Extracellular Vesicles

EV characterization: introducing super-resolution microscopy for single-EV analysis

Introduction

Extracellular vesicles (EVs) are membrane-bound vesicles secreted by all cells that facilitate intercellular communication. EVs are enriched in surface proteins (such as ALIX, flotillin, and tetraspanins) and contain luminal content (including DNA, RNA, and SiRNA Figure 1). Importantly, these small nanosized vesicles that transfer active molecules and genetic materials can be isolated from complex biofluids such as blood, urine, and saliva, which makes them ideal candidates for disease diagnostics and therapeutic applications.

Since EVs resemble the composition of their parental origin, secreted vesicles are becoming a promising source for biomarker discovery, as the specific types of surface proteins and cargo may have profound pathophysiological significance. The ability to identify and measure the presence or abundance of particular components could represent particular "disease signatures". Therefore, characterizing molecular profiles of EVs may yield powerful diagnostic capabilities that more accurately determine the onset of distinct cancers[1], neurodegeneration[2], and cardiovascular diseases[3]. In this application note, we will discuss how super-resolution microscopy can be used as a powerful tool for EV characterization and imaging.

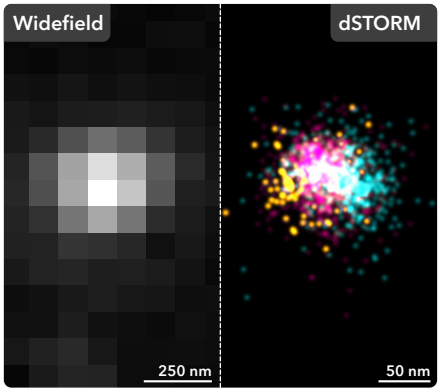

Figure 1 | 3-color widefield vs dSTORM image of CD9-ATTO488, CD63-Alexa Fluor®-647 and CD81-Alexa Fluor®-555 EVs.

Open questions

Owing to their diverse biological heterogeneity, the term EV itself encompasses a number of subpopulations referred to as exosomes, microvesicles, outer membrane vesicles (OMVs) and ectosomes. The myriad of names mentioned throughout the literature[4,5] (Table 1), stems from the cellular origin, separation and isolation abilities as well as molecular profiles of the different EVs. To unify and establish a set of universal guidelines for EV characterization, a list of minimal information for studies of extracellular vesicles (MISEV) was published in 2014 and updated in 2018 by leaders within the field to outline an official set of characterization requirements. This in turn, has greatly contributed to the development and advancement of our understanding of EVs.

