

PARTICLE CHARACTERIZATION BY FLOW CYTOMETRY

CROSS REFERENCES TO RELATED APPLICATIONS

[0001] This application is being filed 10 March 2025 as a PCT International Patent Application and claims the benefit of U.S. Provisional Application No. 63/563,091, filed 08 March 2024, and U.S. Provisional Application No. 63/566,884, filed 18 March 2024, each of which is incorporated by reference in its entirety to the extent appropriate.

INTRODUCTION

[0002] In flow cytometry, particles are arranged in a sample stream, and typically pass one-by-one through one or more excitation light beams with which the particles interact. Light scattered or fluoresced by the particles upon interaction with the one or more excitation beams is collected and analyzed to characterize and differentiate the particles. In a sorting flow cytometer, particles may be extracted out of the sample stream after having been characterized by their interaction with the one or more excitation beams, and thereby sorted into different groups.

[0003] The light scattered by the particles is typically measured in two directions: forward scattered light which is parallel with the excitation light beams, and side scattered light which is orthogonal to the excitation light beams. Relative to forward scattered light signals, the side scattered light signals are weak. Conventional flow cytometers typically include a single detector for detecting the side scattered light under a particular wavelength which limits the usage of the side scattered light signals for characterizing the particles by flow cytometry.

[0004] The CytoFLEX nano is a flow cytometer from Beckman Coulter developed as a nanoparticle analyzer sensitive enough to characterize small particles in suspension, e.g., extracellular vesicles (EVs) in suspension or lipid nano particles (LNPs) in suspension. The CytoFLEX nano is capable of facilitating single nanoparticle detection, count, and immunophenotyping for biological nanoparticles, for example EVs, virus particles, virus-like particles, and LNPs.

[0005] Lipid nano particles (LNPs) have become increasingly valuable research and therapeutic tools. LNPs consist of capsids filled with compounds with pharmaceutical properties such as modified mRNA. Several methods exist for determining LNP quality such as electron microscopy, dynamic light scatter and tracking, AUC, among others.

Most of these techniques, however, require complex sample preparation, expensive instrumentation, and sometimes complex data analysis. Dynamic light scatter, nano particle tracking, and AUC provide assay characterization of the samples, but are lacking with respect to specificity. In an embodiment of the present disclosure, an alternative to current methods is disclosed that utilizes nano flow cytometry to characterize LNPs. This technique is a significant improvement over the existing methods because, for example, it requires minimal sample preparation and provides fast results that can be correlated to functional cell transduction assays, single particle characterization with high throughput up to 5K events per second, and precise counting. In an embodiment of the present disclosure, a flow cytometer capable of characterizing particles less than 100nm, e.g., CytoFLEX nano, is used to characterize LNPs of different sizes and/or have different contents.

[0006] Extracellular vesicles (EVs) are a heterogenous family of membranous particles released by all cells, which provide crucial insights into a variety of fields and diseases. To better understand EVs, researchers must characterize EVs. However, due to their heterogeneity, size, refractive index, and complex nature it makes analysis of EVs challenging. For example, the overall size range of the EVs can range from as small as 20nm, with the recently discovered supermeres and exomeres, to as large as 5µm for the bigger apoptotic bodies. Conventional flow cytometers struggle to characterize biological samples smaller than about 100-150nm or bead/particles smaller than about 80-100nm. CytoFLEX nano is a flow cytometer with high sensitivity capable of detecting nanoparticles via violet side scatter down to 20nm as well as provide better sensitivity and resolution for all 6 fluorescent channels. In an embodiment of the present disclosure, methods are disclosed for immunophenotype characterization using a designed 5 color panel to identify different types of EVs in platelet-poor plasma (PPP) samples. In an embodiment of the present disclosure, a flow cytometer capable of characterizing particles less than 100nm, e.g., CytoFLEX nano, is used to characterize EVs of different sizes and/or have different contents.

[0007] The ability of the CytoFLEX nano to characterize small particles in suspension, e.g., extracellular vesicles (EVs) in suspension or lipid nano particles (LNPs) in suspension, makes it attractive to use in conjunction with other laboratory techniques, e.g., ultra centrifugation, column chromatography, ELISA, ELISPOT, and/or electron microscopy. In an embodiment of the present disclosure, a flow

cytometer capable of characterizing particles less than 100nm, e.g., CytoFLEX nano, is used prior to another laboratory technique, after another laboratory technique, simultaneously with another laboratory technique, or any combination thereof, to confirm another laboratory technique's characterization of small particles and/or further characterize small particles. In an embodiment, the other laboratory technique is ultra centrifugation, column chromatography, electron microscopy, ELISA, ELISPOT, or any combination thereof.

[0008] The ability of the CytoFLEX nano to characterize small particles in suspension, e.g., extracellular vesicles (EVs) in suspension or lipid nano particles (LNPs) in suspension, also makes it attractive for use to detect the degree of modification or insertion of surface markers, targeting ligands, etc. on the surface of small particles, including EVs and LNPs.

SUMMARY

[0009] In general terms, the present disclosure relates to characterizing particles by flow cytometry. Various aspects are described in this disclosure, which include, but are not limited to, the following aspects.

[0010] One aspect relates to a method of characterizing particles by flow cytometry, the method comprising: illuminating the particles with one or more excitation light beams as the particles individually pass through an interrogation zone; collecting light in a plurality of channels from the particles passing through the interrogation zone; and characterizing the particles based on the light collected in the plurality of channels.

[0011] Another aspect relates to a method of using flow cytometry to improve centrifugation, the method comprising: performing centrifugation on a specimen; performing a flow cytometry analysis of the specimen after centrifugation; and adjusting one or more parameters of the centrifugation based on the flow cytometry analysis.

[0012] A variety of additional aspects will be set forth in the description that follows. The aspects can relate to individual features and to combination of features. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the broad inventive concepts upon which the embodiments disclosed herein are based.

[0013] The disclosure provides a method of characterizing particles in a flow cytometer, the method comprising: illuminating the particles with one or more excitation light beams at one or more wavelengths as the particles individually pass through an interrogation zone; collecting side scattered light in a plurality of channels at a plurality of wavelengths from the particles passing through the interrogation zone; identifying the mean side scattered light intensity in each of the plurality of channels; calculating an average calibration factor determined in the same flow cytometer from a plurality of standard particles having known refractive indices and sizes; characterizing the particles based on the side scattered light collected in the plurality of channels; inputting one or more known parameters; inputting initial guess parameters and parameter bounds, and optionally parameter constraints, for the particles; and calculating unknown parameters that minimize loss function and satisfy the bounds and constraints.

[0014] In some cases, the particles are biological particles. In some cases, the particles are nanoparticles. In some cases, the nanoparticles are biological nanoparticles.

[0015] In some cases, the average calibration factor is determined comprising illuminating at one or more wavelengths calibration beads having known size and refractive index; collecting side scattered light from all wavelengths of interest; calculating forward Mie scattering profiles for each input calibration bead diameter; integrating Mie scattering profiles for each calibration bead diameter; calculating calibration factor for each calibration bead size; calculating goodness of fit for current half angle; reporting collection half angle with highest goodness of fit; calculating Mie scattering profile at half angle identified in previous step; and calculating the average calibration factor and CV over all calibration bead sizes for half collection angle from previous step.

[0016] In some cases, the integrating Mie scattering profiles is performed over $90 \text{ deg} \pm \text{current collection half angle}$ or from 0 deg to 90 deg in 0.1 deg steps.

[0017] In some cases, the calculating of forward Mie scattering profile comprises input of a parameter selected from the group consisting of bead diameter, bead refractive index at wavelength, medium refractive index, wavelength, polarization of incident light, and detector polarization sensitivity; calculating profiles in the range of 0 deg - 180 deg , optionally in 0.1 deg steps; and output of Mie scattering intensity as a function

of polar angle; and numerically integrating the forward Mie scattering intensity over an angular range defined by current proposed collection half angle.

[0018] In some cases, the method further comprises calculating calibration factor comprising determining the ratio of measured scattering intensity and the integrated forward Mie intensity to obtain the calibration factor.

[0019] In some cases, the goodness of fit is calculated as 1-CV of all calibration factors.

[0020] In some cases, the CV is calculated over the set of calibration factors:

CV = standard deviation (all calibration factors)/average (all calibration factors).

[0021] In some cases, the calculating the unknown parameters comprises employing a minimization algorithm and initial guess parameters, parameter bounds, and optionally parameter constraints.

[0022] In some cases, the minimization algorithm is Powell's algorithm or constrained trust region minimization algorithm.

[0023] In some cases, the loss function is: loss function = [(integrated, calculated Mie signal) x (calibration factor) – measured signal].

[0024] In some cases, the calculated Mie signal is a function of the particle parameters listed in FIG. 60.

[0025] In some cases, the particles, optionally nanoparticles, are characterized as extracellular vesicles, lipid nanoparticles, virus, virus-like particles, or protein aggregates.

[0026] In some cases, the particles, optionally nanoparticles, are characterized based on an amount of protein loading on a surface of the nanoparticles or an amount of therapeutic payload within the nanoparticles.

[0027] In some cases, the particles, optionally nanoparticles, are characterized based on detection of an empty therapeutic payload, a partial therapeutic payload, or a full therapeutic payload.

[0028] In some cases, the plurality of channels includes a plurality of side scatter channels and a plurality of fluorescence channels.

[0029] In some cases, the particles are characterized based on one or more attributes identified in the plurality of side scatter channels and optionally the plurality of fluorescence channels.

[0030] In some cases, the method further comprises sorting the particles based on their characterization.

[0031] In some cases, the particles are sorted to increase a purity or yield of the nanoparticles.

[0032] In some cases, the particles are sorted based on their secretion.

[0033] In some cases, the empty and full particles are distinguishable based on a detectable increase in scatter.

[0034] In some cases, the excitation light is selected from one or more of, two of, or two or more of VSSC1, VSSC2, BSSC, YSSC, and RSSC.

[0035] In some cases, the increase the scatter is directly correlated to the ability of the particle to transduce cells.

[0036] In some cases, the cells are selected from the group consisting of patient cells and a production cell line, optionally wherein the production cell line is a CHO cell line.

[0037] In some cases, the patient is a human patient.

[0038] In some cases, the characterizing further comprises determining unknown parameters selected from the group consisting of solid particle size, solid particle refractive index, core shell particle shell thickness, core shell particle core diameter, core shell particle shell refractive index, and/or core shell particle core refractive index of the particles.

[0039] In some cases, the characterizing further comprises inputting one or more known parameters selected from the group consisting of solid particle size, solid particle refractive index, core shell particle shell thickness, core shell particle core diameter, core shell particle shell refractive index, and/or core shell particle core refractive index of the particles.

[0040] In some cases, the method further comprises using a flow cytometry method to improve a particle sample preparation prior to the particle characterization, the method comprising: performing a sample preparation technique on a biological specimen; performing a flow cytometry analysis of the specimen after sample preparation; and adjusting one or more parameters of the sample preparation technique based on the flow cytometry analysis. In some cases, this method can be used to perform quality control for manufacturing/preparing particles such as EVs, LNPs, virus, or virus-like particles.

