¹ There have been many recent review articles summarizing microfluidic methods to separation subpopulations of EVs, involving size-based separation, membrane-based filtration, nanowire trapping, nanoscale deterministic lateral displacement sorting, acoustic isolation and viscoelastic flow sorting.^{62,63} Hence, we do not go into details of these approaches but would like to emphasize that these approaches are mainly based on generating novel hydrodynamic effects at the micro- and nanoscale with rationally designed micro- and nanostructures or through the integration of the structures with external actuation principles. They can be readily used to sort EVs of different properties, primarily based on size and shape. Apart from the traditional sorting capability, the concept of digital microfluidics can also be translated to EV research. For instance, the digital qualification of targeted EVs can be realized by using droplet microfluidics, taking the format of digital PCR. To achieve the digital quantification, magnetic beads were used to capture exosomes through sandwich ELISA complexes. They were subsequently encapsulated in micro-droplets to ensure not more than one bead per droplet.⁶⁴ A limit of detection down to 10 enzyme-labelled exosome complexes per microliter ($\sim 10^{-17}$ M) was achieved.

4. Emerging directions and challenges for EV heterogeneity research

Profiling EVs at the single-cell level

Analyzing EVs at single-cell level is crucial to reveal EV heterogeneity as the heterogeneity of EVs may come from their parental cells. Statistical analysis over a number of single cells would provide more information that is often hidden in large cell populations.^{69,70} In this regard, the characteristic dimension of microfluidic devices matching the size of individual cells can help quickly reach the detectable concentration of secreted molecules from a single cell. Thus, effective, sensitive, and quantitative isolation and identification of single cells can be easily achieved,^{61,71} which are difficult to be realized in macroscopic systems.^{72,73} Single-cell EV analysis requires the isolation of single cells, subsequent collection of EVs, and highly sensitive detection methods for composition profiling. In this respect, the reconfigurability of a microfluidic device would provide unmatched advantages for dynamic monitoring of secreted analytes released from single cells. This has been recently presented by trapping antibody-modified sensing microbeads with single cells inside picoliter microcompartments.⁶⁵ With secreted cytokines or exosomes captured by microbeads, binding of secondary antibodies attached to the microbeads causes the change of fluorescence intensity of the microbeads over time, providing the dynamic information of single-cell secretory activity (Fig. 3a).

Furthermore, it is possible to create arrays of single cells for time-lapse studies of exosome secretion and cell behaviours.⁶⁶ Small EVs secreted by each batch of single cells