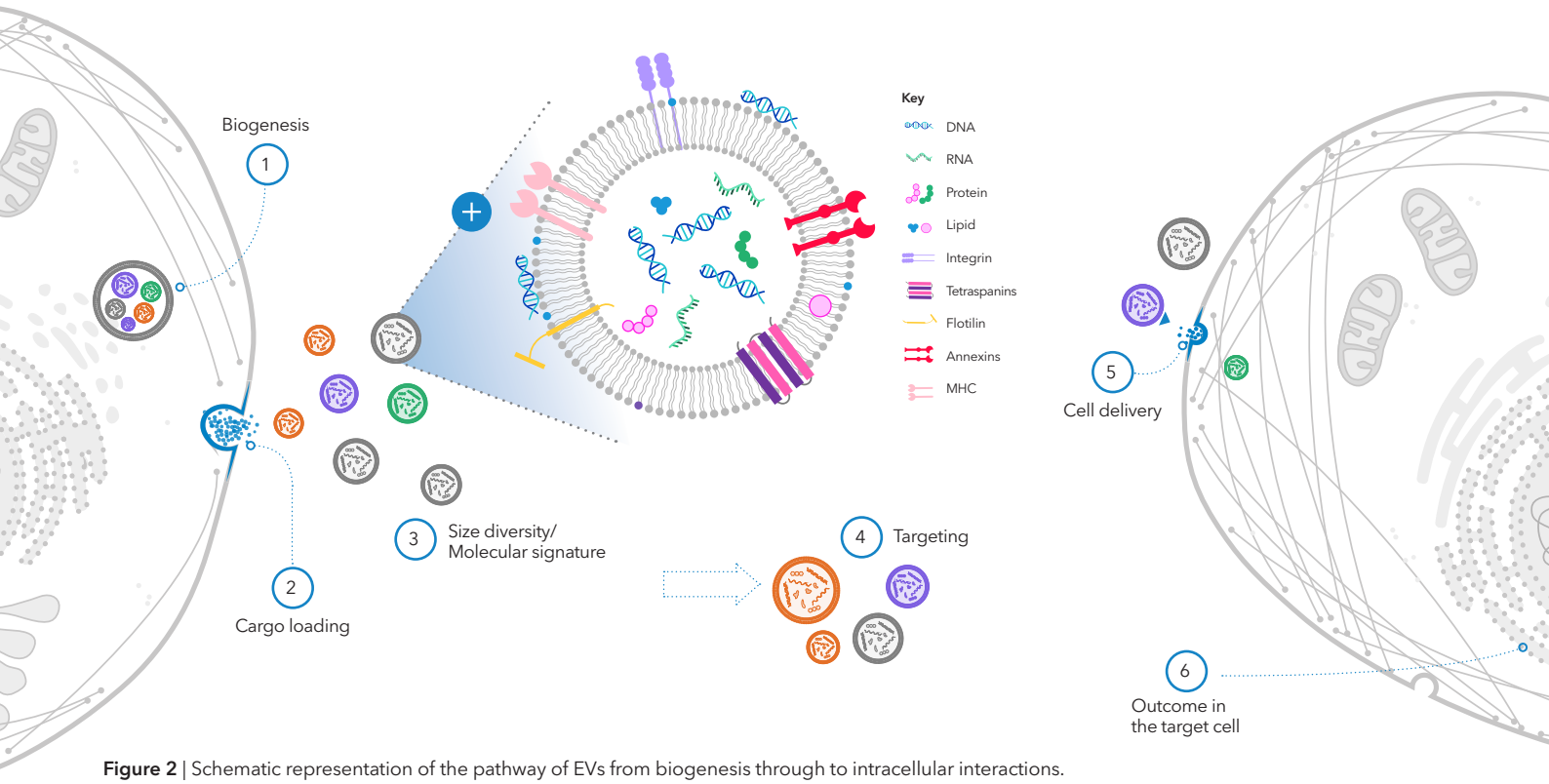

Figure 2 | Schematic representation of the pathway of EVs from biogenesis through to intracellular interactions.

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However, there are still many fundamental unanswered questions surrounding the journey EVs take from biogenesis through to internalization by target cells (Figure 2). For instance, whilst it is understood that EVs selectively capture predefined proteins and nucleic acids, the mechanics that regulate such sorting of materials and cargo loading into the EVs is not. Another open question surrounding the EV journey relates to their distinct targeting capabilities, with studies investigating how differences in cargo and surface proteins influence their respective targets. Interestingly, the amount of cargo transported within an EV has been shown to be very low[6], which formulates the hypothesis that EV targeting needs to be highly efficient and specific, as the precision may be less important when a large number of EVs are released. Delivery is postulated to depend on the very specific surface molecules that function as 'barcodes', recognized by a receptor or "reader" situated on the cell membrane, but how this relates to an EV's target and internalization remains elusive. To answer such questions requires a complete analysis of EVs at a single-molecule level to determine individual molecular profiles of associated components, so we can ultimately harness their properties for diagnostic and therapeutic purposes.

Table 1:Classification of EV subpopulations. Adapted from Borges et al (2013)[7].

	Exosomes	OMVs	Microvesicles	Ectosomes
Size	40-120 nm	100-400 nm	50-1000 nm	100-1000 nm
Cellular origin	All cell types	Gram negative bacteria	Megakaryocytes, blood platelets, monocytes, neutrophils, tumor cells and placenta	All cell types
Known contents	Proteins and nucleic acids (mRNA, miRNA and other non-coding RNAs)	Virulence factors, proteins and nucleic acids (mRNA, miRNA and other non-coding RNAs)	Proteins and nucleic acids (mRNA, miRNA and other non-coding RNAs)	Proteins and nucleic acids (non-coding RNAs)
Common markers	Alix, Annexins, tetraspanins (CD81, CD63, CD9), flotillin	Outer surface proteins (OmpA, OmpC, OmpX, AbompA), PAMPs (LPS)	Integrins, selectins, CD40	Cholesterol, sphingomyelin and ceramide