[0041] In some cases, the sample preparation technique is selected from the group consisting of centrifugation, membrane filtration, precipitation, and chromatographic purification.

[0042] In some cases, one or more parameters of the centrifugation is selected from the group consisting of centrifugation speed, duration, and temperature.

[0043] In some cases, the purity or yield of one or more target particles in the specimen is increased, further optionally wherein the membrane filtration is selected from the group consisting of ultrafiltration, tangential flow filtration, dialysis, and Tangential Flow Filtration (TFF).

[0044] In some cases, the chromatographic purification is selected from the group consisting of size exclusion chromatography (SEC), immunoaffinity capture, affinity chromatography, and ion exchange chromatography.

[0045] In some cases, the particles are nanoparticles.

[0046] In some cases, the particles are biological particles.

[0047] In some cases, the nanoparticles are biological nanoparticles.

[0048] In some cases, there is a system using the methods described herein.

BRIEF DESCRIPTION OF THE FIGURES

[0049] The following drawing figures, which form a part of this application, are illustrative of the described technology and are not meant to limit the scope of the disclosure in any manner.

[0050] FIG. 1 illustrates an example of a system that can be used to perform flow cytometry, the system including a flow cytometer and a workstation.

[0051] FIG. 2 schematically illustrates an example of a detection system of the flow cytometer of FIG. 1.

[0052] FIG. 3 schematically illustrates an example of a method of using flow cytometry performed by the flow cytometer of FIG. 1 to improve sample preparation.

[0053] FIG. 4 schematically illustrates an example of a method of characterizing nanoparticles that can be performed by the flow cytometer of FIG. 1.

[0054] FIG. 5 schematically illustrates an example of a method of characterizing lipid nanoparticles that can be performed by the flow cytometer of FIG. 1.

[0055] FIG. 6 schematically illustrates an example of a computing system for implementing aspects of the system of FIG. 1.

[0056] FIG. 7 shows a histogram of PBS buffer at gain 200 with identification of noise as baseline (Left panel), a histogram of empty LNP sample (middle panel), and a histogram of eGFP-encoded mRNA loaded LNP sample (right panel).

[0057] FIG 8A shows a table listing four mRNA-loaded LNP modified with no, low, medium, and high amounts of monoclonal antibody (mAb) on the LNP surface, and their corresponding histograms (bottom row). Upper right cartoon depicts models of RNA-loaded LNP with no mAb on the LNP surface (left), and RNA-loaded LNP modified with mAb on the LNP surface (right).

[0058] FIG 8B shows a table listing mRNA-loaded LNP modified with no, low, medium, and high amounts of monoclonal antibody (mAb) on the LNP surface (top table) and their corresponding 2D plots using RSSC-H versus VSSC1-H, from left to right: mRNA-loaded LNP (surface naked LNP with no mAb), mRNA-loaded LNP with low mAb on the LNP surface, mRNA-loaded LNP with medium mAb on the LNP surface, mRNA-loaded LNP with high mAb on the LNP surface.

[0059] FIG. 9 discloses an embodiment of the present disclosure where characterization of the sample flow Events Per Second (EPS) of biological samples of different concentrations were acquired at different sample flow rates. More specifically, FIG. 9 discloses HEK293T Microvesicles where the number of events per minute were analyzed as the percentage to that of the first minute.

[0060] FIG. 10 discloses an embodiment of the present disclosure where characterization of the sample flow (EPS) of biological samples of different concentrations were acquired at different sample flow rates. More specifically, FIG. 10 discloses time plots of the triplicates run for HEK293T Microvesicles at 1:1000 dilution ($5.3 \times 10^5 \text{ p/mL} = 530 \text{ p/}\mu\text{l}$) at $1 \mu\text{l/min}$, $3 \mu\text{l/min}$ and $6 \mu\text{l/min}$ (columns), where p = particles.

[0061] FIG. 11 discloses an embodiment of the present disclosure where characterization of the sample flow (EPS) of biological samples of different concentrations were acquired at different sample flow rates. More specifically, FIG. 11 discloses data and bar graphs of the triplicates run for HEK293T Microvesicles at 1:1000 dilution ($5.3 \times 10^5 \text{ p/mL} = 530 \text{ p/}\mu\text{l}$) at $1 \mu\text{l/min}$, $3 \mu\text{l/min}$ and $6 \mu\text{l/min}$ (columns) also disclosed in FIG. 10, where p = particles.

[0062] FIG. 12 discloses an embodiment of the present disclosure where characterization of the sample flow (EPS) of biological samples of different

concentrations were acquired at different sample flow rates. More specifically, FIG. 12 discloses time plots of the triplicates run for HEK293T Microvesicles at 1:500 dilution (1.06×10^6 p/mL = 1060 p/ μ l) at 1 μ l/min, 3 μ l/min and 6 μ l/min (columns), where p = particles.

[0063] FIG. 13 discloses an embodiment of the present disclosure where characterization of the sample flow (EPS) of biological samples of different concentrations were acquired at different sample flow rates. More specifically, FIG. 13 discloses data and bar graphs of the triplicates run for HEK293T Microvesicles at 1:500 dilution (1.06×10^6 p/mL = 1060 p/ μ l) at 1 μ l/min, 3 μ l/min and 6 μ l/min (columns) also disclosed in FIG. 12, where p = particles.

[0064] FIG. 14 discloses an embodiment of the present disclosure where characterization of the sample flow (EPS) of biological samples of different concentrations were acquired at different sample flow rates. More specifically, FIG. 14 disclose time plots of the triplicates run for HEK293T Microvesicles at 1:250 dilution (2.12×10^6 p/mL = 2120 p/ μ l) at 1 μ l/min, 3 μ l/min and 6 μ l/min (columns), where p = particles.

[0065] FIG. 15 discloses an embodiment of the present disclosure where characterization of the sample flow (EPS) of biological samples of different concentrations were acquired at different sample flow rates. More specifically, FIG. 15 discloses data and bar graphs of the triplicates run for HEK293T Microvesicles at 1:250 dilution (2.12×10^6 p/mL = 2120 p/ μ l) at 1 μ l/min, 3 μ l/min and 6 μ l/min (columns) also disclosed in FIG. 14, where p = particles.

[0066] FIG. 16 discloses an embodiment of the present disclosure where characterization of the sample flow (EPS) of biological samples of different concentrations were acquired at different sample flow rates. More specifically, FIG. 16 discloses sfGFP viral particle testing at 1 μ l/min and 3 μ l/min, as sample control. The data analysis is based on all events and GFP positive (B531-H) events. The gating strategy is disclosed in FIG. 16, where p = particles.

[0067] FIG. 17 discloses an embodiment of the present disclosure where characterization of the sample flow (EPS) of biological samples of different concentrations were acquired at different sample flow rates. More specifically, FIG. 17 discloses time plots for sfGFP (where p = particles) viral particle testing at 1 μ l/min and

3 μ l/min, as sample control. The data analysis is based on all events and GFP positive (B531-H) events.

[0068] FIG. 18 discloses an embodiment of the present disclosure where characterization of the sample flow (EPS) of biological samples of different concentrations were acquired at different sample flow rates. More specifically, FIG. 18 discloses data and bar graphs for sfGFP (where p = particles) viral particle testing at 1 μ l/min and 3 μ l/min, as sample control, also disclosed in FIG. 17. The data analysis is based on all events and GFP positive (B531-H) events.

[0069] FIG. 19 discloses an embodiment of the present disclosure demonstrating the volumetric counting of biological particles by flow cytometry. More specifically, FIG. 19 discloses the V5 virus gating strategy.

[0070] FIG. 20 discloses an embodiment of the present disclosure demonstrating the volumetric counting of biological particles by flow cytometry. More specifically, FIG. 20 discloses the MV gating strategy.

[0071] FIG. 21 discloses an embodiment of the present disclosure demonstrating the volumetric counting of biological particles by flow cytometry. More specifically, FIG. 21 discloses the distribution of the event counts at different conditions.

[0072] FIG. 22 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 22 discloses the plots for PBS control to show baseline, and a commercially available rEV-GFP unstained but with GFP label (B531). On the left, the acquisition settings being used for gain include FSC =100, VSSC1= 50, VSSC2 =200, BSSC =100, YSSC =100, RSSC =100, V447 =1000, B531 =1000, Y595 = 1000, R670 = 1000, R710 = 1000, and R792 = 1000, Y595 = 1000, R670 = 1000, R710 =1000, and R792=1000. The first 2 rows are from PBS (sample buffer =blank). The bottom 2 rows are from rEV diluted at 1:1000, which is the dilution selected as best after titration. The rEVs are green endogenously so on the right plots for the B531 channel for both PBS and sample are shown.

[0073] FIG. 23 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 23 discloses that rEV was successfully stained using single staining with CD81 PB, PE, APC and vFRed and detected on a CytoFLEX nano instrument.

[0074] FIG. 24 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 24 discloses that rEV was successfully stained using single staining with CD81 PB, PE, APC and vFRed and detected on a CytoFLEX nano instrument.

[0075] FIG. 25 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 25 discloses that rEV was successfully stained using a combination of the vFRed lipid dye and CD81-PB, detected on a CytoFLEX nano instrument.

[0076] FIG. 26 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 26 discloses that rEV was successfully stained using a combination of the vFRed lipid dye and CD81-APC, and detected on a CytoFLEX nano instrument.

[0077] FIG. 27 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 27 discloses the characterization of the antibodies at the same dilutions as earlier FIGs. to evaluate the contribution of the antibody aggregates that can affect the sample staining evaluation.

[0078] FIG. 28 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 28 discloses the characterization of antibodies and vFRed at the same dilutions as earlier FIGs. to evaluate antibody aggregates and vFRed aggregates contribution to overall analysis.

[0079] FIG. 29 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 29 discloses an example of different fractions of PPP EVs isolated from 1 sample using a qEV70nm SEC column.

[0080] FIG. 30 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 30 discloses the instrument setting and compensation matrix used for the multi-color staining.

[0081] FIG. 31 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 31 discloses single staining with CD81 PB, CD9 FITC, CD63 APC, CD61 PC7 and CD235a PE.

[0082] FIG. 32 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 32 discloses single staining with CD81 PB, CD9 FITC, CD63 APC, CD61 PC7 and CD235a PE.

[0083] FIG. 33 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 33 discloses the staining of the same sample with the full panel of antibodies, diluted at the same dilution in a mix.

[0084] FIG. 34 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 34 discloses the antibody background between PBS buffer, Ab only, unstained sample, and single-stained sample.

[0085] FIG. 35 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 35 discloses the antibody background between PBS buffer, Ab only, unstained sample and multiple-stained (Ab mix) sample.

[0086] FIG. 36 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 36 discloses the data collected from unstained and stained samples with fraction 6 selected from qEV 70nm SEC column.

[0087] FIG. 37 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 37 discloses the data collected from unstained and stained samples with fraction 6 selected from qEV 70nm SEC column.