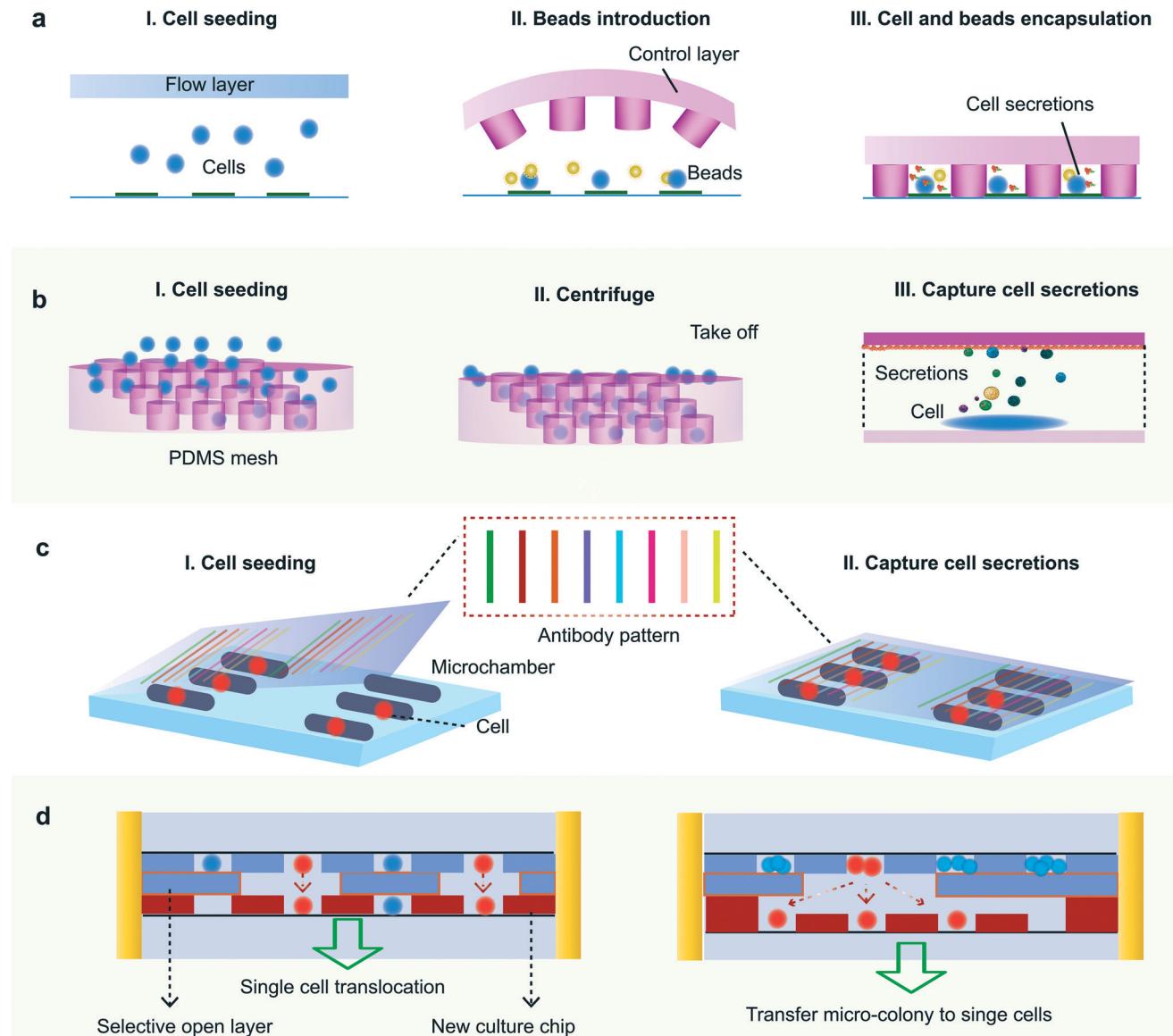


Fig. 3 Profiling EVs at the single-cell level. (a) Microcompartment arrays for isolating single cells and monitoring the release of exosomes over time using sensing beads.⁶⁵ (I) Cells are captured on the micropatterned floor (CD4 Ab, collagen). (II) Microbeads are flowed into the device and become entrapped inside the compartments. (III) Fluorescence increases over time after exosomes are bound on the bead surface. (b) Single-cell microfluidic platform used for analyzing exosome secretion.⁶⁶ (I) Single cells are loaded onto a PDMS culture mesh. (II) The mesh is removed after cell attachment. (III) An antibody-coated glass slide is placed above the cells to capture exosomes. The captured exosomes are labelled with another biotinylated antibody and streptavidin-conjugated quantum dots. (c) Platform for multiplexed profiling of single-cell EV secretion.⁶⁷ (I) A glass slide is patterned with 8 antibodies is superimposed on the high-density microchamber array. (II) The microchamber array accommodates thousands of identical units for isolating single cells and concentrating EVs. (d) Microfluidics platform for single-cell culture and exosome harvesting.⁶⁸ A parallel cell transfer chip consists of a single-cell culture chip – a layer of PDMS through-holes for single cell positioning and culture, and a polyester thin film filter. The targeted cells in each well is translocated to a new single-cell culture chip.

can be captured by antibody-modified glass slides at a certain time point (Fig. 3b). By functionalizing the surface of the glass slide with different capture molecules, it would be feasible to profile an array of different surface markers on EVs (Fig. 3c).⁶⁷ With the capability to simultaneously analyze many markers on the EVs and distinct patterns informing diseased or normal cells, invasive and non-invasive cells would be revealed. Notably, high-density microchambers

arrays allow for large-scale isolation of single cells. The nanoliter volume of each microchamber enabled by microfabrication technologies can concentrate the targets and ensure detection of low-abundance EVs with high sensitivity. Not limiting to analyzing secretions from localized single cells, innovations in microfluidic chips can address the translocation of single cells for downstream analysis (Fig. 3d).⁶⁸ The phenotypes between parental and progeny

cells derived from single cells can be tracked. A collection of holistic EV secretions can be further analyzed by other in-depth sequencing techniques such as digital PCR.

