USE OF CONVENTIONAL FLOW CYTOMETRY TO STUDY EXTRACELLULAR VESICLES

Eli Kraus, Yun Chen, Manish Tandon, Gregg Nyberg

300 North Beacon Street, Watertown, MA 02472

Abstract

1. Extracellular vesicles (EVs), refers to non-replicating, lipid bilayer-delimited particles that are naturally released and intrinsic to all cell types. These heterogenous vesicles are fundamental for intracellular communication and pathophysiology by delivering cargoes carrying a broad range of biomolecules including proteins, nucleic acids, lipids and metabolites. Therefore, they are attractive candidates to exploit for therapeutic benefits.
2. Rapid analysis of these nanoparticles is critical to directly support clinical EV biomanufacturing platforms or to study EVs as carriers of nucleic acids (DNA or RNA) alongside their parental cells. It is a challenge to reliably perform size-based concentration measurements and specific marker-based characterization for nanoparticles such as EVs with currently available analytical methods.
3. Here, we demonstrate the development of a rapid analytical method based on conventional flow cytometry with reliable accuracy, repeatability, linearity, and precision within a defined quantitation range. Utilizing detector configurations with Violet laser (405 nM), nanoparticles ≥100 nm size were resolved. Furthermore, the surface staining of EVs using established biomarkers demonstrated the feasibility of multiplexed characterization of EV phenotype.

Assay development

Resolution of nano beads by Flow Cytometry with VSSC configuration

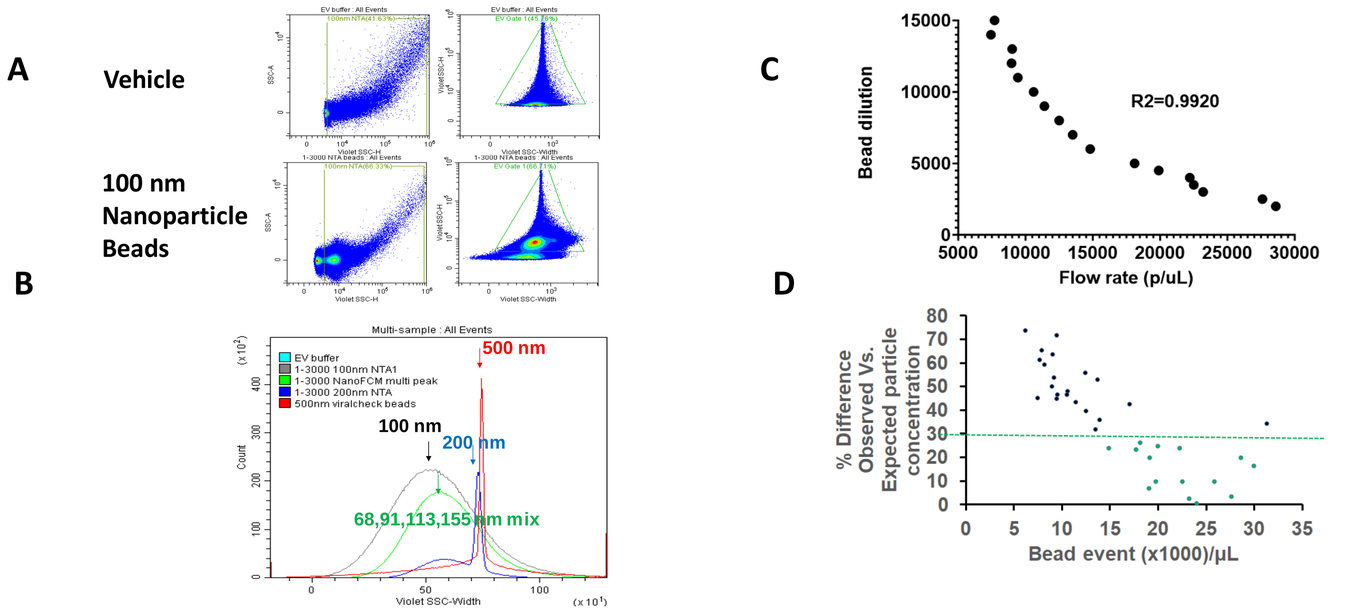
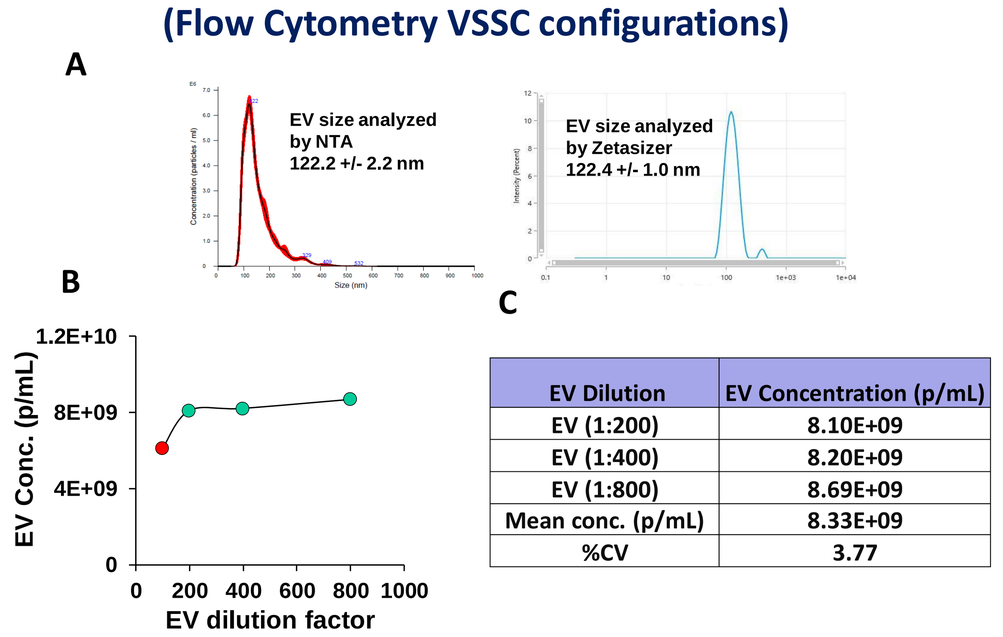


