Characterizing the Light-Scatter Sensitivity of the CytoFLEX Flow Cytometer

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Introduction

Extracellular vesicles (EVs) and other biological nanoparticles (NPs) generally fall within the optical noise of light-scatter-based detection methods, and most flow cytometers are not sensitive enough to effectively detect NPs less than 300nm in diameter. The CytoFLEX is a notable exception to this: it is so sensitive that the SSC detector actually has an attenuation filter to reduce 95% of the scatter signal, adjusting it to a range useful for cells. As an alternative, Violet SSC (VSSC) can be used to bring the CytoFLEX sensitivity well into the nanoparticle range.

In order to better characterize the biological threshold sensitivity of the CytoFLEX using VSSC, we analyzed a variety of NPs of different compositions, including viruses and purified plasma EVs. After acquisition, the median scatter intensity for each EV sample was converted to size or refractive index (RI) using Mie theory.

Materials

Item	Catalog #	Vendor
60nm PS NIST Size Standard	3060A	ThermoFisher
70nm PS NIST Size Standard	3070A	ThermoFisher
80nm PS NIST Size Standard	3080A	ThermoFisher
90nm PS NIST Size Standard	3090A	ThermoFisher
100nm PS NIST Size Standard	3100A	ThermoFisher
125nm PS NIST Size Standard	3125A	ThermoFisher
150nm PS NIST Size Standard	3150A	ThermoFisher
200nm PS NIST Size Standard	3200A	ThermoFisher
300nm PS NIST Size Standard	3300A	ThermoFisher
68.6nm Si NIST Beads	NS-0070A	MSP Corporation
98.6nm Si NIST Beads	NS-0100A	MSP Corporation
110nm Si Beads	803308	Sigma Aldrich
160nm Si Beads	SS02000	Bangs Labs
214nm Si Beads	140140-10	Corpuscular, Inc
293nm Si Beads	SS02001	Bangs Labs

Item	Catalog #	Vendor
Adenovirus, HAdV-5	1060	Vector Biolabs
MV-M-Zero (MLV)	MV-M-Zero	ViroFlow Technologies
HV-H-Zero (HIV-1)	HV-H-Zero	ViroFlow Technologies
Herpes Simplex Virus (HSV-1)	Custom	ViroFlow Technologies
Vaccinia Virus	Custom	ViroFlow Technologies
HPLC Water	WX0008-1	EMD Millipore
Izon qEVsingle/70nm Columns	SP2	Izon Science
0.2μm Acrodisc Syringe Filter	4612	Pall Corporation
CD61-PC7	IM3716	Beckman Coulter
FlowClean Cleaning Agent	A64669	Beckman Coulter
CytoFLEX Sheath	B51503	Beckman Coulter
CytoFLEX-S (N-V-B-R)	B78557	Beckman Coulter
CytExpert Software v2.3	N/A	Beckman Coulter
Kaluza v1.5	B16406	Beckman Coulter
DelsaMax Pro	B29164	Beckman Coulter

Methods

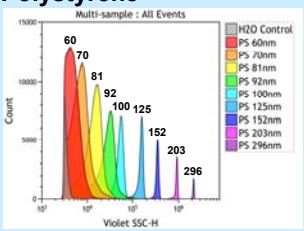
1. Upon startup, the instrument was primed, cleaned and flushed.
2. Polystyrene (PS) and silica (Si) beads were diluted and titrated with HPLC water.
3. Viruses were diluted and titrated with PBS.
4. Extracellular Vesicles (EVs) were prepared from fresh human blood as follows:
   a) 2mL of K3-EDTA blood was first aliquotted into 12x75mm centrifuge tubes. Cells in larger volumes, further away from the max radius, do not pellet as well within a short time frame and larger EVs pellet with longer time frames, so if a larger volume is needed, increasing the number of tubes works better than a greater volume per tube.
   b) The blood was centrifuged for 5 min at 200xg to pellet the majority of cells and platelets.
   c) Roughly 1mL of platelet-poor plasma (PPP) was removed from the top, careful to minimize collection of the platelet-rich plasma near the WBC layer.
   d) The PPP was filtered through a 200nm syringe filter to remove residual large particles.
   e) Finally, the filtered PPP was further purified using Izon size-exclusion chromatography (SEC) columns to remove particles smaller than 70nm.
   f) EVs were incubated with antibodies for 1 hour in the dark at RT, and then diluted 1:1K to 1:4K in PBS + 0.2% PFA prior to acquisition.