However, single-cell isolation is still in low yield (less than 30% in large scale). Single cell culturing in thousands of wells need to be recognized to avoid acquiring inaccurate data. It is labor-intensive and time-consuming to build one-to-one correspondence between single cells with chips. More innovative microfluidic chips are highly recommended to achieve high single cell yield. Formidable challenges of single-cell chips involve the need to address the critical requirement of high-fidelity data acquisition. Most current chips do not eliminate the potential crosstalk of cell debris which could generate artefacts. Long-term cell culture and study of cell behaviors in a more complex physiologic context would be possible by introducing on-chip culture medium exchange and fabricating chips with materials mimicking the native extracellular matrix. Though many single chips have been fabricated, many of them do not lead to new discoveries due to insufficient follow-up analysis. For one reason, single cells are in low secreting yield, difficult for statistical analysis. For another, methods for subsequent on-chip analysis are limited. Immunofluorescence staining is commonly adopted for protein studies in single-cell chips. However, subsequent discoveries are still limited (only surface protein information could be offered). New tools are necessary to increase the dimensions of EV analysis at the single-cell level. Recently, high-quality factor optical sensors were assembled in microfluidics to resolve single-cell EV secretion dynamics.⁷⁴ Compared with immunofluorescence staining, this new type of optical sensor represents one of the efforts devoted to analyzing EV dynamics with much higher spatiotemporal resolution. Droplet microfluidics has been presented as a promising platform for single-cell sorting and analysis.⁷⁵ Besides the information of surface proteins, it also offered information on inner proteins and nucleic acid. Correlation of single-cell omics with the compositional profiling of EVs would establish a crucial database for understanding EV biogenesis, cell communication and disease progression. However, this would require addressing critical challenges to separate individual cells and establish one-to-one correlation with their secreted EVs.

Profiling single EVs

Current methods cannot profile the inherent differences between individual EVs. Addressing this issue may require the development of a single-vesicle analysis tool rather than bulk analysis. There were many advanced tools in single EV profiling methods, like fluorescent nanoparticle tracking analysis,⁸⁰ flow cytometry,^{51,53–55} digital methods^{64,81,82} and fluorescence imaging techniques.^{76–79} The digital method plays a significant part in accurately quantifying nucleic acids and proteins of single EVs. Digital PCR and digital ELISA

dilute the EV sample to a certain concentration, ensuring only one or no molecule in a microdroplet or microchamber, to achieve the specific analysis of proteins or nucleic acid at the single-molecule level.

Using super-resolved microscopy would break the diffraction limit in revealing the fine structure of EVs (Fig. 4b).⁷⁷ Currently available super resolution microscopy techniques include structure illumination microscopy, stimulated emission depletion microscopy, photoactivation localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM) and single-molecule localization microscopy (SMLM), with resolutions in the range of a few tens of nanometres.^{83,84} SMLM has been recently applied to image quantum dot-labelled EVs with a spatial resolution of 30 nm.⁸⁵ PALM and d-STORM were used to study the interactions of EVs with neurons in Alzheimer's disease with a precision of 25 nm.⁸⁶ A current technical trend under development is focused on using super-resolution microscopy to study EV uptake and sizing (Fig. 4a).⁷⁶ Apart from quantum dots, a range of luminescent nanoparticle probes are available, such as lanthanide-doped nanocrystals and carbon dots.⁸⁷ They would be suitable for long-term tracking of individual EVs, but smart functionalization strategies are needed to selectively label EVs with these nanoparticles.

Apart from that, it is still challenging to profile the composition of single EVs, which is beyond the capabilities of state-of-the-art super-resolution microscopy techniques with existing functional probes. Many existing high-resolution microscopies have been applied to resolve the fine subcellular structures. However, small EVs with a much smaller scale of a few tens of nanometers are difficult to resolve with current microscopy techniques. Promising solutions include signal amplification methods such as DNA assisted ligation or extension assays which combine affinity probes (*e.g.* proteins) with amplifiable oligonucleotides.^{88–90} This allows converting protein identities to DNA sequences for protein detection at the level of single molecules or molecular complexes. Very recently, such DNA ligation assays have shown the potential for profiling surface proteins on EVs (Fig. 4c).⁷⁸ Up to 38 different surface proteins on EVs originating from 18 different sources have been analyzed using this approach. The approach is ultimately limited by the specificity of a given protein combination, the coverage and depth of sequencing and the density of surface proteins that may affect the affinity binding of antibodies. Despite DNA-assisted technologies shining some light on the analysis of surface markers of EVs, profiling the inner content of EVs remains elusive. Research along this direction has been devoted to the advancement of instrumentation and labelling strategies to achieve highly sensitive single-EV detection. The microfluidic and nanofluidic technologies can be combined with imaging to achieve more sensitive single EV imaging and tracking (Fig. 4d).⁷⁹ Microfluidic and nanofluidic devices can enable imaging and characterization of small vesicles on a single particle basis.⁹¹ For instance, individual vesicles can be visualized by fluorescence microscopy while passing

