Small particle flow cytometry using 3 light scatter detectors enhances extracellular vesicle analysis in liquid biopsies, highlighting the potential to segregate EVs by refractive index.

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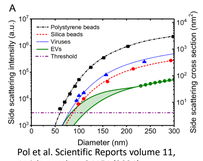
1. Introduction

Extracellular vesicle analysis using “small particle” flow cytometry would be greatly enhanced if data from materials of different refractive index (RI) could be segregated. Likewise, relative sizing of EVs using small particle flow cytometry is confounded by the influence of RI on light scatter. Beads of different composition and refractive index scatter light differently, so that small beads of high RI and large beads of lower RI can have overlapping signals on a two dimension light scatter plot.

As particle size decreases, light scatter intensity profiles eventually merge regardless of refractive index.

In this project, we aimed to demonstrate graphically, (1) the enhancement of EV flow analysis when using an additional angle of light scatter collection (medium angle of light scatter, MALS) to identify different sample components (e.g. lipids, protein, extracellular vesicles) and (2) the practical reality of sample component overlap at different particle sizes.



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2. Methods

An Apogee A60 Micro-Plus outfitted with a third (medium) angle of light scatter (MALS) collection was used to analyze samples typical of EV analysis.

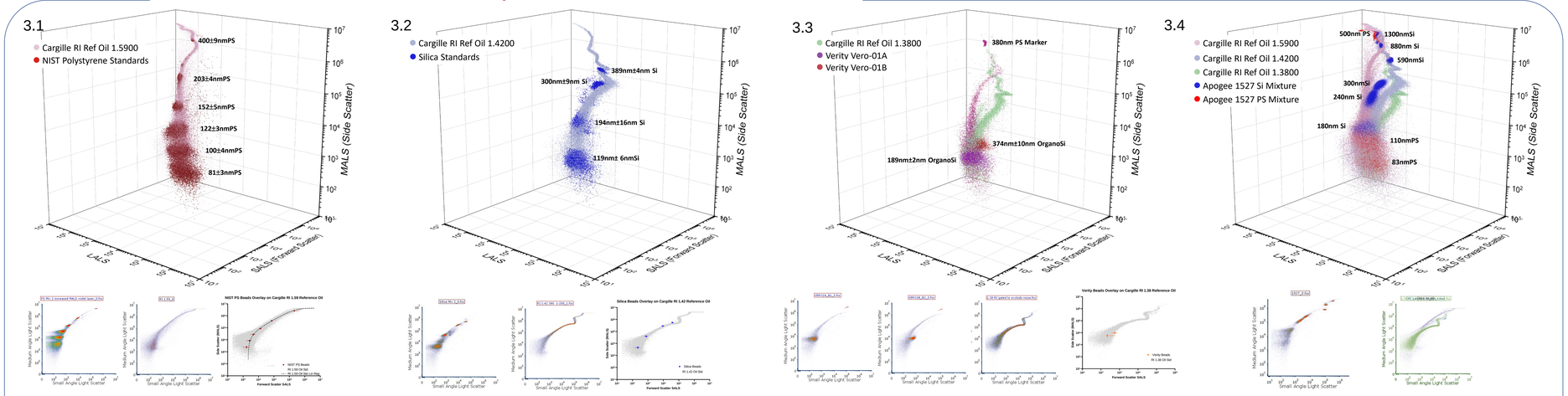
- Events were triggered solely by MALS excited from a 405nm laser.
- NIST bead (70-400nm) and silica bead standard mixtures (100-400nm) were analyzed first to define cytometer settings.
- Refractive index oil emulsion standards (RI 1.38, 1.42, 1.59, Cargille) were prepared to generate a continuum of particle sizes; each emulsion was analyzed separately.
- The multi-angle light scatter data from each RI standard was plotted to yield the distinct pattern of particle size (light scatter intensity) influenced by refractive index.