4. All samples were acquired on a CytoFLEX-S N-V-B-R flow cytometer, and the data were analyzed in CytExpert v2.3.

5. The median sizes of the EV fractions were analyzed by dynamic light scatter (DLS) using cumulant analyses of Brownian motion on a DelsaMax Pro. Each sample was read 10x for 10x 2-second acquisitions per read.

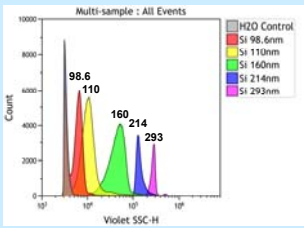
Reference Standards and Viruses

Polystyrene



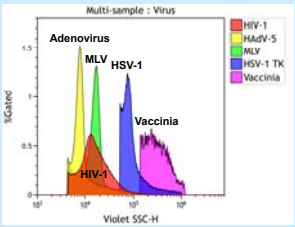
- 60nm PS is detected just above threshold.
- 70nm PS is resolved.
- 80nm PS is the official CytoFLEX spec.

Silica



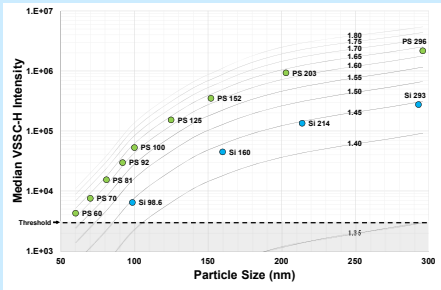
- 98.6nm Si beads are fully resolved.
- The 98.6nm Si beads overlap with plasma EVs in both size and RI.

Viruses



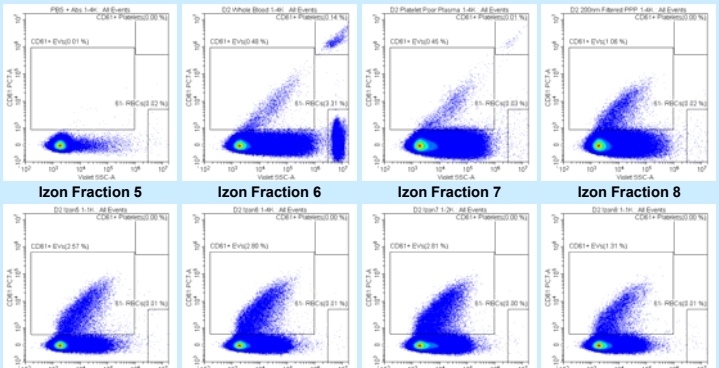
- Adenovirus is 95nm.
- HIV-1 is 100nm.
- MLV is 110nm.

Mie Theory RI Curves Scaled to VSSC-H



Characterization of the Light-Scatter Sensitivity for Detecting Extracellular Vesicles

CD61⁺ Platelet EV Sample Preparation



- CD61⁺ platelet EVs can be easily detected directly in whole blood or plasma.
- To purify EV fractions, the larger particles were first removed by centrifugation and filtration, and then the smaller particles were removed by SEC, narrowing the sample range to approximately 70-200nm.

DLS Size Controls

Si 68.6nm

Read	Cumulant	DYNALS
1	68.3	69.1
2	67.3	70.4
3	66.8	68.8
4	67.2	67.7
5	67.0	66.7
6	68.2	68.6
7	67.7	67.8
8	67.0	66.5
9	67.3	71.2
10	67.8	69.0
Average	67.46	68.58
SD	0.52	1.49
%CV	0.77	2.17
PD Index	0.04	0.03