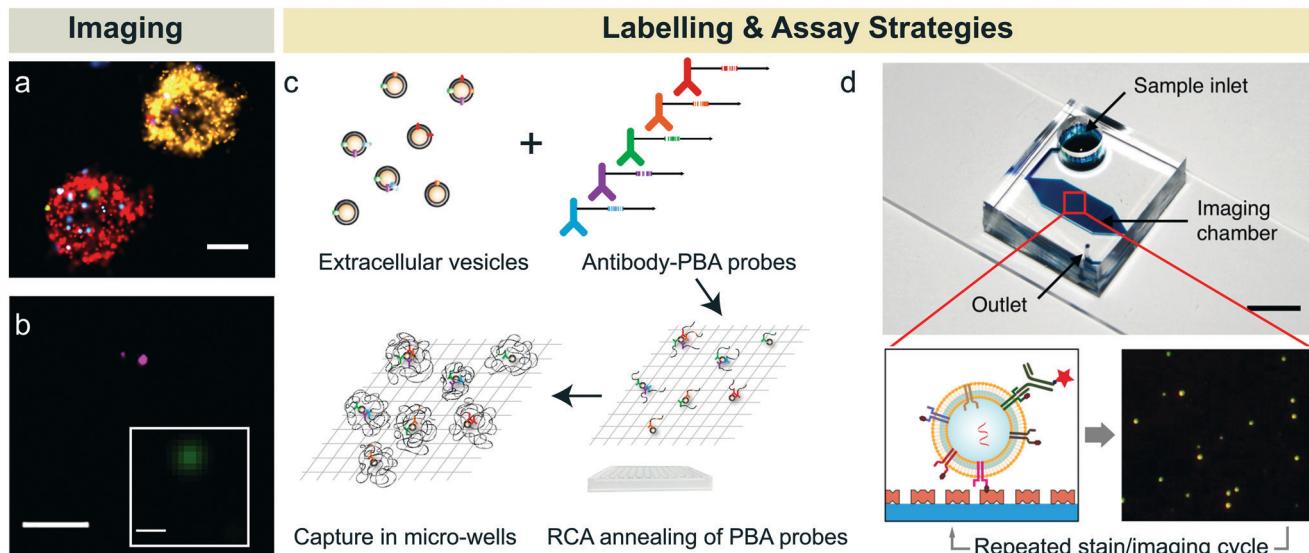


Fig. 4 Single EV imaging. (a) An excellent example of the application of Nanoimager (dSTORM) to investigate EVs.⁷⁶ Nanoimager is the world's first desktop-compatible microscope able to easily visualize EVs with a resolution reaching 20 nm. The ability to track in two colors allows it to characterize and compare two different biomolecules on the EV at the same time. (b) d-STORM imaging of DiD-labelled EVs.⁷⁷ d-STORM (magenta) and reconstructed standard wide field (green). Scale bar: 500 nm. (c) Design and workflow of proximity-dependent barcoding assay (PBA).⁷⁸ Exosomes are first incubated with PBA probes, hybridizing to a unique rolling circle amplification primer. Then, the complex tag incorporates along with a standard sequence motif for enzymatic extension. The identities of proteins on an individual exosome is revealed by analyzing the PCR product. (d) Microfluidic chip for single EV analysis.⁷⁹ EVs are first biotinylated and captured on a neutravidin-coated surface. EVs are then stained by fluorescent antibodies (three colors per step) and imaged by microscopy.

through parallel nanochannels in a pressure-driven flow. It requires very small amounts of sample volume to quantify both the vesicle content and the fluorescence signals emitted by individual vesicles. Future advances of micro- and nanofabrication would enable the study of EV transport and the roles of EVs in cell communications by combining microfluidic and nanofluidic devices with fluorescence imaging techniques.

