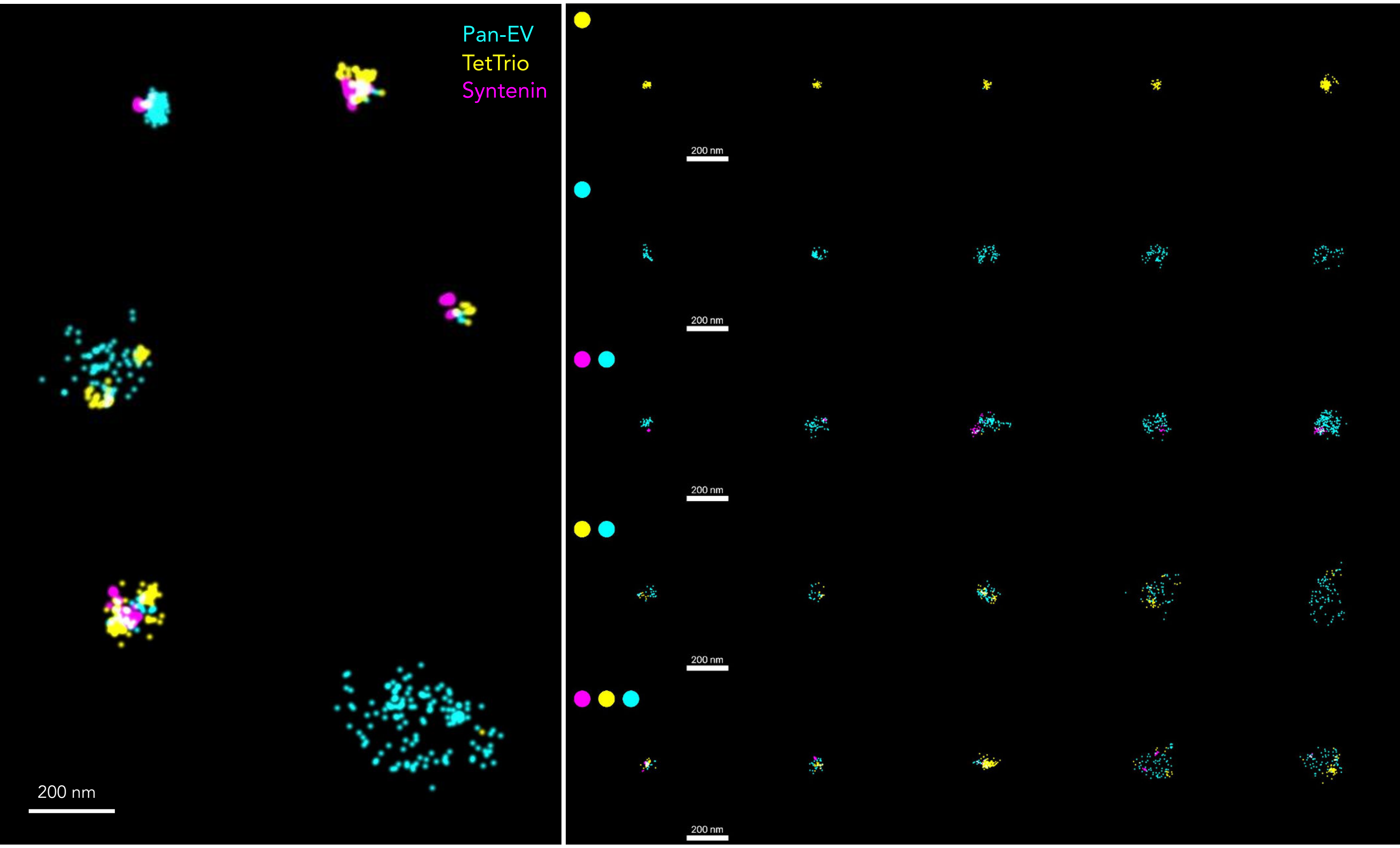
ONI

Extracellular Vesicles

White paper

Profiling Heterogeneity: Automated, AI-driven Analysis of EV populations Down to the Single-EV Level



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SUMMARY

Extracellular vesicles (EVs) represent the next frontier in diagnostics and targeted therapeutics, yet their clinical translation has been hindered by bulk analyses that obscure critical particle heterogeneity, rare subpopulations driving disease pathology, or crucial single-EV-level details.

This white paper leverages the Aplo platform, an end-to-end solution that simplifies EV characterization through Single-Molecule Localization Microscopy (SMLM), to showcase how automated EV sample preparation, imaging, and analysis can deliver consistent results across fields-of-view (FOVs), lanes, chips, and systems. By achieving 20 nm precision, the platform transforms diffraction-limited "spots" to discrete molecular maps of surface biomarkers and internal cargo. All while reducing manual preparation to a 30-minute automated setup, eliminating user variability, delivering high-throughput super-resolution imaging, and AI-driven analysis to transform multidimensional datasets into actionable population-level insights. Through this "digital subpopulation sorting", the Aplo platform enables researchers to identify clinically-relevant EV profiles or subpopulations with unprecedented consistency and scale.