[0088] FIG. 38 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 38 discloses the CytoFLEX nano settings and compensation applied for the data in FIG. 36 and FIG. 37.

[0089] FIG. 39 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 39 discloses the same sample evaluated with different triggers. B531 trigger, targeting CD9 positivity and R670 trigger, targeting CD61 PC7 positive population.

[0090] FIG. 40 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 40 discloses the same sample evaluated with different triggers. B531 trigger, targeting CD9 positivity and R670 trigger, targeting CD61 PC7 positive population.

[0091] FIG. 41 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 41 discloses the data obtained with fraction 6 collected from the qEV 35nm SEC column. Shown are single color staining with CD9 FITC, CD81 PB, CD63 APC, CD61 PC7 and CD235a PE and the same sample stained with the panel of antibodies (mix).

[0092] FIG. 42 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 42 discloses the data obtained with fraction 6 collected from the qEV 35nm SEC column. Shown are single color staining with CD9 FITC, CD81 PB, CD63 APC, CD61 PC7 and CD235a PE and the same sample stained with the panel of antibodies (mix).

[0093] FIG. 43 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 43 discloses the setting and compensation applied to fraction 6 collected from the qEV 35nm SEC column.

[0094] FIG. 44 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow cytometry. More specifically, FIG. 44 discloses the same evaluate with different triggers. B531 trigger, targeting CD9 positivity and R670 trigger, targeting CD61 PC7 positive population.

[0095] FIG. 45 discloses an embodiment of the present disclosure demonstrating the detection and characterization of platelet-poor plasma samples using flow

cytometry. More specifically, FIG. 45 discloses the same evaluate with different triggers. B531 trigger, targeting CD9 positivity and R670 trigger, targeting CD61 PC7 positive population.

[0096] FIG. 46 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 46 discloses unstained GFP-labeled sample.

[0097] FIG. 47 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 47 discloses the CytoFLEX nano settings used in FIG. 46.

[0098] FIG. 48 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 48 discloses single staining done with CD81 antibody targeting the same epitope of the tetraspanin membrane protein in three different colors: PE (first row), APC (second row) and PB (third row) and a single stain with vFRed lipid dye (fourth row).

[0099] FIG. 49 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 49 discloses the combination of CD81APC and vFRed, together with the GFP label that were evaluated with different triggers and compared with slight MFI changes.

[0100] FIG. 50 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 50 discloses the combination of CD81APC and vFRed, together with the GFP label that were evaluated with different triggers and compared with slight MFI changes.

[0101] FIG. 51 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 51 discloses the combination of CD81PB and vFRed, together with the GFP label evaluated with different triggers and compared with slight MFI changes.

[0102] FIG. 52 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant extracellular vesicles (rEVs) using

flow cytometry. More specifically, FIG. 52 discloses the combination of CD81PB and vFRed, together with the GFP label evaluated with different triggers and compared with slight MFI changes.

[0103] FIG. 53 discloses an embodiment of the present disclosure demonstrating characterization of a heterogeneous EVs using a flow cytometer.

[0104] FIG. 54 discloses an embodiment of the present disclosure demonstrating the characterization of a biological sample using a flow cytometer based on protein loading on the surface of the biological sample.

[0105] FIG. 55 discloses an embodiment of the present disclosure demonstrating the characterization of drug loading in liposomes using a flow cytometer.

[0106] FIG. 56 discloses an embodiment of the present disclosure demonstrating the characterization of expression levels of a biomolecule using a flow cytometer.

[0107] FIG. 57 discloses an embodiment of the present disclosure demonstrating the characterization of single particle surface marker interactions using a flow cytometer.

[0108] FIG. 58 discloses an embodiment of the present disclosure demonstrating the characterization of less abundant biomarkers using a flow cytometer.

[0109] FIG. 59 discloses a method of determining a flow cytometer instrument calibration factor including instrument calibration (step 1), empirical data collection (step 2), and post processing step (step 3) to calculate the output parameters that minimize the loss function and satisfy the bounds and constraints.

[0110] FIG. 60 discloses ten different use cases for solid nanoparticles and core-shell nanoparticles along with input parameters and output parameters that can be determined according to methods of the disclosure.

[0111] FIG. 61 discloses algorithm parameters for ten different use cases including the inputs of nanoparticle constraint parameters, initial guess parameters, and parameter bounds.

[0112] FIG. 62 discloses two use case scenarios for solid particles. In use case 1: the refractive index is the known input parameter and size is unknown output parameter. In use case 2: the size is the known input parameter and the refractive index is the unknown output parameter.

[0113] FIG. 63 discloses four use case scenarios for core-shell modeling. Use case 3: refractive index (RI) of the core, shell RI, shell thickness are known input

parameters, core size is unknown output parameter. Use case 4: shell RI, core RI and core size are known input parameters and shell thickness is unknown output parameter. Use case 5: shell RI, shell thickness, and core size are known input parameters and core RI is unknown output parameter. Use case 6: shell thickness, core RI, core size are known input parameters and shell thickness is unknown output parameter.

[0114] FIG. 64 discloses four use case scenarios for core-shell modeling. Use case 7: shell RI, shell thickness are known input parameters; core RI and core size are unknown output parameters. Use case 8: core RI and core size are known input parameters; core RI, core size are unknown output parameters. Use case 9: core RI and shell RI are known input parameters; shell thickness, core size are unknown output parameters. Use case 10: core size and shell thickness are known input parameters; core RI, shell RI are unknown output parameters.

[0115] FIG. 65 discloses a method of instrument calibration to define calibration factor based on identified half angle. Empirical data collection is obtained from a plurality of polystyrene size reference beads having known size and RI. The graphs in the top row show violet VSSC1 and VSSC2 histograms for two sets of polystyrene size reference beads. The Tables at top right show nominal reference bead size, CV% and RI at four wavelengths 405, 488, 561, and 633 nm. Next, the half angle is defined per each instrument for three instruments MP03, MP05 and MP07. The graphs in middle row show confidence of fit % (goodness of fit) as a function of scattering angle; the half collection angle is defined at the highest goodness of fit. Next, the calibration factor is defined based on identified half angle. The table at bottom shows averaged calibration factors over all bead sizes at different wavelengths of scatter for VSSC1 (violet), BSSC (blue), YSSC (yellow), and RSSC (red) for three flow cytometers MP05, MP07, and MP03.

[0116] FIG. 66 discloses an example of Step 2 empirical data collection for a first example of a biological control that is a commercially available MLV virus particle, top row, showing histograms of VSSC1 (a), BSSC(b), YSSC (c), RSSC(d) and dot plot (e) showing VSSC vs. RSSC gating strategy for MLV, and a second example of a biological control that is a commercially available recombinant EVs, bottom row, showing dot plot (a) showing VSSC1 vs. B531 FL, P2 gating on unstained and P1 GFP positive. Dot plot (b) shows BSSC vs. B531 FL. Dot plot (c) shows YSSC vs. B531 FL. Dot plot (d) shows RSSC vs. B531 FL.

[0117] FIG. 67 discloses an example of Step 2 empirical data collection for a third example of a biological control of lipid nanoparticles (LNPs) with Histograms VSSC1 of 20 nm for a) buffer, b) empty LNP, and c) loaded LNP; and for a fourth example of a biological control of LNPs along with histogram overlay of 4 LNPs with polyA surrogate mRNA payloads, approximate mRNA concentration (ug/mL), and mAb concentration of low, medium and high during Click Reaction loading of LNP (mg/mL), as shown in the table.

[0118] FIG. 68 discloses a table of Step 3 post processing parameters for Use case 1 with known RI and unknown diameter. The table identifies instrument, particle type (MLV), channel (VSSC1, BSSC, YSSC, or RSSC), expected core diameter (nm) from Cryo TEM, input parameter of expected core RI, empirical data, output parameter of predicted core diameter (nm), and predicted size error (predicted – expected).

[0119] FIG. 69 discloses a table of Step 3 post processing parameters for Use case 2 with known diameter and unknown RI. The table identifies instrument, particle type (MLV), gain, channel (VSSC1, BSSC, YSSC, or RSSC), input parameter of expected core diameter (nm), expected core RI, empirical data, output parameter of predicted core RI, and predicted RI error (predicted – expected).

[0120] FIG. 70 discloses a table of Step 3 post processing parameters for Use case 5 with known shell RI, shell size, core size, and unknown core RI. The table identifies instrument, particle type (rEV), gain, channel (VSSC1, BSSC, YSSC, or RSSC), input parameters of (i) expected core diameter (nm), (ii) expected shell thickness (nm), and (iii) expected shell RI. The output parameter is predicted core RI. Other parameters include expected core RI, empirical data, and predicted core RI error (predicted core RI – expected core RI).

[0121] FIG. 71 discloses a table of Step 3 post processing parameters for Use case 10 with known shell thickness, and known core size, and unknown shell RI and unknown core RI. The table identifies instrument, particle type (rEV), gain, channel (VSSC1, BSSC, YSSC, or RSSC), input parameter of expected core diameter (nm), input parameter of expected shell thickness (nm); expected core RI, expected shell RI, empirical data, constraint (shell RI > core RI); and output parameter of predicted core RI, predicted core RI error (predicted core RI – expected core RI), output parameter of predicted shell RI, and predicted shell RI error (predicted shell RI – expected shell RI).

[0122] FIG. 72 discloses an example of Step 3 post processing step for Use case 2, with Step A in the algorithm for a nanoparticle having known diameter and unknown RI (left), Step B input known parameters of size for empty LNP nanoparticle and RNA loaded LNP nanoparticle, Step C calculation of RI, and Step D comparing RI between loaded and empty. ($\Delta RI = RI_{\text{loaded}} - RI_{\text{empty}}$). This illustrates that refractive index change from loaded to empty can be correlated to amount of mRNA payload.

[0123] FIG. 73 discloses an example of post processing step (step 3) for Use case 10 or Use case 6 Step A in the algorithm. At left panel, for Use case 10, the shell thickness and core size are known, and the shell RI and core RI are unknown (left); for Use case 6, the shell thickness and core size are known, and shell RI and core RI is unknown. Step B shows input of known parameters of core size and shell thickness for a loaded nanoparticle without mAb on surface and for a loaded nanoparticle having mAb on surface, left middle panel. Step C shows calculation of shell RI and core RI for loaded LNP particles [instrument, particle type loaded LNP, gain 200 for all side scatter channels, channels (VSSC1, BSSC, YSSC, or RSSC), input parameter core size, shell thickness, empirical data for VSSC1 medium scatter intensity, and output parameters of shell RI and core RI] and loaded LNP particles with mAb on surface [instrument, particle type loaded LNP with mAb, gain 200 for all side scatter channels, channels (VSSC1, BSSC, YSSC, or RSSC), input parameters of core size, shell thickness, empirical data for VSSC1 medium scatter intensity, and output parameters of shell RI and core RI]. Step D shows step of comparing RI between loaded LNPs and loaded LNPs with different amount of mAb on surface where ($\Delta \text{shell RI} = \text{shell RI}_{\text{loaded}} + \text{mAb} - \text{shell RI}_{\text{loaded}}$), right panel. The change of shell RI can be correlated to amount of mAb on surface of nanoparticle. The ($\Delta \text{shell RI} = \text{shell RI}_{\text{loaded}} + \text{mAb} - \text{shell RI}_{\text{loaded}}$). The change of refractive index of core can be correlated to amount of mAb on surface of loaded nanoparticle.