Si 98.6nm

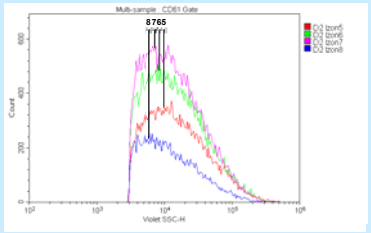
Read	Cumulant	DYNALS
1	99.5	102.4
2	98.5	99.4
3	97.7	98.4
4	96.7	97.9
5	96.8	98.6
6	97.0	98.5
7	96.6	98.0
8	97.0	98.7
9	98.2	98.8
10	96.4	96.5
Average	97.64	98.72
SD	1.06	1.50
%CV	1.09	1.52
PD Index	0.03	0.01

Si 214nm

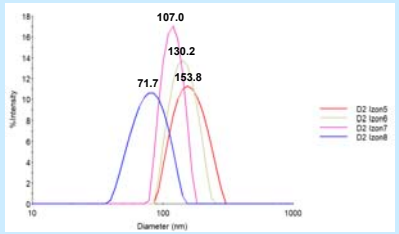
Read	Cumulant	DYNALS
1	212.9	212.3
2	214.9	213.0
3	214.0	213.1
4	209.2	210.1
5	212.0	217.4
6	214.3	216.0
7	212.7	217.0
8	214.9	216.5
9	213.5	214.7
10	216.5	217.5
Average	213.49	214.76
SD	1.99	2.53
%CV	0.93	1.18
PD Index	0.02	0.00

Analyses of Light Scatter vs. Size for Plasma EVs

CytoFLEX-S – VSSC-H



DelsaMax – DLS



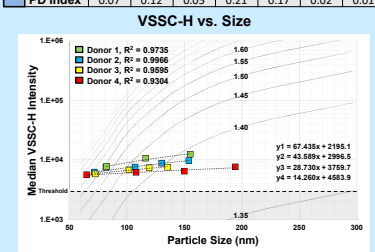
CytoFLEX

	Donor 1				Donor 2				Donor 3				Donor 4			
	F8	F7	F6	F5	F8	F7	F6	F5	F8	F7	F6	F5	F8	F7	F6	F5
VSSC-H	7439.5	7707.1	10578.9	12388.7	6173.0	7532.8	8738.5	9707.6	5764.8	6734.3	7380.8	7479.5	5597.9	6144.1	6415.0	7549.2
SD	40.69	129.66	231.41	100.54	88.96	25.24	111.37	15.71	561.97	380.26	114.16	103.64	244.22	211.50	64.88	404.14
%CV	0.55	1.68	2.19	0.81	1.44	0.34	1.27	0.16	9.75	5.65	1.55	1.39	4.36	3.44	1.01	5.35

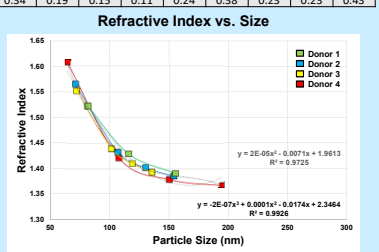
DelsaMax

	Donor 1				Donor 2				Donor 3				Donor 4			
	F8	F7	F6	F5	F8	F7	F6	F5	F8	F7	F6	F5	F8	F7	F6	F5
Size	81.6	82.3	116.0	155.0	71.7	107.0	130.2	153.8	72.5	101.7	119.2	135.4	64.8	107.8	150.0	194.3
SD	3.88	5.18	6.94	12.42	6.66	11.37	8.16	11.73	1.17	2.46	2.11	3.54	2.45	4.07	6.23	12.89
%CV	4.76	6.30	5.98	8.01	9.29	10.63	6.27	7.62	1.62	2.41	1.77	2.61	3.78	3.78	4.15	6.63
PD Index	0.07	0.12	0.05	0.21	0.17	0.02	0.01	0.34	0.19	0.15	0.11	0.24	0.38	0.23	0.23	0.43

VSSC-H vs. Size



Refractive Index vs. Size



Discussion

Ultimately, the CytoFLEX is highly sensitive for nanoparticle detection. We found that the CytoFLEX could fully resolve 70nm PS and 100nm Si NPs, as well as 95nm Adenovirus, 100nm HIV-1, and 110nm MLV. Moreover, we were able to detect platelet EVs at least as small as 65nm in diameter using only a VSSC trigger and CD61 labeling to identify the population. Further analysis revealed that the RI of the smallest EVs increases with decreasing diameter, expanding the lower range of EVs detectable by light scatter. This effect has been observed in literature.