Cytometer Settings

Platform	Apogee A60 MP	S/N 0130		
Parameter	**Setting**		**Parameter**	**Setting**
Flow Rate	0.75 -3.01 µL/min		**Trigger**	MALS
Pressure	150 units		**Sample volume**	10 µL
Acquisition time	60 - 120 sec		**Diluent**	PBS
Sample Dilution	50-10^{6}PBS		**Diluent**	PBS
Channel	**Laser Power (mW)**	**PMT (v)**	**Gain**	**Threshold**
405nm	85	---	---	---
488nm	70	---	---	---
561nm	70	---	---	---
638nm	70	---	---	---
405-SALS	---	370	1.0	---
405-LALS	---	385	1.0	---
405-MALS	---	380	1.0	27
405-Blue	---	505	1.0	---
405-Green	---	575	1.0	---
488-Green	---	480	1.0	---
488-Orange	---	300	1.0	---
488-Red	---	310	1.0	---
561-Orange	---	510	1.0	---
561-Red	---	770	1.0	---
638-Red	---	500	1.0	---
638-Far Red	---	300	1.0	---

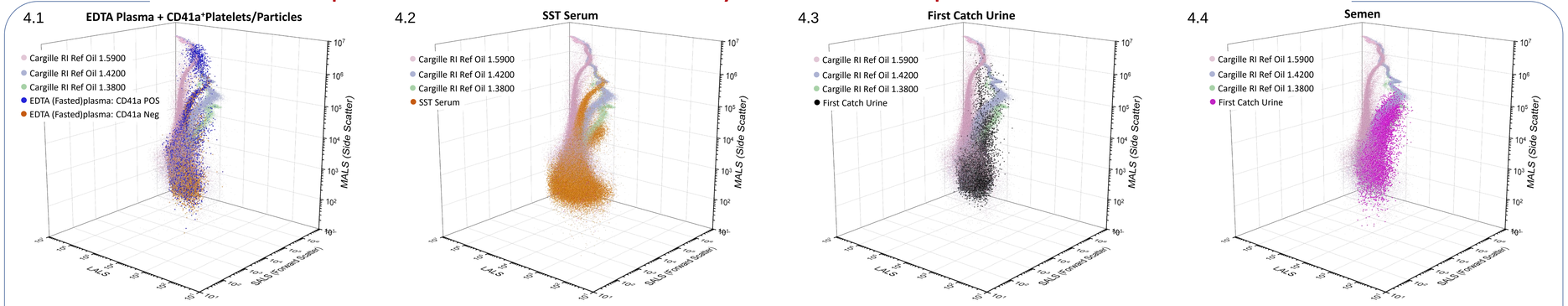
Product	**Product #**	**Attribute**	**Lot #**	**Company**
Calibration Mix	1524	Mixture Si/PS	CAL0143	ApogeeFlow Systems
Monitoring Mix	1527	Mixture Si/PS	CAL0145	ApogeeFlow Systems
NIST 70nm PS	3070A	70nm±3nm	Lot#: 230764	ThermoScientific
NIST 80nm PS	3080A	81nm±3nm	Lot#: 229986	ThermoScientific
NIST 90nm PS	3090A	92nm±3nm	Lot#: 231451	ThermoScientific
NIST 100nm PS	3100A	100nm±4nm	Lot#: 231703	ThermoScientific
NIST 125nm PS	3125A	122nm±3nm	Lot#: 230329	ThermoScientific
NIST 150nm PS	3K-150	152nm±5nm	Lot#: 232375	ThermoScientific
NIST 200nm PS	3200A	203nm±4nm	Lot#: 232366	ThermoScientific
100nm Silica	SISN100	104nm±9nm	Lot# JEA0234	NanoComposix
120nm Silica	SISN120	119nm±6nm	Lot# JRC0354	NanoComposix
200nm Silica	SISN200	194nm±16nm	Lot# JEA0232	NanoComposix
300nm Silica	SISN300	300nm±12nm	Lot# SCM0179	NanoComposix
400nm Silica	SISN400	389nm±4nm	Lot# JRC0354	NanoComposix
Verity Small	vero1A	189nm±2nm	NA	Exometry
Verity Large	vero1B	374nm±10nm	NA	Exometry
Biologicals				
IntraLipid (20%)	I141	20% emulsion	MKCG7221	Sigma Aldrich
Abnormal Lipids	LRC 03	Xs Chol, TGs, Lps	3527	SolomonPark
Excess protein	INT-01P	Albumins, γ-globulins	0314721	Sun Diagnostics
Anti-CD41-SB436	62-0419-41	Primary Ab	2056117	ThermoScientific
Anti-CD63-APC	130-111-020	Primary Ab	52103004403	Miltenyi Biotec
GoatantiMs-AF488	A31620A	Secondary Ab	2342829	Invitrogen
Plasma	APCaRI donor	EDTA plasma		Nanostics
Serum	APCaRI donor	SST serum		Nanostics
Urine	APCaRI donor	First catch		Nanostics
Semen	APCaRI donor	Standard sample		Nanostics
Oil Emulsions				
Refractive Index Oil	1803	Series AAA 1.38000	0521	Cargille
Refractive Index Oil	1806	Series AAA 1.42000	0321	Cargille
Refractive Index Oil	1809	Series AAA 1.59000	0920	Cargille