Table of contents

INTRODUCTION

Extracellular vesicles (EVs) are lipid-bilayer-enclosed nanoparticles naturally secreted by virtually all cell types into the extracellular space. Redefined by high-resolution analytical technologies as a sophisticated system of intercellular communication, EVs facilitate the transfer of bioactive cargo, including mRNA, miRNA, DNA, proteins, metabolites, and lipids, to effectively modulate the physiological and pathological states of recipient cells. Together with surface components and targeting molecules, including tetraspanins, EV cargo provides a complex molecular architecture to EVs that enables functional versatility, with specific vesicle docking and internationalization to ensure precise cargo delivery [1-2].

Because EV composition reflects the metabolic and genetic state of the parent cell, analyzing these circulating signals has profound implications for non-invasive diagnostics in biofluids like blood plasma and urine. However, the heterogeneous nature of EV populations, their small size (30-1000 nm) falling below the optical diffraction limit (250 nm), and low protein copy numbers (10-100 copies) pose significant challenges for precise EV population analysis. Standard "bulk" methods, such as Western Blotting or conventional Flow Cytometry, often aggregate measurements, masking the biological "truth" held within rare, highly active EV subpopulations. To truly unlock EV-based applications requires the sensitivity to detect EV signals against background noise and the ability to visualize and quantify individual vesicles with nanometer precision [3-4].

Super-resolution microscopy provides ten times better resolution than traditional microscopy, allowing imaging of single EVs and their biomarkers with 20 nm precision. Single-molecule localization microscopy (SMLM), an umbrella term for a variety of techniques that enable super-resolution imaging of EVs, allows researchers to gain insight into EV size and shape, while simultaneously detecting internal cargo or surface biomarkers, providing insights into EV biogenesis and uptake pathways. This evolution has enabled clinical application, such as identifying cancer-associated thrombosis risk through the use of single-EV analysis to detect and quantify rare subpopulations of small EVs in blood plasma 5, the early detection of Parkinson disease through the quantification of membrane-associated α-synuclein on individual EVs [6], or profiling of urinary EV biomarkers in prostate cancer patients through the characterization, quantification, and size profile of urinary EVs [7].

Beyond diagnostics, EV are ideal candidates for next-generation therapeutic delivery systems thanks to their innate biocompatibility and programmable targeting capabilities. By engineering producer cells that display specific targeting molecules on the surface, e.g., chimeric antigen receptors (CARs) [8], researchers can direct EVs to deliver therapeutic agents to precise cellular targets. In these contexts, single-EV analysis is indispensable for verifying engineering efficiency and molecular distribution across EV populations and accurately identifying different EV subpopulations.

Despite this enormous potential, the clinical translation of EVs is hindered by complex analytical workflows. The inherent heterogeneity of EVs in terms of size, morphology, and molecular content necessitates rigorous, standardized characterization protocols. These need to ensure that purified EVs from a range of samples can be analyzed without perturbing their morphology while confirming their identity compared to non-specific particles. Advanced microscopy requires purified EVs to be selectively immobilized onto a high-purity surface to minimize non-specific binding and maintain the signal-to-noise ratio required for single-molecule detection. EVs are typically labeled with primary antibodies (and fluorescently-labeled secondary antibodies) targeting specific surface or intralumenal vesicle components, including various permeabilization and blocking steps to accurately label internal cargo versus surface biomarkers without compromising vesicle integrity. The complexity of sample preparation scales with the depth of characterization and the number of samples being analyzed, with manual preparation risking often increasing sample-to-sample variability. In addition, mastering sub-diffraction instrumentation and ensuring minimal setting variability across samples remains a significant hurdle for many users.

Integrated automated workflows that ensure longitudinal reproducibility are crucial for detecting EV population heterogeneity and cross-population differences with single EVs and nanoscale precision. ONI's Aplo platform delivers an end-to-end EV characterization solution that leverages automated fluidics and imaging to provide a user-friendly workflow for all EV researchers (**Figure 1**). Automated EV sample preparation with Aplo Flow and the EV Profiler 2 kit reduces bench time to a 30-minute setup. It eliminates user-to-user variability and ensures selective, reproducible EV capture (using phosphatidylserine (PS) or antibody-based capture) and labeling, through a Pan-EV membrane stain and specific protein labels for surface and cargo detection. Subsequent imaging is performed on the Aplo Scope, engineered for high-throughput single-molecule analysis, with fast multi-channel imaging, and a field of view three times larger than the previous generation to maximize data acquisition and rare phenotype detection. Automated image acquisition is managed by AutoEV, a framework for acquisition and analysis that automates and simplifies sample focus, illumination, and calibration into "walk-away" experience with real-time feedback.