5. Perspectives

Table 1 provides an overview of current and emerging approaches for isolation and analysis of EV populations, which highlights their key capabilities that have led to a few major scientific discoveries. Critical questions about the roles of different EV subtypes in physiology remain to be addressed in the development of EV research. We note that the majority of current tools have focused on presenting new assay methodologies and in-depth studies on cell culture conditions. However, examining clinical samples with these tools are still needed to generate translational impact. Furthermore, the advancement of basic research on EVs in cell biology requires continuously advancing the associated technologies. This requires interdisciplinary research efforts to collectively contribute to the technological demand. From this perspective, we expect that future technologies would address a few key capabilities for EV analysis (Fig. 5), which is detailed as follows.

New material-based bioassays

New material-based bioassays (*e.g.* quantum dots, upconversion nanoparticles, polymer dots, fluorescent nanodiamonds, carbon-based nanodots and nonfluorescent surface-enhanced Raman scattering nanoparticles) provide abundant material resources to solve labelling issues associated with fluorescent bleaching and surface marker heterogeneity. Direct labelling often suffers from false-positive results by excess and free dyes and lower the signal-to-noise ratio. A large number of fluorescent dyes with various chemical and photonic properties can be applied to multiplexing diagnostics by using multi-channel colors to examine different analytes. However, such spectral multiplexing is often impractical: (i) images may be blurred; (ii) available fluorescence markers are limited; (iii) bleaching and loss of fluorescence intensity may happen with prolonged exposure to fluorescent light; (iv) the complex body fluids may create various difficulties for spectral multiplexing. Therefore, fluorescent dyes are not an ideal choice for multiplexing labelling. In recent years, the development of immunoassays has been strongly driven by new material-based luminescent nanoparticles as they are chemically and physically stable and their properties can be tuned by physical, chemical and biological properties with enhanced performance. In addition, new labelling materials show great potential in long-term tracking of single molecules and real-time super-resolution imaging of subcellular structures. Among those, fluorescent

Table 1 Comparison of current isolation methods and detection methods on EV heterogeneity

	Isolation methods	Discoveries	Ref.
EV subpopulation isolation	Ultracentrifuge	A novel population of EVs (P200 fraction) smaller than exosomes promoting cell proliferation was identified	27
	Density gradients	(a) Specific exosomal (CD63, CD81 or CD9) and non-exosomal subpopulations existing within small EVs; (b) Argonaute 1–4 and major vault proteins were released free of small EVs and annexin A1 is a specific marker of MVs	23, 41
	Sequential centrifugal ultrafiltration	ILIM1863 colon cancer cell derived large EVs promoted invasion to 3-fold than small EVs	59
	Asymmetric field flow fractionation	Discovery of exosomes	50
	Immunoaffinity capture	(a) NK cell-derived EVs and platelet-derived EVs are devoid of CD9 or CD81; (b) small EVs do not contain DNA and active secretion of cytosolic DNA occurs through an amphipathic-dependent mechanism	41, 49
	Analytical methods	Capabilities	
EV profiling from single cells	Sensing beads	Monitoring the dynamics of single-cell secretory activity	65
	ELISA	Time-lapse studies of exosome secretion and cell behaviors	66
	Translocation and secretion assay	Time-lapse molecular cargo analysis and sorting of EVs secreted from single cells	68
Single EV characterization	Multiplexed ELISA	High-throughput detection and biomarker correlations	67
	Flow cytometry	Detection of small EVs with size below 100 nm	51
		Sizing individual EVs down to 40 nm	52
	Imaging	Profiling two different biomolecules on the EV at the same time	111
		Imaging single EVs with precision down to 30 nm	112
		Single EV enumeration and multiplexed profiling	79
	Digital	Rapid isothermal nucleic acid detection assay of specific single EV surface biomarker	81, 82
		Single bead encapsulated in a droplet, enabling absolute counting of cancer-specific exosomes	64

upconversion nanoparticles have attracted attention due to their superior optical properties, including large anti-Stokes shifts, sharp emission bandwidth, high quantum yield, low toxicity, great photostability, and high chemical stability.^{92,93} Such unique luminescent nanoprobes may offer a reliable alternative to traditional dyes for EV research.