Figure 1. Assessment of nanoparticle enumeration with Flow Cytometry VSSC configuration. (A) 100 nm nanoparticle beads (*Malvern Panalytical*) were analyzed by Flow Cytometry Violet laser Side Scatter (VSSC) (*CytoFLEX S*) configurations together with vehicle control. The scatter plots are shown. **(B)** The histogram plots of nanoparticles with variable sizes analyzed by Flow Cytometry VSSC configuration. The results indicated that the limit of resolution for nanoparticle size is 100 nm with VSSC configuration. **(C)** The titration of 100 nm beads at different dilutions is indicated. The correlation (R^2) of the bead concentration and dilution was observed to be =0.9920. **(D)** The % difference between counts observed by Flow Cytometry and expected concentration (1.7E11 p/mL) by Nanoparticle Tracking Analysis is shown. The green cut-off at 30% difference is suggested as a putative accuracy criteria. This data suggest that nanoparticle bead concentration should be within the Flow Cytometry instrument detection range for an accurate nano particle enumeration.

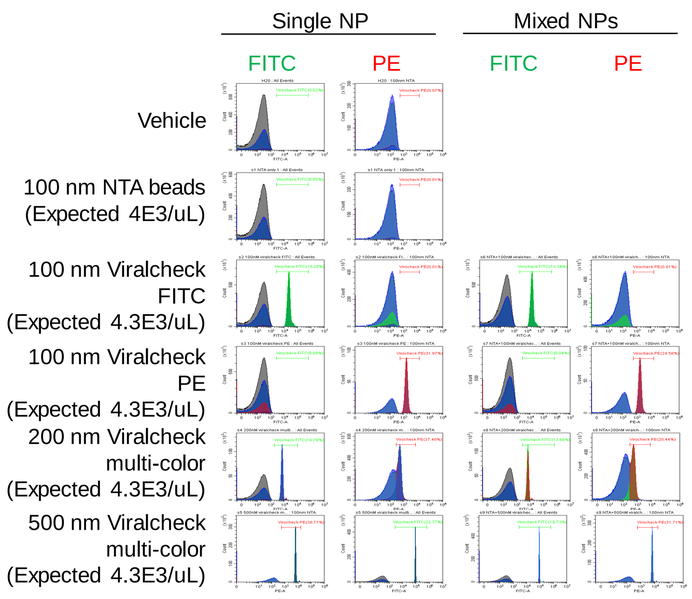
Reliable assessment of Extracellular Vesicles (EV) (Flow Cytometry VSSC configurations)



EV Dilution	EV Concentration (p/mL)
EV (1:200)	8.10E+09
EV (1:400)	8.20E+09
EV (1:800)	8.69E+09
Mean conc. (p/mL)	8.33E+09
%CV	3.77

Figure 2. A representative EV sample tested with Flow Cytometry VSSC configuration. (A) The size of representative EV (>100nm) was confirmed by two orthogonal instruments, *Nanosight (NTA 3.4)* and *Zetasizer Ultra,* based on laser light scattering. **(B)** Using the 100 nm beads for size gating, the serially diluted EVs were analyzed by Flow Cytometry. **(C)** The tabulated result demonstrate that the EV concentration within the linear range with 200- to 800-fold dilution (%CV=3.77).