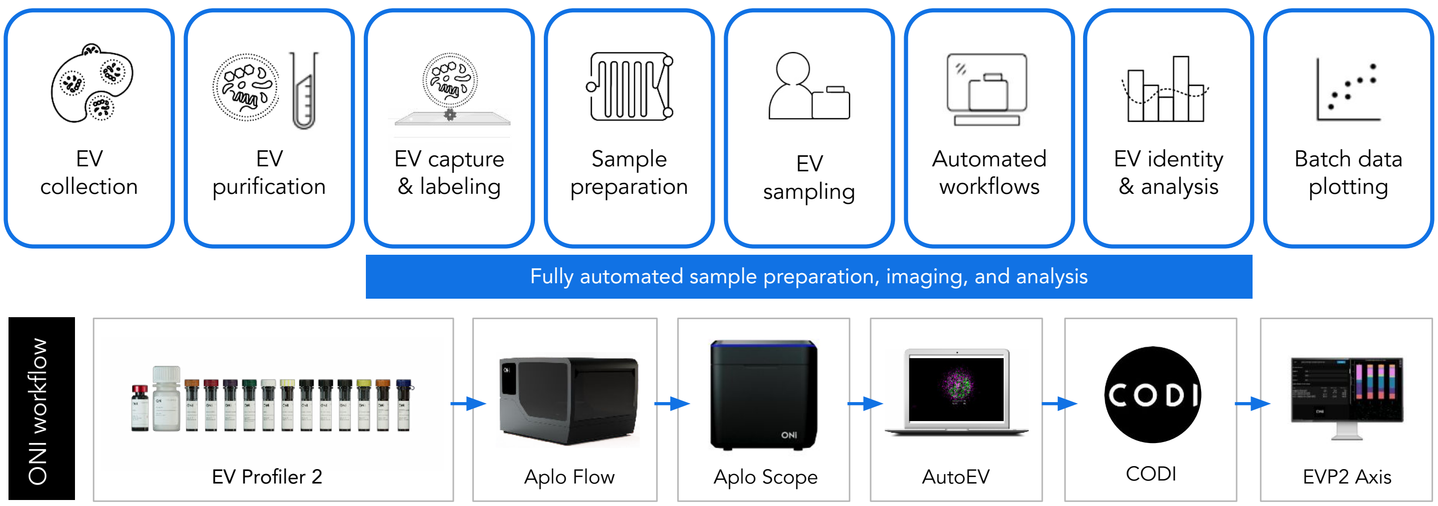

Figure 1 | ONI's workflow for end-to-end EV characterization is powered by the Aplo platform. Including automated EV sample preparation with Aplo Flow and the EVP Profiler 2 kit, automated image acquisition with Aplo Scope and AutoEV, and data processing and batch analysis using CODI and EVP2 Axis for population-level analyses and reporting.

The transition from raw images to biological insights is managed by CODI, ONI's cloud-based analysis and collaboration platform. Integrated into AutoEV, CODI automates SMLM workflows, including localization filtering, drift correction, and clustering, to map biomarker distribution on EVs with nanometer precision. Its AI EV Profiling tool uses machine learning to identify clusters of interest, removing the need for manual clustering expertise. CODI generates final reports detailing biomarker distribution, EV counts, size distributions, and population montages for seamless cross-dataset analysis. As the final step, the EVP2 Axis tool provides a free, open-source environment to generate standardized and custom plots, and extract population-level insights across several fields of view (FOVs) and samples.

This white paper showcases how automated EV sample preparation, imaging, and data analysis through the Aplo Platform delivers consistent results across FOVs, lanes, chips, and systems. The data obtained from CODI was processed using the EVP2 Axis tool, enabling side-side population-level comparisons to turn multidimensional EV datasets into actionable insights.

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METHODS

HEK EV Standard Control from ONI was diluted 1:10 in EV Wash Buffer from ONI's Application Kit™: EV Profiler 2. EVs were captured using either PS capture (phosphatidylserine), Tetraspanin Trio capture (anti-CD9 + anti-CD63 + anti-CD81), or anti-CD9 capture. EVs were then stained with either the 3 color tetraspanin modality (anti-CD9, anti-CD63, and anti-CD81 conjugated to 488, 561, and 647 fluorophores, respectively) or the Pan-EV + Tetraspanin Trio + custom marker detection modality. Syntenin was detected using an Alexa Fluor® 647 Anti-Syntenin antibody purchased from Abcam [EPR8102].

Imaging was performed on an Aplo Scope microscope using AutoEV in CODI using the recommended laser settings in the user guide: 30 ms exposure with 250 mW power at the sample for the 638 laser, 92 mW for the 561 laser, and 240 mW for the 488 laser. For the Pan-EV + Tetraspanin Trio + custom marker modality, 1000 frames were taken for Syntenin (638 laser) and TetTrio (561 laser) and 3000 frames were taken for Pan-EV (488 laser). For the 3 color tetraspanin modality, 1000 frames were taken for each of the three channels.

Analysis was performed using ONI's CODI software with the AI EV Profiling App. This app is a step-by-step analysis workflow for EV characterization. For the Pan-EV + Tetraspanin Trio + custom marker modality, the default "Pan-EV (488), TetTrio (561), and Custom Biomarker (647)" settings in the AI EV Profiling app were used. For the tetraspanin modality, the default "3-Color Tetraspanin" settings in the AI EV Profiling app were used. In brief, the AI EV Profiling app consists of several steps: [1] Drift correction, [2] Filtering, [3] Machine-learning-based clustering tool, and [4] Localization-based counting tool, which are applied sequentially to the datasets.

The open-source EVP2 Axis 2.0 tool from ONI was used to plot cross-dataset comparisons directly using output files from CODI's AI EV Profiling app. The EVP2 Axis 2.0 tool creates auto-generated cluster count and biomarker positivity plots, calculates summary statistics for each sample, and allows for custom scatterplot, violin plot, and histogram generation.