EVs and super-resolution microscopy

Current methods of EV characterization include flow cytometry, nanoparticle tracking analysis (NTA), epifluorescence imaging techniques and electron microscopy (EM). However, despite the commonplace of these techniques, they often fail to give the necessary resolution to accurately characterize individual EVs or, in the case of EM, struggle to visualize multiple markers in a single-experiment, limiting the ability to assess the molecular signatures of EVs.

Super-resolution imaging techniques, featured in the MISEV18 guidelines, can overcome the resolution limits associated with conventional light microscopy. Through Single-molecule localization microscopy (SMLM) techniques, such as dSTORM and PALM, EV biomarkers are labeled with with a class of fluorophores of which the photochemical properties can be exploited to stochastically switch them between a dark and an emissive state. This allows small subsets of fluorophores to be detected in isolation and the localization of each fluorescent molecule to be fitted with a Gaussian function. By imaging in this way, SMLM retains the advantage associated with traditional fluorescence imaging techniques while circumventing the diffraction-limit, providing an achievable resolution exceeding 20 nm and allowing the spatial distribution of EV markers to be visualized with single-molecule sensitivity.

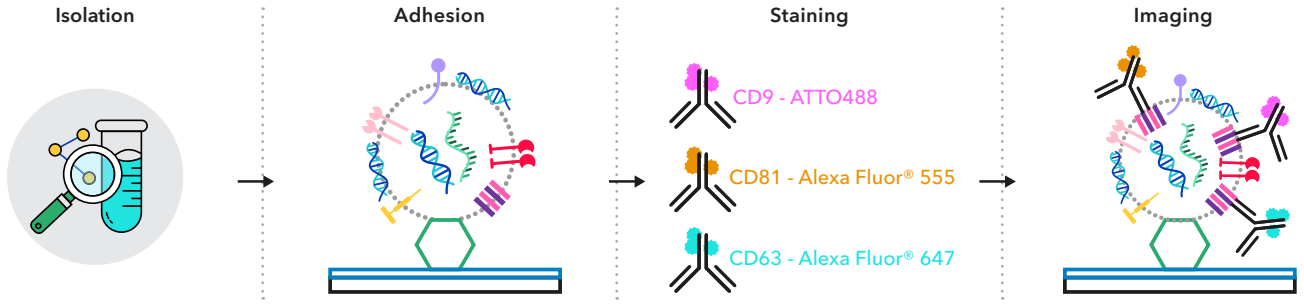

Figure 3 | Schematic representation of isolated and purified EVs, adhered to a glass slide and stained with tetraspanin markers on their surface.

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The Nanoimager is a comprehensive SMLM platform for precise and detailed molecular characterization of EVs. With a multitude of imaging modalities available, this data-driven platform facilitates exploration into composition and behavior of EVs, to provide deeper insights into vesicle subpopulation signaling pathways and intracellular interactions from both a fixed sample and live-cell perspective. To demonstrate the capabilities of SMLM on the Nanoimager in EV characterization, HCT116 EVs were isolated, purified and immunostained with commercially available antibodies against surface membrane tetraspanins, CD9, CD63 and CD81 (Figure 3) and imaged using dSTORM (Figure 4). The results demonstrated a heterogeneous population of EVs that were either single (A-C), double (D-F) or triple (G) positive for tetraspanins. Subsequent analysis would allow for the size profiles and relative abundance of each of the markers to be quantified across the EV population. This data highlights the multi-factor characterization capabilities that follow SMLM imaging, enabling researchers to assess unique protein signatures or changes in biomarker number across populations, which could have important connotations for early disease diagnostics and better understanding EV functionality.