[0124] FIG. 74 discloses an algorithm workflow for instrument calibration from calculating forward Mie scattering profile for each input bead diameter 5915, integrating Mie scattering profiles over $90 \text{ deg} \pm \text{current collection half angle}$ for each bead diameter, calculating calibration factor for each bead size 5925, calculating goodness of fit (1-CV of all calibration factors) for current collection half angle 5930, and reporting collection half angle with highest goodness of fit. A loop over considered collection half angles from 0 deg to 90 deg in 0.1 deg steps is performed between steps

5920, 5925, and 5930. The calibration is determined as calibration factor = measured signal/integrated calculated signal. A graph of Normalized calibration factor vs bead diameter (nm) is shown at bottom. A graph of goodness of fit vs. collection half angle showing best fit at collection half angle = 50.8 deg is shown at upper right.

[0125] FIG. 75 discloses step 1 of calculation of the forward Mie scattering profiles, step 5915. The pySCATMECH library was used. Profiles were calculated in the range of 0-180 deg in 0.1 deg steps. The profiles do not depend on the collection half angle and so are calculated only once. Inputs include bead diameter, bead refractive index at wavelength, medium refractive index at wavelength, wavelength, polarization of incident light, and detector polarization sensitivity. Output is Mie scattering intensity as a function of polar angle. At right is a graph of calculated Mie scattering intensity vs. angle (deg) for polystyrene beads of 44 nm, 80 nm, 141 nm, 304 nm, 600 nm, and 1000 nm diameter.

[0126] FIG. 76 discloses step 2 of the integration of the calculated scattering intensity 5920. Simpson's Rule was used to numerically integrate the Mie scattering profiles over an angular range that is defined by the current proposed collection angle. The integrand needs to be weighted by $\sin \theta$. Example outputs are shown in the four graphs at right of Mie scattering intensity vs angle (deg) at collection half angles of 20 deg (upper left), 40 deg (upper right), 60 deg (lower left) and 80 deg (lower right).

[0127] FIG. 77 discloses step 3 of the calculating calibration factors for each bead size 5925. The calibration factor is defined as the ratio of the measured scattered intensity and the integrated forward Mie intensity that was calculated in step 2. The calibration factor is calculated for each bead diameter. The measured signal for 44 nm beads is divided by the forward Mie scattering model integrated over the entire collection angle of 44 nm beads to obtain the calibration factor for the 44 nm beads. The measured signal for 80 nm beads is divided by the forward Mie scattering model integrated over the entire collection angle of 80 nm beads to obtain the calibration factor for the 80 nm beads. The measured signal for 100 nm beads is divided by the forward Mie scattering model integrated over the entire collection angle of 100 nm beads to obtain the calibration factor for the 100 nm beads. The measured signal for 144 nm beads is divided by the forward Mie scattering model integrated over the entire collection angle of 144 nm beads to obtain the calibration factor for the 144 nm beads.

[0128] FIG. 78 illustrates step 4: goodness of fit. The output of step 3 is a set of calibration factors for each input bead diameter for a single proposed collection half angle. First, the Coefficient of Variation (CV) is calculated over the set of calibration factors:

$CV = \text{standard deviation (all calibration factors)} / \text{average (all calibration factors)}$.

Second, the "goodness of fit" for the current proposed collection half-angle is calculated: $\text{goodness of fit} = 1 - CV$. Since $CV \geq 0$, the goodness of fit is ≤ 1 .

[0129] FIG. 79 illustrates step 5: Identification of the most probable collection angle 5935. Step 4 is repeated for each collection half-angle that is considered. The output is a "goodness of fit" for each potential collection half-angle. The calibration factor is an instrument parameter and should not depend on the bead size, so ideally, the CV calculated over multiple bead sizes would be 0. There is some bead-to-bead variation due to measurement uncertainty, and so the CV is non-zero. The most probable collection half-angle is identified as the angle with the smallest CV, since this most closely aligns with the ideal case. To calculate it, the collection half-angle with the largest goodness of fit is chosen, since the definition of $1 - CV$ means that this is the angle with the CV closest to 0.

[0130] FIG. 80 shows three graphs of confidence of fit % vs scattering angle for three flow cytometer instruments MP03, MP05, and MP07, excluding 44 nm and 103 nm data. The half angles at maximum confidence are 50.7 deg, 49.4 deg, and 52.1 deg, respectively, as shown in the table. The half angle is instrument specific but gain independent.

[0131] FIG. 81 shows comparison of half angle calculations excluding 44 nm and 103 nm data (left graphs) and including 44 nm and 103 nm data (right graphs) for three instruments MP03, MP05, and MP07, upper, middle, and lower graphs, respectively. By excluding the 103 nm the confidence is increased of matching empirical data with theoretical simulations.

[0132] FIG. 82 illustrates an algorithm workflow to calculate the average calibration factor and CV over all bead sizes. When the correct collection angle is identified, there is little variation in the calibration factor, i.e., the calibration factor will not depend on the bead size since it is an instrument parameter. Example outputs are shown in four graphs of normalized calibration factor vs bead diameter for collection

half angles of 20 deg, 40 deg, 60 deg, and 80 deg. Least variation in normalized calibration factor is exhibited at 60 deg collection half angle.

[0133] FIG. 83 shows a generic algorithm for all use cases with some variations between the parameters of the algorithm. All use cases use the same loss function: Loss function = [(integrated, calculated Mie signal) x (calibration factor) – measured signal]. The calculated Mie signal is a function of the particle parameters listed in FIG. 60.

[0134] FIG. 84 illustrates LNP1 solid modeling Mie calculations for empty and mRNA loaded LNPs. Histogram at left shows LNP1-empty. The histogram at right shows LNP1-eGFP. Table shows population results for LNP1-empty and LNP1-eGFP for median solid of RI values of 1.470, 1.450, 1.435, 1.430, and 1.420 (left to right), and sizes of loaded LNPs > size empty LNPs.

Scenario #1

- 1) Empty: RI is known -> calculate size,
- 2) If Size is not changed for loaded -> calculate RI,
- 3) Delta RI=RI (loaded)-RI(empty).

Scenario #2

- 1) Empty: Size is known -> calculate RI,
- 2) If RI is not changed for loaded -> calculate size,
- 3) Delta Size=Size (loaded)-Size(empty).

Scenario #3: both size and RI changed.

[0135] FIG. 85 shows a flow diagram for an example using loaded LNPs and loaded LNPs with different amounts of antibodies. Steps include illuminating with 1 or more excitation light beams, collecting light in a plurality of scatters and FL channels, extracting MSI data for loaded LNPs and loaded LNPs with different amounts of mAbs, using algorithm with known input parameters to obtain unknown parameters, and calculating change in unknown parameter between loaded LNP and loaded LNP with mAb. In some cases, algorithm and input of size is used to calculate RI of loaded. In some cases, algorithm and input of RI is used to calculate size of loaded.

[0136] In some cases, algorithm and input of loaded size, RI, shell thickness of stained sample, is used to calculate the RI of the shell. Calculate delta RI=RI(shell)-RI(buffer) = RI(shell)-RI(buffer) - Sensitivity of the instrument to determine protein loading.

[0137] In some cases, algorithm and input of loaded size and RI, to calculate shell thickness and shell RI. Calculate $\Delta RI = RI(\text{shell}) - RI(\text{buffer})$ - Thickness of the shell, Sensitivity of the instrument to determine protein loading.

[0138] In some cases, algorithm and input of loaded RI, RI of shell of stained sample is used to calculate shell thickness, sensitivity of instrument to determine protein loading.

[0139] In some cases, algorithm and input of loaded size and RI is used to calculate shell thickness and shell RI. Calculate $\Delta RI = RI(\text{shell}) - RI(\text{buffer})$, thickness of shell, sensitivity of instrument to determine protein loading, sensitivity of instrument to determine protein loading.

[0140] FIG. 86 shows a flow diagram for an example using unstained and stained samples. Steps include illuminating with 1 or more excitation light beams, collecting light in a plurality of scatters and FL channels, extracting MSI data for unstained and stained samples. In some cases, algorithm and input of loaded size and RI is used to calculate thickness of the shell and shell RI. Calculate $\Delta RI = RI(\text{shell}) - RI(\text{buffer})$ - Thickness of the shell, Sensitivity of the instrument to determine protein loading. In some cases, algorithm and input of stained shell thickness and shell RI is used to calculate unstained size and RI. In some cases, algorithm and input of stained shell thickness and shell RI is used to calculate shell RI and unstained RI. In some cases, algorithm and input of stained shell thickness and shell RI is used to calculate shell thickness and unstained size.

DETAILED DESCRIPTION

[0141] Various embodiments will be described in detail with reference to the drawings, wherein like reference numerals represent like parts and assemblies throughout the several views. Reference to various embodiments does not limit the scope of the claims attached hereto. Additionally, any examples set forth in this specification are not intended to be limiting and merely set forth some of the many possible embodiments for the appended claims.

Definitions

[0142] The singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0143] The term "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items.

[0144] The term "about," when referring to a measurable value such as an amount of a compound, dose, time, temperature, and the like, is meant to encompass variations of +/-10%, 5%, 1%, 0.5%, or even 0.1% of the specified amount.

[0145] The term "particle size" refers to the overall measurement of a particles dimension expressed as the diameter of a sphere with the same volume or the same surface area as the particle. Unless otherwise specified, the term "particle size" refers to the overall measurement of a particles dimension expressed as the diameter of a sphere with the same volume as the particle

[0146] The term "particle diameter" refers to the straight-line distance across a particle.