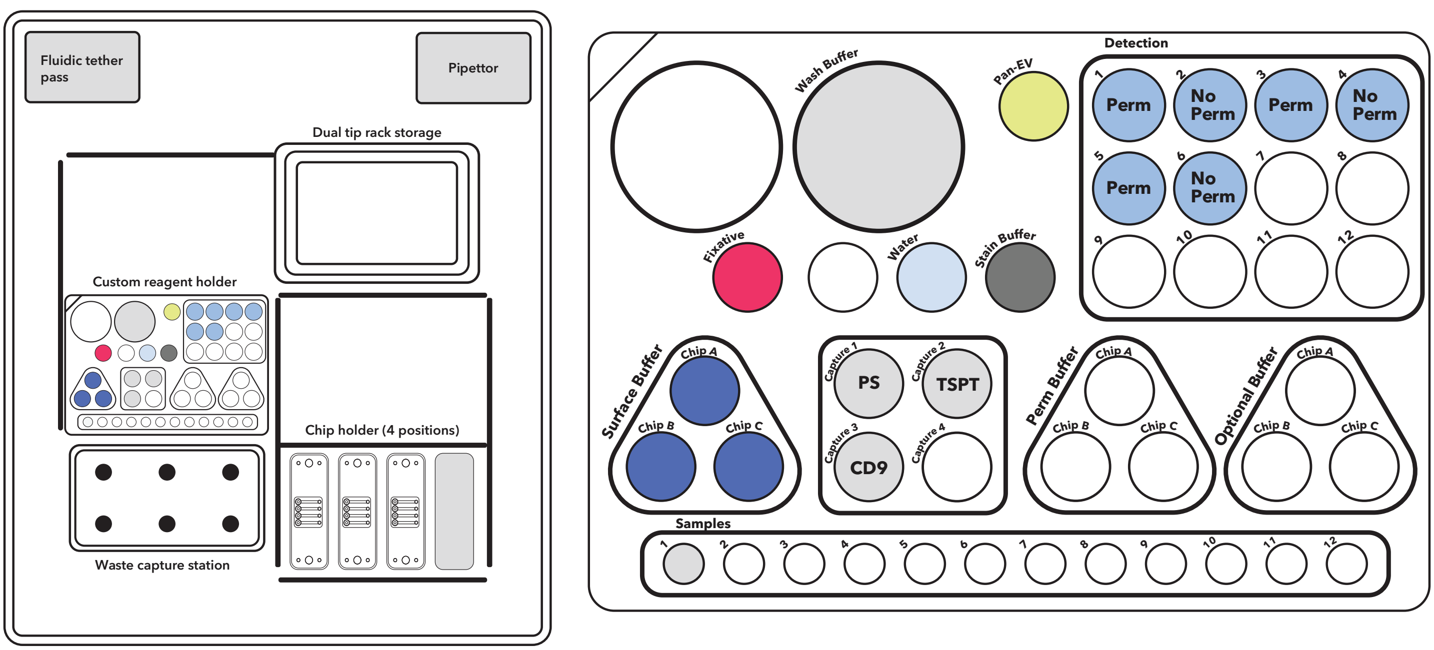


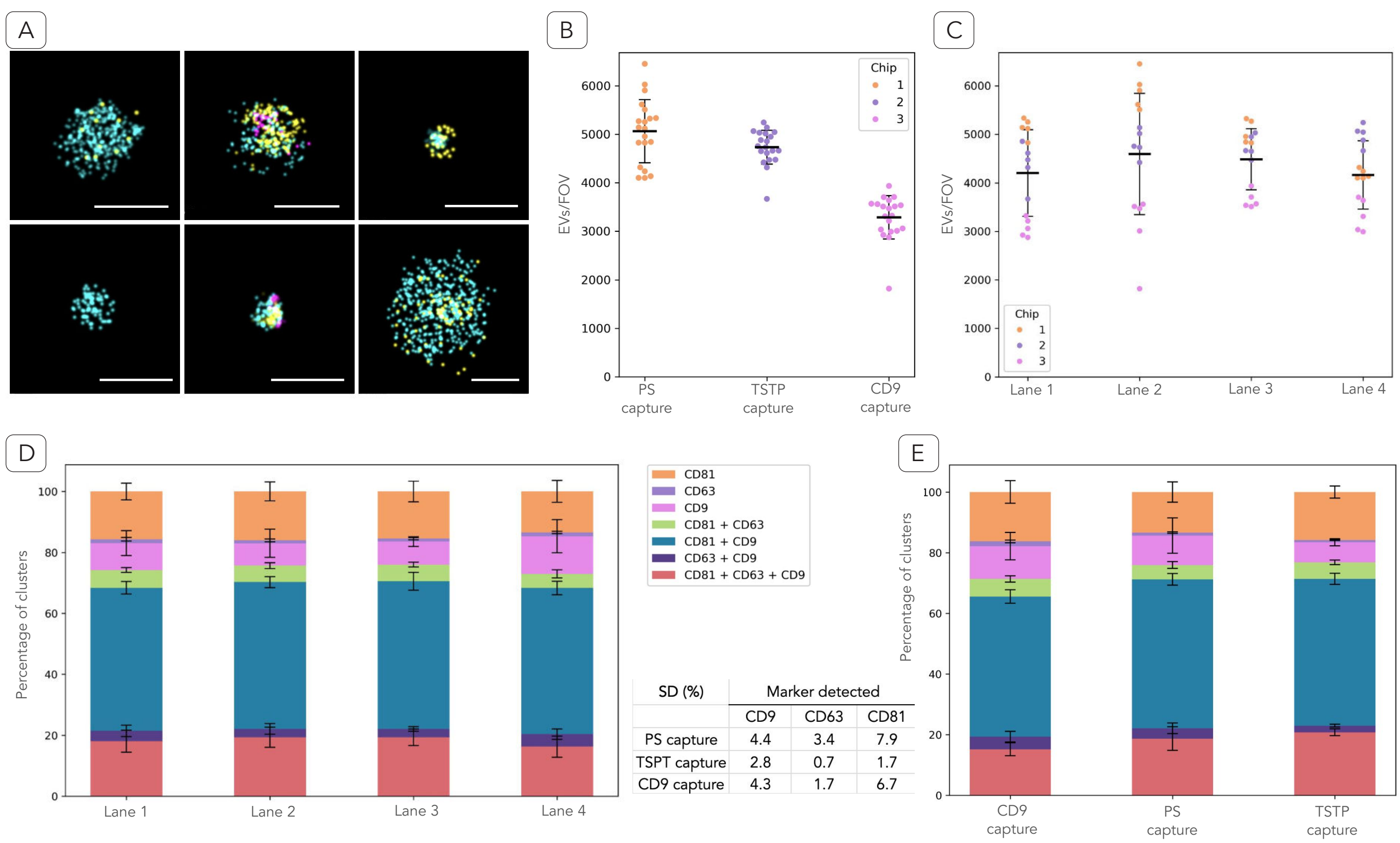
Figure 2 | Aplo Flow EV prep set up schematic. Purified EVs were placed in the sample holder (bottom, marked as 1), while the different surface, capture, fixative, wash, and stain solutions were added in the indicated locations of the Aplo Flow deck. In this case, lanes 1-2 were permeabilized for cargo detection, while lanes 3-4 were not. While in a parallel experiment (three tetraspanin detection), all lanes were treated equally for each chip. In both cases, different EV capture methods, namely phosphatidyl serine (PS) capture, tetraspanin trio (TSPT) capture, and anti-CD9 antibody (CD9) capture, were used.