Three-dimensional super-resolution microscopy

Three-dimensional super-resolution microscopy to specifically recognize unique epitopes of one single EV is suggested to be involved in EV heterogeneity studies. How many epitopes are expressed in one single EV, in which site do they express the specific antigen, and relationships between epitopes and parental cells are far from known. Three-dimensional super-resolution microscopy would fill the gap to provide the 3D information of epitopes in a single EV.

Optical tweezing

Optical tweezing could trap and precisely manipulate small particles and is expected to play an increasingly important role to deliver molecules to cells in a highly controlled manner.^{94,95} Recently, optical manipulation was used to directly image EV-cell interactions and to determine in a quantitative manner the contribution of surface co-receptors

and extracellular protein modulators to the contacts.⁹⁶ Optical tweezing is emerging as a promising technique in revealing the spatiotemporal properties of single EVs, including the abundance of biomarkers expressed on the EV surface or the dynamics of EV uptake and release, which are not achievable by traditional methods.

New library of functional linkers

Current approaches to isolate EVs, including ultracentrifugation, polymer-based precipitation and filtration, could lead to high heterogeneity, contamination and EV damage. This has hampered the downstream analysis of EV heterogeneity with high fidelity. Effective strategies are crucially needed to bypass the expensive or bulky experiments. Designing functional linkers that are capable of highly efficient capture of EVs and subsequent release would be highly desirable. For example, a functional lipid nanoprobe composed of a labelling probe and a capture probe has been synthesized for the labelling of the lipid bilayer of EVs.⁹⁷ With optimized labelling efficiency, the probes embodied by biotin-tagged 1,2-distearoyl-sn-glycero-3-phosphethanolamine-poly(ethylene glycol) can shorten the EV isolation procedures from hours to 15 min. A type of responsive materials such as allylated psoralen functionalized