[0147] The term "nanoparticle" as used herein refers to a nanoparticle that is from 1 nm to 1,000 nm in size. In some cases, the nanoparticle is 10 nm to 1,000 nm in size. In some cases, the nanoparticle is 5 nm to 500 nm in size. In some cases, the nanoparticle is 1 nm to 100 nm in size. In some cases, the nanoparticle is 10 nm to 100 nm in size. In some cases, the nanoparticle is a biological nanoparticle. The term "biological nanoparticle" refers to a nanoparticle that is a naturally occurring or engineered particle. In some cases, the biological nanoparticle is 10 nm to 1,000 nm in size. In some cases, the biological nanoparticle is 5 nm to 500 nm in size. In some cases, the biological nanoparticle is 1 nm to 100 nm in size. In some cases, the biological nanoparticle is 10 nm to 100 nm in size. The nanoparticle can be a solid nanoparticle. The term "solid nanoparticle" is a nanoparticle comprising a solid material. The nanoparticle can be a "core-shell nanoparticle" having a core and an exterior shell surrounding the core. In some cases, the core-shell particle refers to a composite particle comprising a core and a shell, wherein the core is formed from a first material and the shell is formed from a second material that fully or partially surrounds the core. In some cases, an "empty core" within a core shell structure means the core is hollow, essentially containing an empty space inside the shell. In some cases, the core has a different composition than the exterior shell. In some cases, the nanoparticles are biological nanoparticles that can be isolated, for example, from a cell, a culture, a tissue, or a subject. In some cases, the nanoparticles can be prepared synthetically. In some cases, the nanoparticles are commercially available. The choice of materials for the core and shell can be tailored to impart specific physical, chemical, or biological properties to the core-shell particle, such as enhanced stability, targeted functionality,

or controlled release characteristics. For instance, when the lipid nanoparticle is filled with mRNA inside, the lipid layer is the shell and the mRNA inside the lipid layer is the core. For instance, when the lipid nanoparticle is filled with mRNA inside and the surface is modified with antibody, the entire lipid nanoparticle filled with mRNA is the core and the antibody on the surface of lipid nanoparticle is the shell. In some cases, a core can be an "empty core" within a core shell structure means the core is hollow, essentially containing an empty space inside the shell. For instance, a lipid nanoparticle has no cargo loaded inside, the lipid layer is the shell and the core is considered as an empty core as it is only filled with water or diluent buffer.

[0148] In some cases, the particles (e.g., nanoparticles) are spherical or primarily spherical in shape.

[0149] In some cases, the particles (e.g., nanoparticles) are solid particles (e.g., solid nanoparticles).

[0150] In some cases, the particles (e.g., nanoparticles) are core shell particles (e.g., core shell nanoparticles).

[0151] In some cases, the particles (e.g., nanoparticles) can be employed to carry a "therapeutic payload." In some cases, the therapeutic payload can be selected from the group consisting of a nucleic acid, e.g., RNA, mRNA, DNA; a protein; an antibody; an antigen-binding fragment of an antibody; a vaccine; a biomolecule; and a drug. In some cases, the drug can be a nucleic acid, a protein, an isolated natural product, a synthetic molecule, a semi-synthetic molecule, or a combination thereof. In some cases, the therapeutic payload is a chemotherapeutic agent, an antibiotic, or nucleic acid. The antibody or antigen-binding fragment of an antibody can be selected based on the affinity and specificity for binding a target antigen. The target antigen can be any appropriate therapeutic target.

[0152] The term "extracellular vesicle" (EV) as used herein refers to membrane enclosed biological particle secreted by cells and present in all biological fluids. EVs are heterogeneous in size. The three main types of EVs are exosomes, microvesicles (MVs), and apoptotic bodies. EVs can be broadly divided into small EVs, 30-200 nm, or 30-150 nm, commonly referred as exosomes, and larger EVs – 30-1,000 nm, 100-1,000 nm, or 250 nm to 1,000 nm or larger, also known as microvesicles (MVs). MVs can form by direct outward budding, or pinching, of the cell's plasma membrane. Apoptotic bodies typically range in size from 50 nm to 5000 nm in diameter. Apoptotic

bodies are released by dying cells into the extracellular space. In some cases, EVs are recombinant EVs (rEVs). EVs can carry nucleic acids such as miRNA, mRNA and proteins and play an important role in cellular communication both in normal and disease states. In some cases, EVs are shed by cells and carry markers of cellular origin and are studied as potential biomarkers of different diseases as well as therapeutic delivery vesicles. EVs can be isolated via any known technique, for example, size exclusion chromatography (SEC), ultrafiltration, or differential ultracentrifugation. Recombinant EVs are commercially available. In some cases, EVs are lipid bound vesicles secreted by cells into the extracellular space. The content of EVs can include lipids, nucleic acids, and proteins, for example, proteins associated with the plasma membrane, cytosol, and lipid metabolism. Doyle et al., 2019, *Cells*, 8, 727. Exosomes can act as carriers of biomarkers for diseases. Analysis of EVs can include any suitable technique known in the art, such as, for example, nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), electron microscopy such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM), tunable resistive pulse sensing (tRPS), immunodetection methods such as flow cytometry, Western blotting, immune-based microfluidic isolation, thermophoretic profiling, and/or mass spectrometry (MS)-based proteomic analysis.

[0153] The term “lipid nanoparticles” (LNPs) as used herein refers to very small typically spherical particles, having an average diameter of about 10 nm to about 1000 nm, composed primarily of lipids. Lipid nanoparticles are described, for example, by Mehta et al., 2023, *ACS Materials*, Au 2023, 3, 600-619. The lipid nanoparticles can include any appropriate lipid materials. In some cases, the lipid nanoparticle can comprise one or more of a phospholipid, cholesterol, cationic and ionizable lipids, and PEGylated lipids. In some cases, the LNPs are selected from liposomes, nanoemulsions, solid lipid nanoparticles, nanostructured lipid carriers, and lipid polymer hybrid nanoparticles. In some cases, the liposome has a phospholipid bilayer shell and an aqueous core. In some cases, the nanoparticle is a solid lipid nanoparticle (SLN). SLNs are prepared from lipids that are solid at room temperature as well as 37 deg C. In some cases, the solid lipids used in SLNs are selected from, for example, tripalmitin, cetyl alcohol, glycerol monostearate, trimyristin, tristearin, stearic acid, and the like.

[0154] In some cases, the nanoemulsions have a surfactant exterior and an oil core (o/w) or an aqueous core (w/o). In some cases, the solid lipid nanoparticles have a surfactant exterior shell and a solid lipid core. In some cases, the nanostructured lipid carriers include a surfactant shell layer, and a core comprising a solid lipid and a liquid lipid. In some cases, the lipid polymer hybrid nanoparticle includes a lipid shell layer and a polymer core. In some cases, the lipid nanoparticles can be employed to carry a “therapeutic payload.” (as described above). In some cases, the lipid nanoparticle surface can be modified with targeting moieties such as proteins, antibodies, peptides, DNA, small targeting molecules, etc. for delivered purposes to certain tissues and cells.

[0155] The term “virus-like particles” (VLPs) refers to nanostructures assembled from capsid proteins of viruses. VLPs are virus-derived structures made up of one or more different molecules with the ability to self-assemble, mimicking the form and size of a virus, but lacking the genetic material so they are not capable of infecting the host cell. Three main classes of VLPs are enveloped, non-enveloped, and chimeric. VLPs can be used, for example, for vaccine development and intracellular delivery of biomolecules and compounds. VLPs can be used, for example, for encapsulating a “therapeutic payload” (as described above), inorganic nanoparticles, polymers, and fluorescent dye molecules. VLPs surface can be modified with targeting moieties such as proteins, antibodies, peptides, DNA, small targeting molecules, etc. for delivered purposes to certain tissues and cells. VLPs are described in, for example, Nooraei et al., *J Nanobiotechnol* (2021) 19:59; and Travossos et al., *Int J Mol Sci* 2024, 25, 6699.

[0156] The term “forward scatter” (FSC) refers to a physical parameter measured in flow cytometry that detects scatter along the path of the laser and indicates relative size of a cell. FSC intensity is proportional to the diameter of a cell and is primarily due to diffraction around the cell.

[0157] The term “side scatter” (SSC) refers to a measurement in flow cytometry that detects scatter at a ninety-degree angle relative to the laser and can help provide information about the internal complexity (i.e., granularity) of the cell, which is proportional to the number of organelles and other components inside the cell’s cytoplasm.

[0158] The term “violet side scatter” (VSSC) refers to SSC using a violet laser at 405 nm. “VSSC1” and “VSSC2” refer to violet side scatter used to detect side scatter signals generated by small particles being analyzed when they are illuminated by a

violet laser (405 nm), with the difference between VSSC1 and VSSC2 being the amount of scatter light that is collected. The VSSC1 channel captures 95% of the side scatter light generated by the particles being analyzed when they are illuminated by a violet laser (405 nm), providing information about the granularity and complexity of the small particles. VSSC1 typically, detects smaller particles with low refractive index or less scattering intensity. The VSSC2 channel captures 5% of side scatter light compared to VSSC1, offering additional granular information and potentially helping to distinguish between different subpopulations of particles based on their light scattering properties. VSSC2 typically detects signals from slightly larger particles and/or those with higher refractive index.

[0159] The term “blue side scatter” (BSSC) refers to SSC using a blue laser at 488 nm.

[0160] The term “yellow side scatter” (YSSC) refers to SSC using a yellow laser at 561 nm.

[0161] The term “red side scatter” (RSSC) refers to SSC using a red laser at 633 nm.

[0162] The term “polar angle” refers to the angle at which scattered light is collected and measured. In some cases, forward scatter (FSC) is collected along the lasers path (0 degrees). In some cases, side scatter (SSC) is collected at 90 degrees.

[0163] The term “fluorescent dye” refers to any dye, molecule, complex, or particle that may be excited by a given wavelength of electromagnetic radiation and emit photons of another wavelength. The terms “fluorescent dye”, “fluorescent probe”, “fluorescent molecule”, “fluorophore”, “chromophore”, “fluorochrome”, “fluorescent nanocrystal”, and grammatical equivalents thereof, may be used interchangeably herein to refer to a fluorescent dye.

[0164] The term “optical standard particles” or “optical particles” refers to particles that can be used to calibrate and measure the size and number of particles in a sample. As used herein, particle and bead are used interchangeably to mean the same thing and refer to any solid particle, of virtually any shape, suitable for measurement by a fluorescent instrument. Various types of particles can be employed, for example, silica beads, polystyrene beads, magnetic beads, latex beads, biological particles, protein particles, virus-like particles, liposome particles, gold nanoparticles, lipid nanoparticles (LNP), hydrogel particles, poly(methylmethacrylate) (PMMA) particles. In some instances, the bead is a silica bead. In some instances, the bead is a polystyrene bead. The particle size can span a range from a few nanometers to micrometers, depending on

the application. In some cases, the average size of the optical particles or beads is in a range of from about 10 nm to about 2 μ m, about 10 nm to about 900 nm, about 20 nm to about 2 μ m, about 900 nm to about 2 μ m, about 20 nm to about 900 nm, about 30 nm to about 800 nm, about 40 nm to about 500 nm, about 50 nm to about 400 nm, or about 80 nm to about 300 nm. In some cases, the average size of the optical particles or beads is in a range of from about 10 nm to about 900 nm, about 20 nm to about 2 μ m, about 900 nm to about 2 μ m, about 20 nm to about 900 nm, about 30 nm to about 800 nm, about 40 nm to about 500 nm, about 50 nm to about 400 nm, or about 80 nm to about 300 nm.