RESULTS

Aplo Flow EV prep delivers consistent results across chips, lanes, and FOV

For this study, three separate chips containing 4 lanes each were operated in parallel, whereby the fractions of the same EV sample were captured using different capture methods, namely phosphatidyl serine (PS) capture for Chip 1, tetraspanin trio (TSPT) capture (anti-CD9 + anti-CD63 + anti-CD81) for Chip 2, and anti-CD9 antibody (CD9) capture for Chip 3. The automation of EV sample preparation, including EV capture and labeling resulted in highly consistent results across chips, lanes, and FOV, as seen by the preservation of EV integrity and high-resolution single-EV detection (**Figure 3A**), as well as EV counts which showed a lane-to-lane average CV of 7.3%, and a FOV-to-FOV average CV of 6.5% (**Figures 3B-C**).

The lower capture efficiency when using anti-CD9 capture is expected when compared to the more generic TSPT and PS capture methods. Highly consistent results across lanes were also observed in terms of biomarker positivity distributions across EVs (**Figures 3D-E**). The lane-to-lane SD (standard deviations) for the different capture methods were: for PS capture 4.2% CD9, 3.2% CD63, and 1.5% CD81; for TSPT capture 7.% CD9, 4.7% CD63, and 3.1% CD81; and for CD9 capture 3.0% CD9, 3.3% CD63, and 1.6% CD81 (see table in **Figure 3**).



SD (%)	Marker detected		
	CD9	CD63	CD81
PS capture	4.4	3.4	7.9
TSPT capture	2.8	0.7	1.7
CD9 capture	4.3	1.7	6.7

Figure 3 | Aplo Flow EV prep delivers consistent results across chips, lanes, and FOV. A) Representative examples of single EVs imaged using dSTORM on Aplo Flow, labeled with anti-CD9-488 (cyan), anti-CD63-561 (yellow), and anti-CD81-CF647 (magenta). Scale bars = 200 nm. B-C) EV cluster counts per FOV (field of view) across all 3 chips (color-coded) and sorted by capture type in B, or by lane number in C. D-E) Normalized biotype distribution graphs showing the percentage of EV clusters positive for either of the three probed tetraspanin markers, including single-, double-, and triple-positive EVs across lanes in D, and across capture types in E. Standardized plots were automatically generated using the EVP2 Axis tool. Error bars in graphs show SD. The table shows the lane-to-lane SD values for the percentage of tetraspanin detection across capture types.

Side-by-side population-level comparison reveals EV size differences across populations

SMLM imaging of EV populations enables the study of EV samples and different experimental cohorts across scales, from the EV population level down to single EVs. Using all the single-molecule imaging data gathered across samples, the EVP2 Axis tool enables EV researchers to carry out cross-sample comparison generating both standardized and custom plots, including violin plots and scatter plots which allow side-by-side comparison. Custom plots can be generated on a standard or logarithmic scale using one parameter or comparing two different EV parameters such as circularity, density, skew, area, radius of gyration, diameter, number of localizations, or marker positivity. In addition, a split parameter can be selected to split the data within each plot to facilitate comparison based on a specific parameter. For instance, violin plots can be extremely helpful to compare the different capture types and the overall EV diameter observed, and to further split the plot by biomarker positivity, as shown in **Figure 4A**, whereby CD9-positive EVs appear to be larger than CD9-negative EVs across samples. Another example would be the scatter plot in **Figure 4B** showing the comparison of EV diameter vs number of single-molecule localizations, split by biotype (single-, double-, or triple-positives for the three tetraspanins used), where distinct subtypes can be observed.