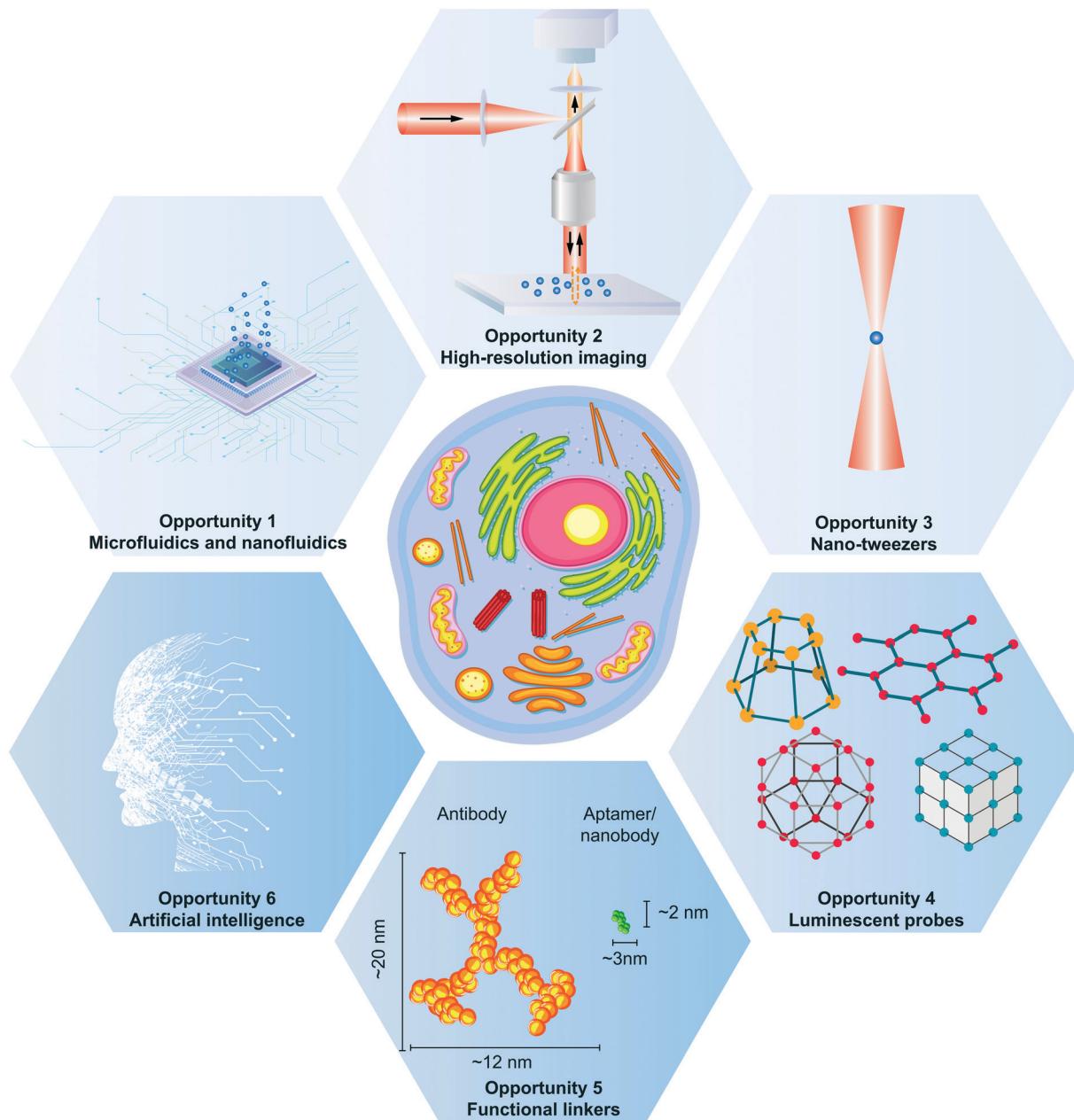


Fig. 5 Overview of key opportunities in developing new capacities for EV heterogeneity research.

polyacrylamide gel are used to capture and release target nucleic acids spanning a wide range of lengths upon UV irradiation within 1 min.⁹⁸ Such responsive linkers should be possible for their use in the surface modification of microfluidic devices, micro- and nanocarriers for EV isolation. Only a few responsive linkers are currently available, but a range of responsive linkers should be possible through the rational synthetic design of functional groups for targeting EV surface markers and those responding to external stimuli, such as light, temperature and pH.

Aptamers (~ 15 kDa) and nanobodies (~ 13 kDa) are smaller than conventional antibodies (~ 150 kDa). They could

break the limitations of heterogeneity analysis associated with steric hindrance and self-aggregation of labelling agents. In conventional immunostaining, the primary/secondary antibody complex can have a linear length of ~ 20 nm, complicating their penetration into tissue and reducing the number of epitopes detected. Aptamers are short single-stranded nucleic acid sequences capable of binding to target molecules in a way similar to antibodies.^{99,100} The main advantages of aptamers in advanced microscopy lie in monomeric binding, small size, short generation time, low batch-to-batch variability, low/no immunogenicity, high modifiability and high target potential.^{101–103} Nanobodies are recombinant, antigen-specific, single-domain, variable

fragments of camelid heavy chain-only antibodies.^{104,105} Nanobodies are superior as a renewable source of affinity reagents, possessing advantageous features such as minimal size, great stability, reversible refolding and ability to specifically recognize unique epitopes with sub-nanomolar affinity.¹⁰⁶ We anticipate that aptamers and nanobodies would play an increasingly important role in labeling biomolecules in advanced microscopy for EV heterogeneity research.

Artificial intelligence

Artificial intelligence is emerging as a promising technique in helping to process big data and classify complicated data based on patterns submerged inside to overcome the complexity and heterogeneity. It can easily detect disease signatures that typically contain large data sets and enables to predict specific disease states.^{107–109} Artificial intelligence including machine learning or deep learning provides an unprecedented opportunity to extract information from complex or big data sets in chromatography, mass spectrometry, nuclear magnetic resonance, and spectroscopy previously.¹¹⁰ In addressing the complexity in heterogeneity, artificial intelligence is expected to offer a powerful tool in big data processing to decode the complexity of cell multi-omics and EV compositional profiling.

With the above capabilities, we would be more likely to expand a few key points of EVs, including: (1) how EV heterogeneity contributes to cell heterogeneity; (2) what factors contribute to EV heterogeneity and if they can be controlled for therapeutics, *e.g.* drug delivery; (3) how EV heterogeneity patterns inform disease status and may ultimately contribute to clinical diagnostics. This would require technology developers and end-users (biologists and clinicians) to work more closely to address common biological questions. By filling the significant gap between physicists, engineers, biologists and clinicians, more in-depth and precise investigations on the underlying complexity of EV heterogeneity would accelerate the advancement of EV-based early diagnostics and personalized therapeutics.

Conflicts of interest

The authors declare no conflict of interest.

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