[0165] In some cases, the term “size reference beads” or “calibration beads” refers to nonfluorescent microsphere suspensions to serve as size references for flow cytometry or particle analyzer. For example, size reference beads can be unstained microspheres, such as unstained polystyrene microspheres, of known diameters, e.g., as determined by transmission electron microscopy. In some cases, the size reference beads are polystyrene beads. In some cases, the size reference beads also called calibration beads can be obtained commercially, for example, nanoViS Nanoscale Sizing Standards (Beckman Coulter, Inc.). In some cases, the size reference beads comprise a plurality of sizes. In some cases, the calibration beads comprise a plurality of calibration beads having two or more diameters. In some cases, the calibration beads comprise a plurality of bead sizes of 40 to 1000 nm in diameter, 80-1000 nm in diameter, 100-1000 nm in diameter, 40-600 nm in diameter, 40-300 nm in diameter, 40-150 nm in diameter. In some cases, the calibration beads comprise a plurality of sizes selected from 44 nm, 80 nm, 105 nm, 144 nm, 300 nm, 600 nm, and 1000 nm $\pm$ 10%. In some cases, the size reference beads also called calibration beads include one or more, two or more of 44 nm, 80 nm, 105 nm, and 144 nm. In some cases, the size reference beads also called calibration beads include one or more, two or more of 144 nm, 300 nm, 600 nm, and 1000 nm. In some cases, the size of cells in an experimental sample can be estimated by comparing the side scatter (SSC) signals with those of the size reference beads also called calibration beads. The size reference beads also called calibration beads can be intermixed with the experimental sample or used in parallel runs.

[0166] The term “Simpson’s Rule” refers to a numerical integration method that can be used to approximate the definite integral of a function as seen in the context of Mie

scattering calculations. Cartwright, 2017, J of Mathematical Sciences & Mathematics Education, Vol. 12, No. 2, 1-9.

[0167] The term “Powell’s algorithm” also known as Powell Method refers to a minimization algorithm for finding a local minimum of a function. Vassiliadis, V.S., Conejeros, R. (2001). Powell Method. In: Floudas, C.A., Pardalos, P.M. (eds) Encyclopedia of Optimization. Springer, Boston, MA. https://doi.org/10.1007/0-306-48332-7_393.

[0168] The term “Constrained trust-region” refers to a minimization algorithm optimization technique that finds the minimum of a function while respecting constraints, iteratively refining approximations within dynamically adjusted trust regions. Byrd et al., 1987, SIAM Journal on Numerical Analysis, pp. 1152-1170.

[0169] The term “pySCATMECH library” refers to a python interface to SCATMECH: polarized light scattering C++ class library. SCATMECH is an object-oriented C++ class library developed to distribute models for light scattering applications. See <https://pages.nist.gov/SCATMECH/index.htm> for SCATMECH applications. The pySCATMECH package provides a Python interface to many features of this library. pages.nist.gov/pySCATMECH/index.html. Current version 0.1.9 (Dec. 19, 2024) requires Python 3.6 or greater. In some cases, the Scatterer module is employed for Mie scattering or Mie scattering with a coating.

[0170] The term “room temperature” (RT) refers to 23 deg C $\pm$ 2 deg C.

[0171] In some instances, hydrophilic polymers can be, for example, PEG, a protein, a peptide sequence, a peptoid, a DNA sequence, an RNA, a carbohydrate, an oxazoline, a polyol, a dendron, a dendritic polyglycerol, a cellulose, a chitosan, or a derivative thereof.

[0172] In flow cytometry, the “half angle” refers to the angular range of light collected by the side collection unit 130, which is the cone of light gathered around the particle passing through the interrogation zone 18, with the half angle representing half of the cone's apex angle. A larger half angle means more scattered light is captured from a wider range of angles around the particle. Modern flow cytometers typically have an SSC collection half-angle (ϵ) of about 50° which means the collected SSC light of each of the particles passing through the interrogation zone 18 would be the sum of all angles from 40° to 140°. However, the half angle of the side collection unit 130 may vary from instrument to instrument due to alignment of the side collection unit 130

relative to the interrogation zone 18. In some examples, the half angle may vary by about ± 10 degrees. In some cases, the half angle is calculated for each instrument.

[0173] In some cases, identification of the half angle can include comparing empirical side scatter light intensity data to simulated side scatter light intensity data over a range of about $\pm 10^\circ\text{C}$ around a nominal half angle of about 54°C to identify the half angle of the side collection unit 130. For example, the half angle of the side collection unit 130 is identified when the empirical side scatter light intensity is closest to the simulated side scatter light intensity for a given half angle value.

[0174] The term “Mie scattering” refers to the scattering of plane waves of light by spherical particles. Mie scattering can be applied to particles of any size. In some cases, the particles are smaller than the wavelength of light. In some cases, the scattering pattern depends on the size, refractive index of the particles, polarization of incident light, refractive index of the medium, and wavelength of incident light. Mie scattering does not have a strong dependence on wavelength. It is applicable across a broad range of wavelengths. To produce calculations, Mie scattering relies on certain dependencies, such as the input and output parameters provided in Step 3 of FIG. 59. In some cases, the input parameters include one or more, or a plurality of particle size, particle refractive index, shell thickness, shell refractive index, wavelength, buffer material of medium, buffer material or medium refractive index, half collection angle, calibration factor. In some cases, the input parameters include one or more, or a plurality of particle size, particle refractive index, shell thickness, shell refractive index, core size, core refractive index, wavelength, buffer material of medium, buffer material or medium refractive index, half collection angle, calibration factor.

[0175] The term “calibration factor” refers to the ratio of the measured scattered intensity and the calculated Mie scatter intensity that was calculated in Step 2 of the algorithm shown in FIG. 59.

[0176] The term “goodness of fit” refers to 1 minus the Coefficient of Variation (CV) of the calibration factor calculated for each bead.

[0177] The term “particle refractive index” refers to the measure of how much light bends when passing through a particle, essentially indicating how much the speed of light changes when entering a particle compared to the surrounding medium

[0178] The term “core refractive index” refers to refractive index of the particle that is close to the center of the particle.

[0179] The term “core size” refers to diameter of the particle that is close to the center.

[0180] The term “shell thickness” refers to thickness of the exterior layer around the core of a core-shell particles (e.g., nanoparticle).

[0181] In the context of a core-shell model, the term “shell size” refers to the thickness or dimension of the outer layer, or shell, that surrounds the inner core of a particle (e.g., nanoparticle).

[0182] In the context of a core-shell model, the term “shell thickness” refers to the measure of the distance between the outer surface of the core and the outer surface of the shell of a particle (e.g., nanoparticle).

[0183] The term “shell refractive index” refers to refractive index of the exterior layer of the core of a core-shell particle (e.g., nanoparticle).

[0184] The term “excitation wavelength” refers to wavelengths that been used to scatter the light from the particle.

[0185] The term “buffer refractive index” refers to refractive index of the diluent that been used to dilute the sample.

[0186] The term “output parameters” refers to the parameters obtained after calculations in Steps 1-3 in algorithm shown in FIG. 59. In some cases, the output parameter is one or more of particle diameter, particle refractive index, core diameter, shell thickness, core refractive index, shell refractive index, core diameter and core refractive index, shell thickness and shell refractive index, core diameter and shell thickness, or core refractive index and shell refractive index.

[0187] The term “initial guess parameters” refers to parameters used as the starting point for calculating unknown parameters comprising employing a minimization algorithm, such as Powell’s algorithm or constrained trust region minimization algorithm, in order to avoid local minimums and find a single solution close to what it should be. Customers may know the initial guess parameters based on their own previous data, or they can use initial guess parameters based on the data set forth in FIG. 61 created for particles, such as viruses, EVs, and LNPs. In some cases, the initial guess parameter for solid particle RI is 1.4. In some cases, the initial guess parameter for solid particle size is 100 nm. In some cases, the initial guess parameter for core shell particle core size is 100 nm. In some cases, the initial guess parameter for shell size is 10 nm. In some cases, the initial guess parameter for core RI is 1.4. In some cases, the initial guess parameter for shell RI is 1.4. In some cases, the initial guess

parameter for core RI is 1.4 and core diameter is 50 nm. In some cases, the initial guess parameter for shell RI is 1.4 and shell thickness is 10 nm. In some cases, the initial guess parameter for core diameter is 50 nm and shell thickness is 75 nm. In some cases, the initial guess parameter for core diameter is 100 nm and shell thickness is 10 nm. In some cases, the initial guess parameter for core RI is 1.4 and shell RI is 1.45. In some cases, the initial guess parameter for core RI is 1.45 and shell RI is 1.4.

[0188] The term “loss function” is an expression used to measure how close the predicted value is to the actual value. The loss function can be calculated as follows: loss function = [(integrated, calculated Mie signal) x (calibration factor) – measured signal].

[0189] The term “bounds and constraints” refers to boundary conditions for each parameter, for example, bounds of refractive index can be between 1.33-2.5. In some cases, the constraint comprises shell thickness > core diameter. In some cases, the constraint comprises shell thickness < core diameter. In some cases, the constraint comprises shell RI > core RI. In some cases, the constraint comprises shell RI < core RI. In some cases, the bounds for solid particle RI are [1.33, 2.5]. In some cases, the bounds for solid particle size are [10 nm, 1500 nm]. In some cases, the bounds for core size are [10 nm, 1500 nm]. In some cases, the bounds for shell size are [0 nm, 1500 nm]. In some cases, the bounds for core RI are [1.33, 2.5]. In some cases, the bounds for shell RI are [1.33, 2.5].

[0190] The following acronyms are used in the disclosure.

[0191] AA700 refers to Alexa Fluor 700. AA750 refers to Alex Fluor 750. Ab refers to antibody. ALS refers to Amyotrophic Lateral Sclerosis. APC refers to Allophycocyanin. AUC refers to Analytical Ultra Centrifuge. B531 refers to a specific bandpass filter designed to isolate and detect light at a wavelength around 531 nm when excited with a Blue laser 488 nm in CytoFlex nano. BIOS refers to Basic Input/Output System. BFF refers to Back and Forth Flush. BV refers to Brilliant Violet™ (Thermo Fisher Scientific Inc.). CHO refers to Chinese Hamster Ovaries. CPU refers to Central Processing Unit. CV refers to Coefficient of Variation. EDTA refers to Ethylenediaminetetraacetic Acid. ELISA refers to Enzyme Linked Immunosorbent Assay. ELISPOT refers to Enzyme-linked immunosorbent spot. EPS refers to Events Per Second. EV refers to extracellular vesicle. rEV refers to recombinant extracellular vesicle. FACS refers to Fluorescence -Activated Cell Sorting.