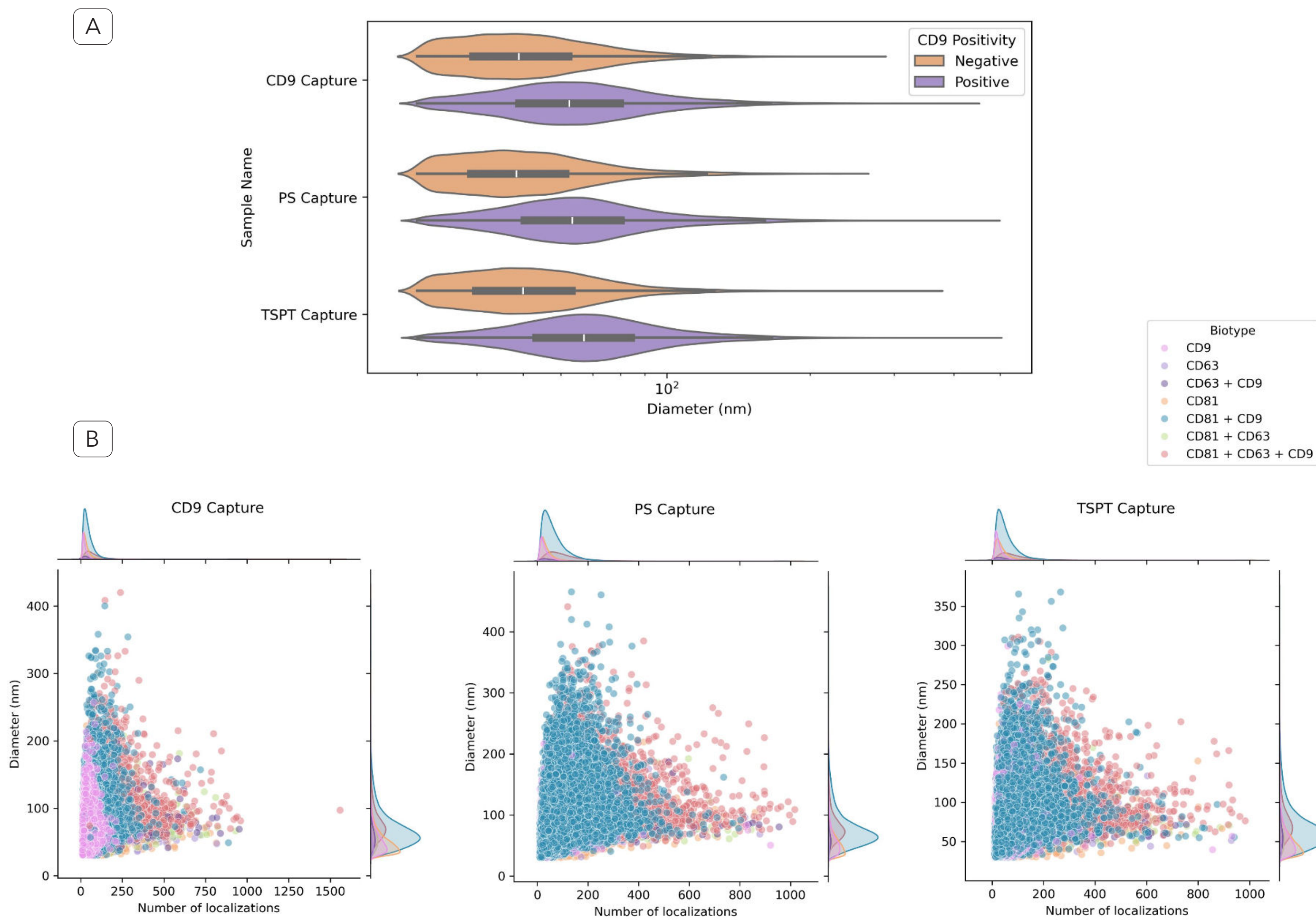


Figure 4 | Customized plots enable side-side population-level comparison across EV samples. A) Violin plots showing EV population differences based on capture type, EV diameter, and CD9 positivity (as shown in the legend). B) Scatter plots showing cross-sample differences based on number of localizations, diameter, and biomarker positivity (biotype, as shown in the legend). All custom plots were generated using the EVP2 Axis tool.

Verifying EV morphology and intra-EV cargo detection across capture types

In a parallel experiment, the same EV samples were captured on three separate chips using the same three capture methods as above (PS, TSPT, and CD9) but labeled with the Pan-EV + Tetraspanin Trio + custom marker detection modality. Using the EV Profiler 2 Pan-EV stain enables the accurate assessment of EV morphology and size, in parallel to the use of the Tetraspanin Trio cocktail (CD9, CD63, and CD81) to further confirm EV identity and determine biotype distribution. The third SMLM detection channel was used to detect the EV cargo molecule Syntenin following a permeabilization step treatment. A non-permeabilized control was also included in the experiment (**Figure 5A**).