Assessment of nanoparticle fluorescence parameters for accuracy using Flow Cytometry with VSSC configuration

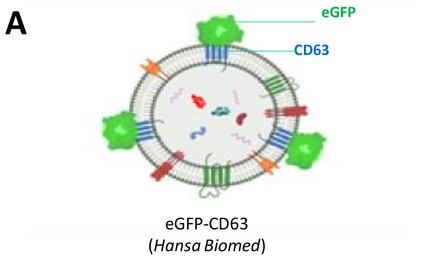


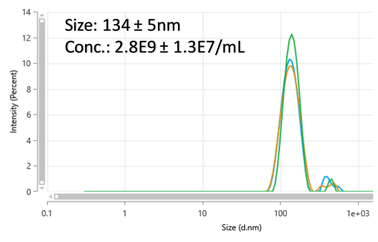
B **Recovery rate of enumeration by EV flow using sizing and/or fluorescent gating**

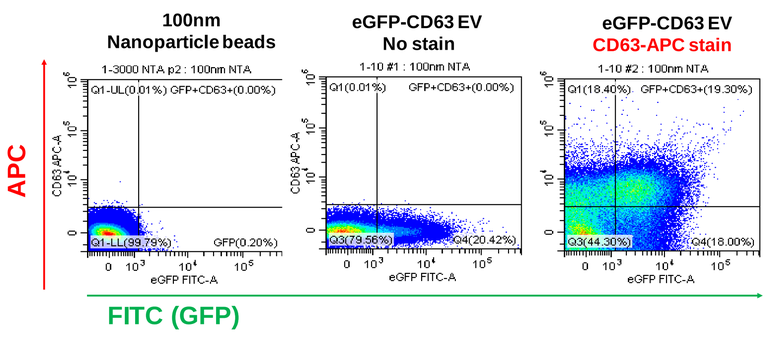
Nanoparticle with different fluorescence and size	Recovery rate (Enumeration by EV flow vs. expected nanoparticle concentration)											
	Test1		Test2			Test3			Mean of % Recovery (fluo and sizing)	%CV (fluo and sizing)	Mean of % Recovery (sizing)	%CV (sizing)
	Fluo (mix)	Sizing (100nm)	Fluo (solo)	Fluo (mix)	Sizing (100nm)	Fluo (solo)	Fluo (mix)	Sizing (100nm)				
100nm NTA beads	N/A	135	N/A	N/A	125	N/A	N/A	125	128	4.4	128	4.4
100nm Viralcheck-FITC	53	76	54	54	70	52	N/A	N/A	60	17.6	73	5.8
100nm Viralcheck-PE	76	78	70	73	87	77	78	83	78	6.8	82	8.0
200nm Viralcheck multi	N/A	N/A	62	60	77	64	59	89	69	17.6	77	5.2
500nm Viralcheck multi	N/A	N/A	86	82	68	88	80	80	81	8.6	74	11.7

Figure 3. Fluorescent parameters with VSSC configurations with *Viralcheck* nanoparticle beads using sizing and fluorescent gating. (A) Monodispersed fluorescent nanoparticle beads of different sizes (*Viralcheck, Thermo Fisher*) were mixed with non-fluorescent 100 nm beads and analyzed by Flow Cytometry using size gating. The resolution of fluorescent peaks (FITC and PE) is indicated. **(B)** The recovery of bead concentration observed by Flow Cytometry (percentage of observed conc./expected conc) was calculated in three independent tests. The precision and accuracy are indicated based on fluorescence + sizing (in green) or sizing alone (in blue). Note: The expected concentration was obtained from the nanoparticle bead manufacture's certificate of analysis.

Assay verification







D **The performance of nanoparticle Flow Cytometry for eGFP-CD63 Fluorescent Extracellular Vesicles**

Nanoparticle samples	100nm sizing Events/µL(V)	Dilution factor	EV conc. (p/mL)	EV conc. (Zetasizer)	% Difference between Flow and Zetasizer analysis
Fluo-EV	4.70E+03	500	2.35E+09	2.80E+09	16.1

Sample ID	% FITC(GFP)	% CD63-APC	% GFP + CD63 +	% Difference between stained CD63-APC and native eGFP
Fluo-EV No stain	21	0	0	N/A
Fluo-EV stained with CD63-APC	23	23	19	9.2

Figure 4. Assessment of a commercial EV product by Flow Cytometry with VSSC configuration. (A) The schema of a commercial EV product (eGFP-CD63, *Hansa Biomed,* produced in HEK293T cells) with fluorescent bio-marker. **(B)** The EV size and concentration were confirmed by *Zetasizer Ultra.* **(C)** The pseudo color plots for eGFP-CD63 Fluo-EV as analyzed by Flow Cytometry with FITC (GFP) and APC (CD63) fluorescent channels is shown. **(D)** The performance of fluorescent EV Flow Cytometry for eGFP-CD63 is presented as % difference between Flow and *Zetasizer* analysis. The duplex analysis by Flow Cytometry indicated <10% difference among the two channels (green and red fluorescence).

Conclusion and Applications

- The conventional Flow Cytometers can provide biological insights into therapeutic Extracellular Vesicle products.
- The Extracellular Vesicles (≥100 nm size) can be reliably and accurately quantified by conventional Flow Cytometry
  - Size, Concentration and Biomarkers
- This method is applicable for real-time monitoring of different EV process development areas- scale-up and downstream.
- The method attributes are applicable for quality-controlled release of EV products.