FITC refers to Fluorescein isothiocyanate. FL refers to fluorescent. vFRed™ refers to a fluorogenic membrane dye used to measure extracellular vesicles (Cellarcus, vFC™ kit). GFP refers to Green Fluorescent Protein. HEK refers to Human Embryonic Kidney Cells. IFU refers to Instructions For Use. IL refers to Interleukin. LNP refers to lipid nanoparticle. MFI refers to Mean Fluorescent Intensity. MV refers to Microvesicles. PB refers to Pacific Blue™ (Thermo Fisher Scientific Inc.). PBS refers to Phosphate-Buffered Solution. PC7 refers to R-phycoerythrin-Cyanine7 Dye. PE refers to R-phycoerythrin. PPP refers to platelet-poor plasma. qEV® refers to a Size Exclusion Chromatography useful, for example, to separate exosomes and other extracellular vesicles (Izon Science Ltd.). R670 refers to a specific bandpass filter designed to isolate and detect light at a wavelength around 670 nm when excited with a Red laser 638 nm in CytoFlex nano. RAM refers to Random Access Memory. RT refers to Room Temperature. SEC refers to Size Exclusion Chromatography. sfGFP refers to superfolder GFP. VEGF refers to Vascular Endothelial Growth Factor. VSSC refers to Violet Side Scatter. VSV refers to Vesicular Stomatitis Virus. V refers to V5 tag expressed on MLV virus surface. V447 refers to a specific bandpass filter designed to isolate and detect light at a wavelength around 447 nm when excited with a Violet laser 405 nm in CytoFlex nano. WBC refers to White Blood Cell. Y595 refers to a specific bandpass filter designed to isolate and detect light at a wavelength around 595 nm when excited with a Yellow laser 561 nm in CytoFlex nano.

[0192] All patents, patent applications and publications referred to herein are incorporated by reference in their entirety.

[0193] The embodiments described in one aspect of the present disclosure are not limited to the aspect described. The embodiments may also be applied to a different aspect of the disclosure as long as the embodiments do not prevent these aspects of the disclosure from operating for its intended purpose.

[0194] FIG. 1 illustrates an example of a system 10 that can be used to perform flow cytometry. The system 10 includes a flow cytometer 100 and a workstation 200. In some instances, the flow cytometer 100 incorporates aspects described in PCT International Patent Application No. PCT/CN2022/099413, filed June 17, 2022, U.S. Provisional Patent Application No. 63/595,928, filed on November 3, 2023, and U.S. Provisional Patent Application No. 63/561,405, filed on March 5, 2024, the disclosures of which are herein incorporated by reference in their entireties.

[0195] In general, the flow cytometer 100 is an analytical instrument that detects physical and chemical properties of samples of cells or particles. The flow cytometer can be any flow cytometer. In some cases, the flow cytometer is a CytoFlex flow cytometer or a CytoFlex nano flow cytometer (both available from Beckman Coulter, Inc.) The flow cytometer 100 is designed to capture robust and high quality data for characterizing biologically relevant particles (e.g., nanoparticles). The flow cytometer 100 is a single instrument that provides simultaneous assessment of particle (e.g., nanoparticle) size, concentration, and cargo to understand biological mechanisms of action and nanoparticle origins. The flow cytometer 100 can collect data from millions of particles or cells in a matter of minutes for display in a variety of formats on a display monitor 204 of the workstation 200.

[0196] The flow cytometer 100 includes a housing 101 having a sample station 104 which receives a container 106 containing a sample of cells and/or particles. In some examples, the container 106 contains a sample of particle (e.g., nanoparticles) such as extracellular vesicles (EVs), virus, and LNPs. A user of the system 10 can manually load the container 106 into the sample station 104. Once loaded in the sample station 104, the flow cytometer 100 can acquire the sample from the container 106 to perform the flow cytometry experiment. In some examples, the container 106 is a sample tube such as a 1.5-mL or 2-mL microtube, and/or has a diameter of 12 mm and a height of 75 mm.

[0197] The flow cytometer 100 can further include a sheath fluid container 107 for holding sheath fluid that is mixed with the sample during the flow cytometry experiment. The sheath fluid is pumped into the flow cytometer 100 causing a laminar flow. The sample is injected at a higher pressure into the center of the laminar flow of the sheath fluid. Hydrodynamic focusing causes the particles to align, single file in the direction of the laminar flow. The sheath fluid container 107 is connected to the flow cytometer 100 via tubing 109.

[0198] The flow cytometer 100 can further include a waste container 108 for collecting waste fluid. The waste container 108 is connected to the flow cytometer 100 via tubing 109.

[0199] The workstation 200 connects to the flow cytometer 100 via a wired or wireless connection to receive data from the flow cytometer 100 for display on the display monitor 204. The workstation 200 includes one or more user input devices such

as a mouse 206 and a keyboard 208 allowing a user of the system 10 to enter data and information, control the flow cytometer 100, and alter the display of the data on the display monitor 204.

[0200] The workstation 200 further includes a computing device 202. In some examples, the workstation 200 utilizes the computing device 202 to process raw data received from the flow cytometer 100. Alternatively, or additionally, the flow cytometer 100 can include a computing device to process data collected from flow cytometry. In such examples, the flow cytometer 100 sends processed data to the workstation 200 for display on the display monitor 204.

[0201] As further shown in FIG. 1, the system 10 further includes a sample preparation component 150 that can be used to prepare and purify biological particle (e.g., nanoparticles) such as extracellular vesicles (EVs), lipid nanoparticles (LNPs), and virus or virus-like particles from collected samples. The sample preparation component 150 can be a centrifuge, a membrane filtration system, a chromatography system, or a precipitation device. In some cases, biological particles (e.g., nanoparticles) can be prepared and purified using a sample preparation technique, for example, selected from the group consisting of centrifugation, membrane filtration, precipitation, and chromatographic purification. The sample preparation comprises using a sample preparation component 150 such as centrifugation, for example, Differential Centrifugation, Density Gradient Centrifugation; membrane filtration system, for example, ultrafiltration or tangential flow filtration; a chromatography system, for example, size exclusion chromatography (SEC), immunoaffinity capture, affinity chromatography, and or ion exchange chromatography, and/or a precipitation device. Extracellular Vesicles (EVs) can be prepared and purified using Differential Centrifugation: Sequential centrifugation steps with increasing speeds to remove cells, debris, and larger particles, followed by ultracentrifugation to pellet EVs. Extracellular Vesicles (EVs) can be prepared and purified using Density Gradient Centrifugation: Using sucrose or iodixanol gradients to separate EVs based on their buoyant density. Extracellular Vesicles (EVs) can be prepared and purified using Ultrafiltration: Filtration through specific pore-sized membranes to concentrate EVs and remove contaminants. Extracellular Vesicles (EVs) can be prepared and purified using Size Exclusion Chromatography (SEC): Separation of EVs from other components based on size exclusion principles. Extracellular Vesicles (EVs) can be prepared and purified

using Immunoaffinity Capture: Using antibodies targeting specific EV surface markers to capture EVs on a solid matrix. Extracellular Vesicles (EVs) can be prepared and purified using Precipitation: Utilizing polymers like polyethylene glycol (PEG) to precipitate EVs from the solution. Lipid Nanoparticles (LNPs) can be prepared and purified using Dialysis: Removing small molecules and solvents from LNPs by dialysis against a large volume of buffer. Lipid Nanoparticles (LNPs) can be prepared and purified using Ultracentrifugation: High-speed centrifugation to pellet LNPs while removing free lipids and other impurities. Lipid Nanoparticles (LNPs) can be prepared and purified using Size Exclusion Chromatography (SEC): Separation based on particle size to obtain a uniform LNP population. Lipid Nanoparticles (LNPs) can be prepared and purified using Tangential Flow Filtration (TFF): Filtration technique to concentrate and diafilter LNPs, improving purity and uniformity. Lipid Nanoparticles (LNPs) can be prepared and purified using Gradient Centrifugation: Utilizing a density gradient to purify LNPs based on their buoyant density. Virus-like particles can be prepared and purified using Differential Centrifugation: Sequential centrifugation to remove cells and debris, followed by ultracentrifugation to pellet viruses. Virus-like particles can be prepared and purified using Density Gradient Centrifugation: Using sucrose or cesium chloride gradients to purify viruses based on their buoyant density. Virus-like particles can be prepared and purified using PEG Precipitation: Polyethylene glycol-mediated precipitation to concentrate virus particles. Virus-like particles can be prepared and purified using Ultrafiltration: Concentrating virus particles using membranes with specific molecular weight cut-offs. Virus-like particles can be prepared and purified using Affinity Chromatography: Using specific ligands or antibodies to capture and purify viruses based on their surface proteins. Virus-like particles can be prepared and purified using Anion Exchange Chromatography: Utilizing the charge properties of virus particles to separate them from contaminants.

[0202] In some examples, the sample preparation component 150 is a centrifuge configured to perform analytical ultracentrifugation. As will be described in more detail, in at least some examples, the centrifuge 150 performs centrifugation on a container 106 having a sample, and the sample is then analyzed by the flow cytometer 100 after completion of the centrifugation. In such examples, the analysis performed by flow cytometer 100 is used to adjust one or more operations of the centrifuge 150 to maximize purity, yield, or enrichment of targeted particles acquired from a sample via

the centrifugation performed by the centrifuge 150. Accordingly, the flow cytometer 100 can be used as an analytical tool to improve the centrifugation performed by the centrifuge 150 for acquiring targeted particles.

[0203] The workstation 200 can be communicatively coupled to the flow cytometer 100 and the centrifuge 150 via a network 160 that connects and exchanges data between the workstation 200 and the flow cytometer 100 and the centrifuge 150. The network 160 can include any type of wired or wireless connections, or any combinations thereof. In some examples, the wireless connections can be accomplished using Wi-Fi, ultra-wideband (UWB), Bluetooth, and the like. In some examples, the network 160 is an Internet of things (IoT) network.

[0204] In additional embodiments where the system 10 includes a centrifuge 150, the centrifuge is used to perform characterization on a sample after the sample is analyzed by flow cytometer 100 and the analysis by the centrifuge is used to adjust one or more operations of the flow cytometer to maximize characterization of particles in the sample. Accordingly, centrifuge 150 can be used as a tool to improve the characterization of particles in a sample by a flow cytometer 100. In additional embodiments where the system 10 includes a centrifuge 150, a flow cytometer 100 is used simultaneously with a centrifuge 150 to characterize particles and/or maximize purity, yield, or enrichment of targeted particles.

[0205] The above discussion is presented in the context of a centrifuge, but the present disclosure contemplates using a flow cytometer in conjunction with any laboratory technique/instrument to improve the characterization of particles and/or maximize purity, yield, or enrichment of targeted particles. This includes, but is not limited to, electron microscopy, column chromatography ELISA, ELISPOT, and other laboratory techniques that are used to characterize and/or purify particles. While this contemplates particles of any size, this is particularly relevant to particles greater than about 20nm, greater than about 40nm, greater than about 60nm, greater than about 70nm, greater than about 80nm, less than about 100nm, less than about 90nm, less than about 80nm, less than about 70nm, less than about 60 nm, less than about 50nm, or any combination thereof. In embodiments, and less than 100nm, or less than 80nm, or less than 70nm, or less than 60nm, or any combination thereof.

[0206] Extracellular vesicles (EVs) are a heterogenous family of membranous particles released by all cells, which provide crucial insights into a variety of fields and

diseases. To better understand EVs, researchers must characterize EVs. However, due to their heterogeneity, size, refractive index, and complex nature makes their analysis challenging. For instance, the overall size range of the EVs can range from 20 nm to 5 μ m of the bigger apoptotic bodies.

[0207] In some cases, the flow cytometer 100 has high sensitivity capable of detecting nanoparticles of about 40nm via violet side scatter, and also provides better sensitivity and resolution via six fluorescent channels.