3. Common EV Bead Standards Overlay with Refractive Index Emulsion Oils



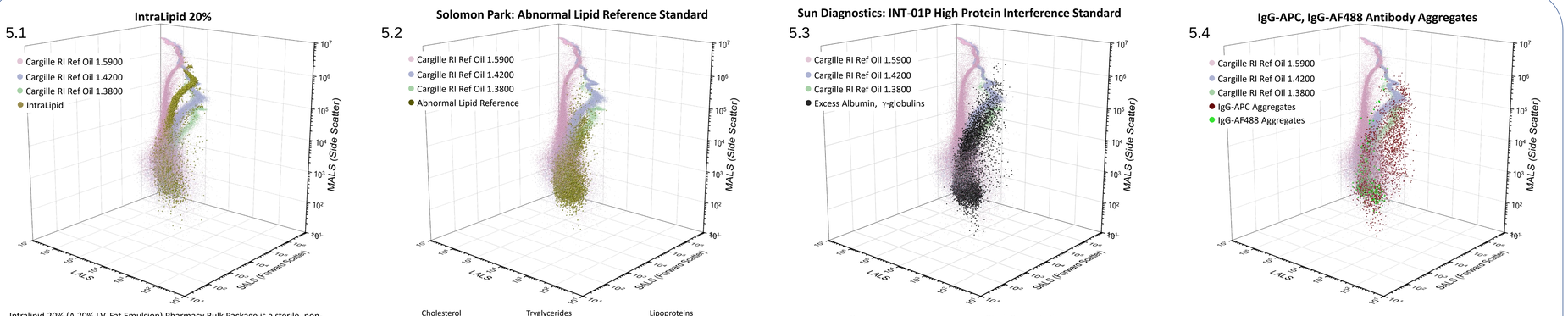
NIST bead (70 -200nm) and silica bead standard mixtures (100-400nm) were analyzed first to define cytometer settings. Refractive index (Cargille) oil emulsion standards (RI 1.38, 1.42, 1.59) were prepared to generate a continuum of particle sizes; each emulsion was analyzed separately. The multi-angle light scatter data from each RI standard was plotted to yield the distinct pattern of particle size influenced by refractive index. NIST (polystyrene) bead data overlaid almost perfectly with the 1.59 RI standard emulsion data (3.1). Similarly, silica bead standards followed the pattern of the 1.42 RI reference emulsions (3.2). Analyzed in the same manner, Verity beads (Exometry) overlaid atop the 1.38 RI reference emulsions (3.3). Apogee 1527 Si and Polystyrene beads overlaid with the expected emulsion profiles (3.4). Emulsion profiles for the refractive indices 1.38 and 1.42 appeared to be clearly separated between ~180nm Si – 590nm Si (3.4); refractive indices 1.38 and 1.59 appeared to be clearly separated between ~180nm Si – 880nm Si (3.4); below 180nm Si all profiles merge (3.4).