The increase in the percentage of synthenin clusters detected after permeabilization was significantly different ($p<0.0001$) between permeabilized and non-permeabilized conditions (**Figure 5B**) as well as across different capture methods (data not shown). While slight variations in the percentage of syntenin detection and EV population size distribution (**Figure 5C**) were observed across the different capture methods, overall, the syntenin-positive populations tended to correlate with higher EV density (**Figure 5D**).

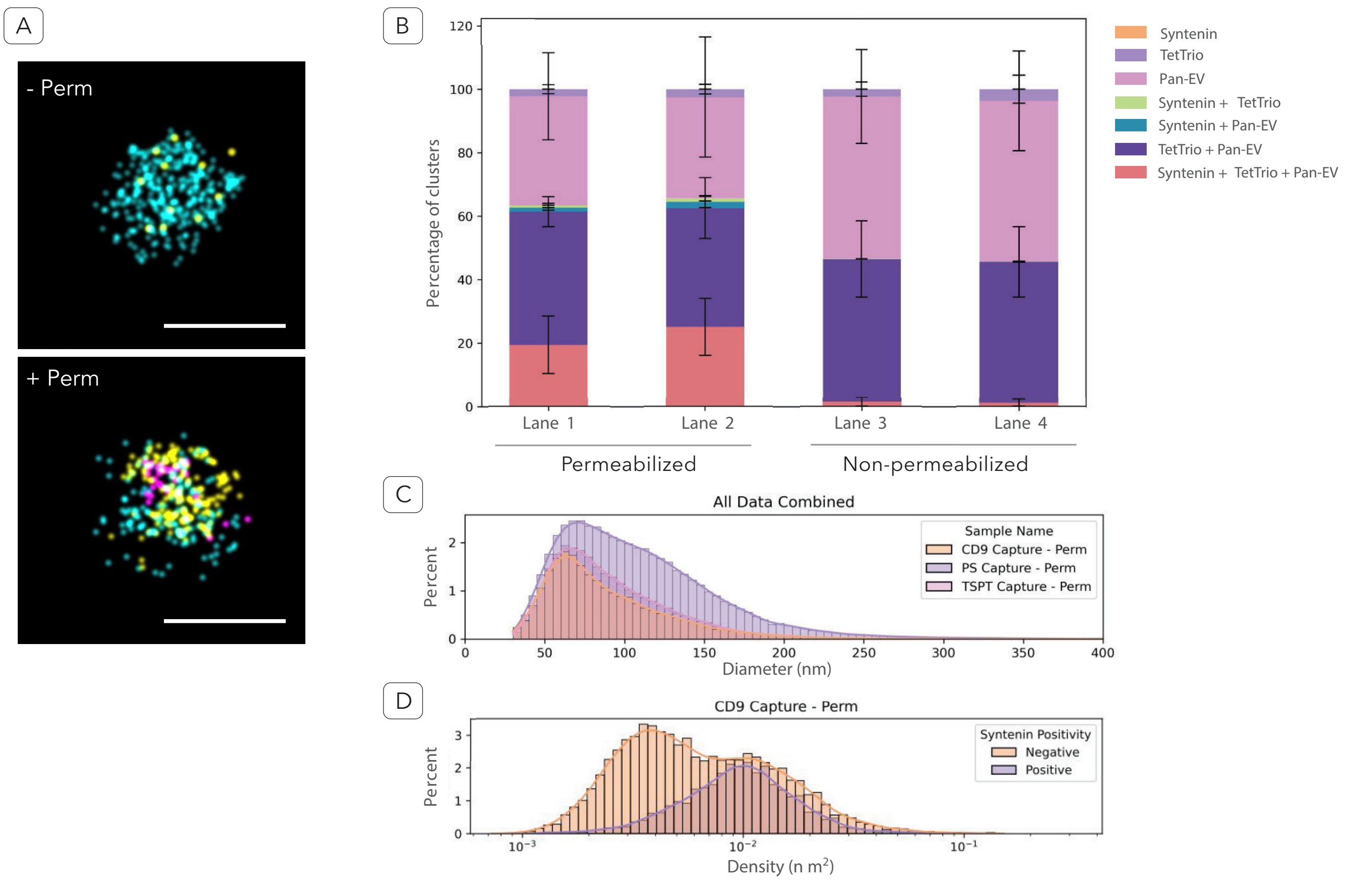
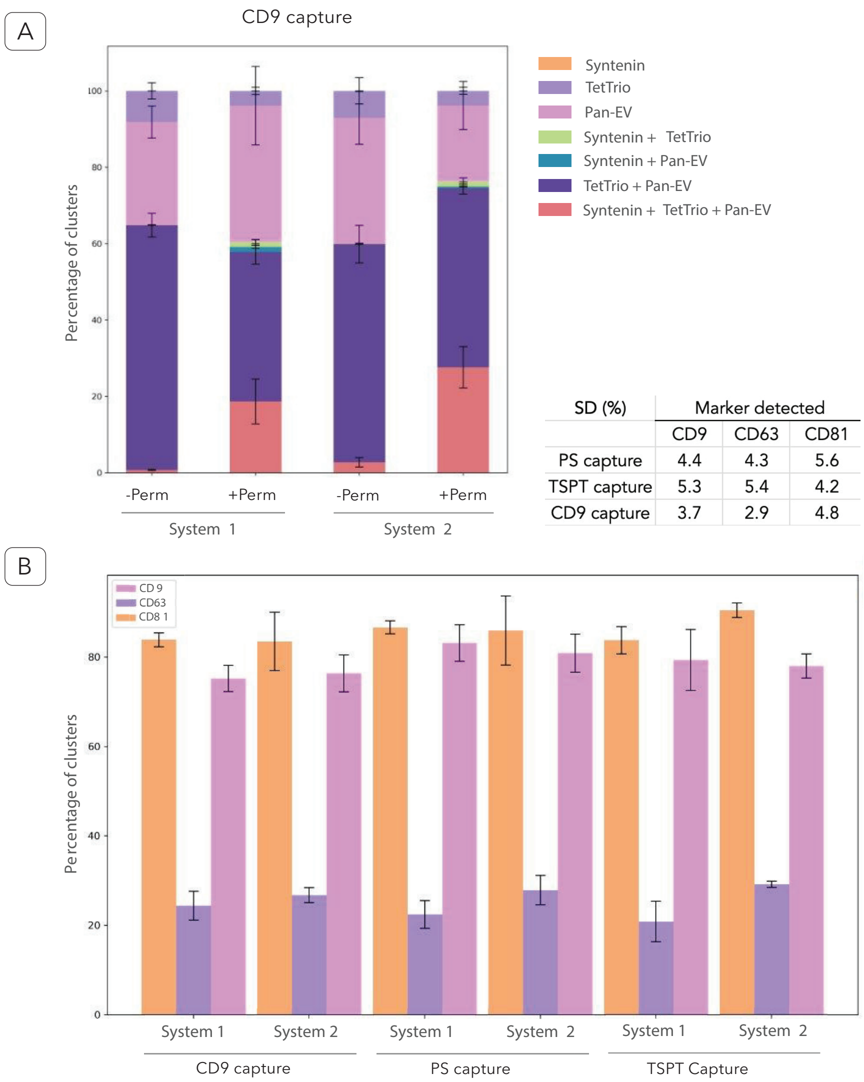