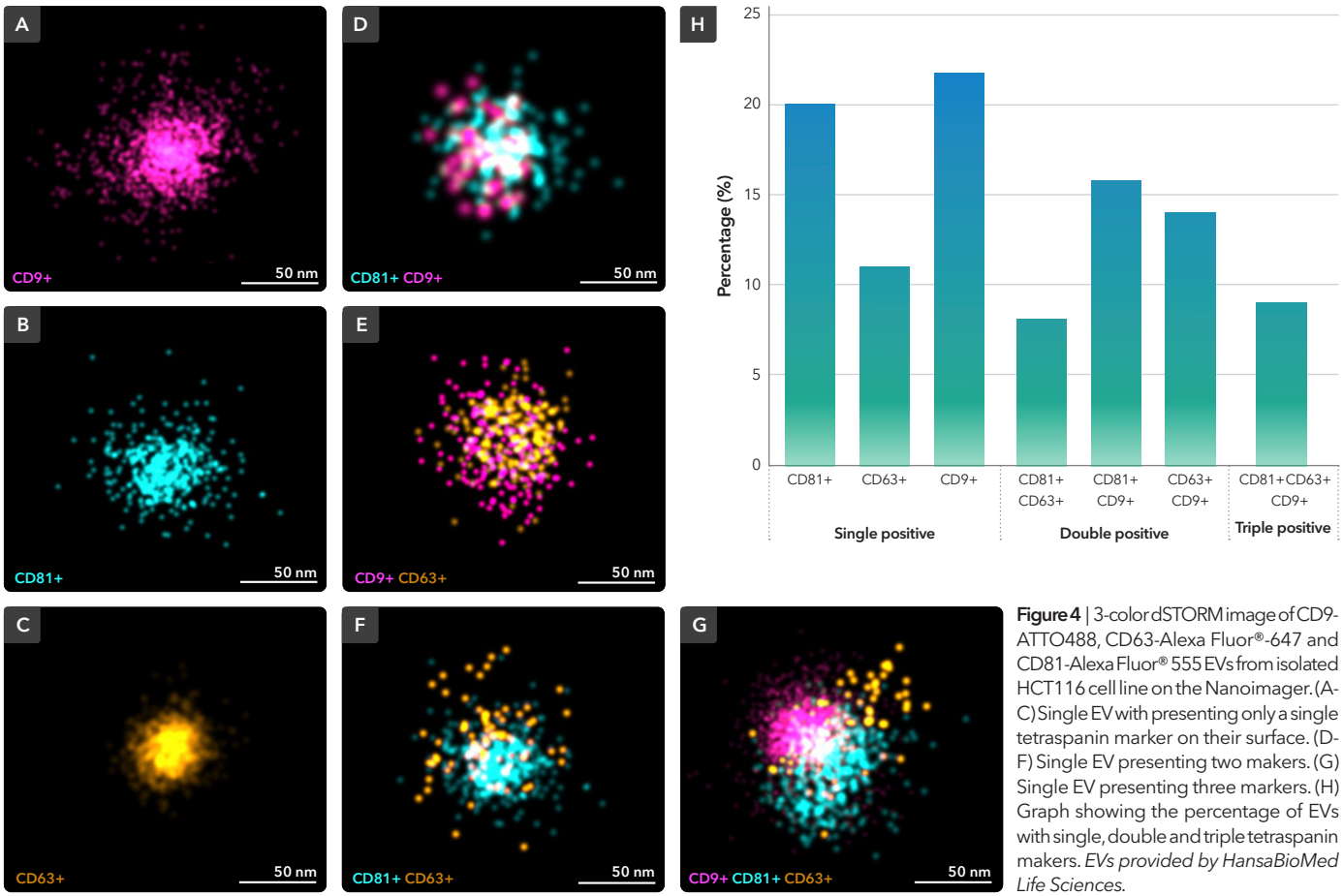

Figure 4 | 3-color dSTORM image of CD9-ATTO488, CD63-Alexa Fluor®-647 and CD81-Alexa Fluor® 555 EVs from isolated HCT116 cell line on the Nanoimager. (A-C) Single EV with presenting only a single tetraspanin marker on their surface. (D-F) Single EV presenting two makers. (G) Single EV presenting three markers. (H) Graph showing the percentage of EVs with single, double and triple tetraspanin makers. *EVs provided by HansaBioMed Life Sciences.*

References

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