4. Common EV Sample Matrices Show Clear Patterns of Overlay & Distinct Areas of Separation with Refractive Index Emulsion Oils



Samples commonly used for EV analyses were compared to the profiles of refractive index oil emulsions. (4.1) Fasted plasma stained for CD41a (a common platelet marker) showed that platelets and platelet derived particles were integrated with both the RI1.38 profile and between the RI 1.42 and 1.59 emulsion profiles. Again, particles below ~180nm Si appear to merged with all RI emulsions. Serum (4.2) gave a similar data as plasma. First catch urine (4.3) yielded more smaller particles that appeared to be immersed between the RI1.38 and 1.42 emulsions, bigger particles appeared to have a higher refractive index. Small particles from semen samples (4.4) were consistently between RI 1.42 and 1.38 emulsion profiles. All particles from any sample below the ~180nm Silica size appeared to be merged within each of the emulsion profiles in this range.

5. Biological Controls used for Diagnostics Show Clear Patterns of Overlay & Distinct Areas of Separation with Refractive Index Emulsion Oils



Intralipid 20% (A 20% I.V. Fat Emulsion) Pharmacy Bulk Package is a sterile, non-pyrogenic fat emulsion intended as a source of calories and essential fatty acids for use in a pharmacy admixture program. It is made up of 20% Soybean Oil, 1.2% Egg Yolk Phospholipids, 2.25% Glycerin, and Water. Intralipid is commonly used to assess lipid interference in diagnostic assays.

All values were established at the NorthWest Lipid Reference Laboratory (NWLRL), an established reference laboratory through the Cholesterol Reference Method Laboratory Network (CRMLN) and certifies manufacturers of clinical diagnostic products that measure total cholesterol, HDL-C, and LDL-C.

Cholesterol
- TCh =287.5mg/dL
- HDL = 68.0 mg/dL
- LDL = 179.0 mg/dL

Tryglycerides
- TG (total) =151.5mg/dL
- TG (net) = 149.5 mg/dL
- TG(free) = 2.0 mg/dL

Lipoproteins
- ApoA1 =198.0mg/dL
- ApoB= 155.5 mg/dL
- Lp(a)) = 41.0 mg/dL

Source
- Human serum albumin
- Human γ-globulins

Stock Concentration
- >20g/dL

Excess protein is commonly used to assess protein interference in diagnostic assays.

Antibody aggregates are often considered a common source of false positives in EV flow cytometry assays.

To generate aggregates, aliquots of expired antibody (IgG-APC, IgG-AF488) were incubated at 50°C for 90 minutes prior to analysis by flow cytometry. Technique based upon Malvern Pananalytical application note AN140303 Aggregation in Proteins)

Biological control samples demonstrated unique profiles. Intralipid (5.1) yielded a profile very similar to plasma (4.1) and serum (4.2). The abnormal lipid mixture (5.2) had an excess of smaller particles that closely resembled the semen sample profile (4.4). The high protein sample standard looked more closely like the profile of EVs in urine (4.3) or semen (4.4). Finally the antibody aggregates (5.4) mostly resided in the area of merged emulsion profiles (<180nm Silica, (3.4)), but did not really have a cleanly defined profile matching either of the emulsions standards.

6. Conclusions

- Reference emulsion oils profile matched the refractive index profiles for bead standards commonly used as size references (section 3).
- Samples commonly used in extracellular vesicle analysis showed both distinct refractive index profiles as well as overlapping profiles (section 4).
- Biological controls for lipids and lipid mixtures showed unique profiles independent of the three reference oils tested (section 5).
- As light scatter intensity decreases with particle size the effective separation of particles of different RI is confounded by the overlap of signals.
- **Addition of a 3rd light scatter collection significantly enhances the analysis of EVs by small particle flow cytometry, but practical thresholds still exist.**

7. Links

Scan the QR code for a link to this poster and access to additional data. Any questions? Email the first author at:
desmond.pink@nanosticsdx.com