Figure 5 | EV morphology and intra-EV cargo detection across capture types. A) Representative examples of single EVs imaged using dSTORM on Aplo Flow either following permeabilization (+Perm) or not (-Perm) and labeled with Pan-EV stain (cyan), Tetraspanin Trio (yellow), and Syntenin (magenta). Scale bars = 200 nm. B) Normalized biotype distribution graphs showing the percentage of EV clusters single-, double-, and triple-positive for Syntenin, Pan-EV, and/or Tetraspanin Trio. Syntenin positivity increased significantly after permeabilization to detect intra-EV molecules. Error bars show SD. C-D) Overlapping histogram plots showing: in C, the distribution (in percentage) of EV diameters across the different populations, and in D, the percentage of syntenin-positive or -negative EV based on density distribution.

The Aplo platform end-to-end EV workflow shows consistent results across labs

The Aplo platform has been engineered to deliver automation to EV workflows, with a particular focus on reliability and consistency. In a preliminary study to confirm result consistency across systems and users, we tested our end-to-end EV characterization platform operated at two independent laboratory locations, using two different Aplo Flow and Aplo Scope systems. Because almost all workflow steps are automated, we considered user-to-user variability to be negligible (albeit this was not tested). Using either the Pan-EV + TetTrio + Syntenin setup or the three separate Tetraspanin detection setup resulted in consistent results observed across systems, in terms of the level of cargo detected after permeabilization, and biomarker positivity levels (**Figure 6**). In addition, chip-to-chip variations remained consistent across systems, with SDs of 40 FOVs across systems remaining around or under 5% (see SD table across capture methods in **Figure 6**). Further experiments will be necessary to precisely confirm reproducibility across systems.



SD (%)	Marker detected		
	CD9	CD63	CD81
PS capture	4.4	4.3	5.6
TSPT capture	5.3	5.4	4.2
CD9 capture	3.7	2.9	4.8

Figure 6 | The Aplo platform EV workflow shows consistent results across systems. Normalized stacked graph bar showing the percentage of EV clusters detected across systems with the: A) Pan-EV + TetTrio + Syntenin detection setup with CD9 capture, and B) Tetraspanin detection setup with different capture methods. Error bars show SD. The table shows the chip-to-chip SDs for all FOVs across both systems for the different capture methods across tetraspanin detection levels (a total of 40 FOVs for each).

CONCLUSIONS

The work presented here validates ONI's end-to-end EV characterization workflow powered by the Aplo platform as a reliable tool to profile EV populations and report cross-dataset differences in a seamless manner. Using automation for EV sample preparation, image acquisition, and analysis, researchers can perform EV profiling reliably from the population level down to single EVs, extracting key parameters such as EV counts, biomarker distribution, morphology assessment, cargo density, and more through both standardized and customized data plotting.

This super-resolution workflow enables a "digital" form of population sorting. Unlike mechanical isolation techniques that may compromise vesicle integrity, single-molecule data allow for the post-acquisition stratification of EVs based on precise physical and molecular parameters. Subpopulations can be selectively analyzed, for example, comparing vesicles smaller than 100 nm against those exceeding 100 nm, to evaluate their respective biomarker signatures. Overall, these multidimensional insights can enable the comparison of experimental cohorts, even when subtle molecular heterogeneity dictates EV functionality, and help identify clinically relevant shifts in EV profiles to unlock early disease diagnostics and novel nanoparticle-based therapeutics.

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