[0208] FIG. 2 schematically illustrates an example of a detection system 12 of the flow cytometer 100. The detection system 12 detects and analyzes particles on the nanoscopic scale such as particles having a diameter less than 100 nanometers (nm). Also, the detection system 12 can detect and analyze particles having a larger size such as particles having a diameter greater than 100 nm. The detection system 12 includes a light emitting unit 110, and a light collection unit 120 that detects characteristics of particles passing through a flow chamber 15.

[0209] The light emitting unit 110 emits one or more excitation light beams for projection onto particles flowing through an interrogation zone 18 in the flow chamber 15. The light collection unit 120 collects light scattered or emitted from the particles that flow through the interrogation zone 18 for analysis by a computing device 600 (see FIG. 6).

[0210] The light emitting unit 110 includes a plurality of light sources 111a-111d such as a first light source 111a, a second light source 111b, a third light source 111c, and a fourth light source 111d. The plurality of light sources 111a-111d can include more than four light sources, or fewer than four light sources. The plurality of light sources 111a-111d can include lasers.

[0211] The plurality of light sources 111a-111d emit excitation light beams within a range of about 300 nm to about 825 nm. As a further example, the light sources 111a-111d emit excitation light beams within a range of about 325 nm to about 808 nm. Each of the plurality of light sources 111a-111d emits an excitation light beam of a particular wavelength. As an illustrative example, the first light source 111a emits an excitation light beam in the red light spectrum (e.g., 625-825 nm), the second light source 111b emits an excitation light beam in the yellow light spectrum (e.g., 560-590 nm), the third light source 111c emits an excitation light beam in the blue light spectrum (e.g., 450-

490 nm), and the fourth light source 111d emits an excitation light beam in the violet light spectrum (e.g., 300-450 nm).

[0212] In the example shown in FIG. 2, the light sources 111a-111d are arranged in parallel. It should be understood that the number, the type, and the arrangement of the light sources are not limited to the example shown and described herein, and may be changed as needed. For example, the system may include three, five, six, or any other suitable number of light sources.

[0213] The light emitting unit 110 further includes a focal lens 119. The focal lens 119 is configured to focus the excitation light beams for high scatter intensity detection of particles. For example, the excitation light beams emitted by the light sources 111a-111d pass through the focal lens 119, which focuses the excitation light beams to the interrogation zone 18 of the flow chamber 15. The interrogation zone 18 may also be referred to as a focus point where the focused excitation light beams meet the core sample stream in the flow cytometer 100.

[0214] Dichroic mirrors 117a, 117b, 117c, and 117d are arranged between the focal lens 119 and the respective light sources 111a-111d. Each of the dichroic mirrors 117a-117d is configured to reflect a light beam of a corresponding one of the light sources 111a-111d and transmit the light beams of the other light sources. The dichroic mirrors 117a-117d are selected and configured according to the wavelengths of the light beams emitted by the respective light sources 111a-111d. For example, the dichroic mirror 117a reflects light of the wavelength emitted by the light source 111a toward the focal lens 119, the dichroic mirror 117b reflects light of the wavelength emitted by the light source 111b toward the focal lens 119 and transmits light of the wavelength emitted by the light source 111a, the dichroic mirror 117c reflects light of the wavelength emitted by the light source 111c toward the focal lens 119 and transmits light of the wavelengths emitted by the light sources 111a and 111b, and the dichroic mirror 117d reflects light of the wavelength emitted by the light source 111d toward the focal lens 119 and transmits light of the wavelengths emitted by the light sources 111a, 111b, and 111c.

[0215] The light beams emitted by the light sources 111a-111d are reflected by or transmitted through the dichroic mirrors 117a-117d to form collinear beams. The collinear beams share an optical axis, and provide a confocal point of multiple light sources by focusing on the same interrogation point. The dichroic mirrors 117a-117d

are adjustable in their positions or orientations, such that they can be used to adjust the position of the focus point of the light beams, especially, the position on a plane perpendicular to the optical axis.

[0216] Lenses 115a-115d are arranged between the respective light sources 111a-111d and the respective dichroic mirrors 117a-117d. In some examples, the lenses 115a-115d are long-focus lens. In some examples, the lenses 115a-115d are spherical lenses. In other examples, the lenses 115a-115d are aspheric lenses. Each of the lenses 115a-115d can convert light beams into parallel beams. In the example shown in FIG. 2, each of the lenses 115a-115d is in the form of planoconvex lens with a flat surface and a convex surface opposite to each other.

[0217] The lenses 115a-115d are adjustable in their positions or orientations, so as to adjust the position of the focus point of the light beams, especially, the position on the plane perpendicular to the optical axis. Generally, the dichroic mirrors 117a-117d can be used to roughly adjust the position of the focus point of the light beams, whereas the lenses 115a-115d can be used to finely adjust the position of the focus point of the light beams.

[0218] It should be understood that the number, the type, and the arrangement of the dichroic mirrors 117a-117d and the lenses 115a-115d may be changed as needed, and are not limited to the example illustrated herein. Also, the dichroic mirrors 117a-117d and the lenses 115a-115d can be replaced with other optical elements or optical modules with similar functions.

[0219] Beam expanders 113a-113d are arranged between the respective light sources 111a-111d and the respective lenses 115a-115d. Each of the beam expanders 113a-113d can change a sectional dimension and a divergence angle of a light beam. As such, each of the beam expanders 113a-113d are configurable according to a desired size of a spot of a light beam.

[0220] The light beams irradiated on the particles by the focal lens 119 have a spot size that allows for more concentrated light beams with a higher power density. This can increase intensity of the light beams irradiated on the particles, and ultimately the intensity of the optical signals collected from the particles. This can improve the efficiency of collecting the optical signals, and thereby provide higher resolution and higher sensitivity for nanoparticle detection.

[0221] In the example shown in FIG. 2, the light sources 111a-111d are in the form of lasers that include respective laser diodes 112a-112d. As further shown in the example of FIG. 2, half-wave plates 116a-116d are provided between the dichroic mirrors 117a-117d and the lenses 115a-115d, respectively. The spot of the light beam can be reduced by orientation of the light sources 111a-111d and by use of the half-wave plates 116a-116d.

[0222] As further shown in FIG. 2, cylindrical lenses 114a-114d are provided between the respective beam expanders 113a-113d and the respective lenses 115a-115d. The horizontal size of the spot of the light beam focused in the flow chamber 15 can be adjusted by replacing the cylindrical lenses 114a-114d with replacement cylindrical lenses having different curvatures. The power of some or all of the light sources 111a-111d can also be increased. The increased power of the light sources 111a-111d can also improve detection sensitivity.

[0223] Each of the beam expanders 113a-113d is formed of a first optical part and a second optical part. In the example shown in FIG. 2, each of the beam expanders 113a-113d includes a concave lens adjacent to the corresponding light source as the first optical part, and further includes a convex lens away from the corresponding light source as the second optical part. It should be understood that each of the beam expanders 113a-113d is not limited to the example shown in FIG. 2. The beam expanders 113a-113d may be formed of any suitable optical lens or lens group. For example, each of the first optical part and the second optical part can be selected from one of a convex lens, a convex lens group, a concave lens, and a concave lens group.

[0224] For each of the beam expanders 113a-113d, the distance between the first optical part (e.g., the concave lens) and the second optical part (e.g., the convex lens) is adjustable. This allows for adjustment of a waist position (the focus point) of the light beam on the optical axis.

[0225] As described above, by adjusting the dichroic mirrors 117a-117d, the lenses 115a-115d, and the beam expanders 113a-113d, the individual light beams can be focused at the desired interrogation point, and multiple light beams can be focused at the same interrogation point. It should be understood that the position of the focus point of the light beams may be adjusted by adopting any other optical element or in any other adjustment manner. One or more adjustments to the dichroic mirrors 117a-117d, the lenses 115a-115d, and the beam expanders 113a-113d may be made manually, or

may be made electronically using a computing device (e.g., a controller) that is associated with one or more actuators coupled to these components.

[0226] The light collection unit 120 includes a side collection unit 130 and a forward collection unit 152. The side collection unit 130 collects side scattered light and fluorescent light emitted from the particles in the sample as they are irradiated by the excitation light beams while passing through the flow chamber 15. The optical axis of light beams collected from the particles by the side collection unit 130 is approximately perpendicular to, or about 90 degrees, from the optical axis of the light beams emitted from the light sources 111a-111d and directed by the dichroic mirrors 117a-117d toward the flow chamber 15.

[0227] The forward collection unit 152 collects forward scattered light from the particles. The optical axis of light beams collected from the particles by the forward collection unit 152 may be approximately parallel to, or about 0 degrees from, the optical axis of the light beams that are directed toward the flow chamber 15. The side collection unit 130 and the forward collection unit 152 are described in further detail below.

[0228] The side collection unit 130 includes an optical focusing lens group including a concave mirror 134 and an aspheric lens 135, a collection fiber 136, a beam splitter 133, a first wavelength division multiplexer 131, and a second wavelength division multiplexer 132. The concave mirror 134 reflects the side scattered light and the fluorescent light that diverge in various directions at the interrogation zone 18.

[0229] The concave mirror 134 and the aspheric lens 135 focus the reflected light onto the collection fiber 136 by focusing on the same point of the collection fiber 136 as shown in the dotted block 139 in FIG. 2. The concave mirror 134 can focus the reflected light on the fiber, while the aspheric lens 135 can make the focal point smaller (i.e., reduce the aberration).

[0230] To prevent crosstalk, the beam splitter 133 is arranged to separate the scattered light that has high intensity from the fluorescent light that has low intensity. The side scattered light is directed toward the first wavelength division multiplexer 131 via a first fiber 137, and the fluoresced light is directed toward the second wavelength division multiplexer 132 via a second fiber 138. Optical signals with different wavelengths are separated in the first wavelength division multiplexer 131 and the second wavelength division multiplexer 132 for analysis.

[0231] The beam splitter 133 includes a dichroic mirror 532 and a notch filter 534. Collected light is directed into the beam splitter 133 toward the dichroic mirror 532 by the collection fiber 136, which may be oriented such that the light beam is directed toward the dichroic mirror 532 at an incident angle of, for example, 45 degrees. The dichroic mirror 532 reflects the side scattered light coming out of the collection fiber 136 such that the side scattered light enters the first wavelength division multiplexer 131 through the first fiber 137.

[0232] The fluorescent light coming out of the collection fiber 136 passes through dichroic mirror 532, and is incident to the notch filter 534 at an incident angle of about 90 degrees and then passes through the notch filter 534. The fluorescent light enters the second wavelength division multiplexer 132 through the second fiber 138.

[0233] The dichroic mirror 532 and the notch filter 534 can each have multiple bands according to the arrangement of the light sources 111a-111d. In the example shown in FIG. 2, the dichroic mirror 532 and the notch filter 534 both have four bands that block four laser wavelengths. The number of bands on the dichroic mirror 532 and the notch filter 534 can correspond to the number of the light sources 111a-111d.

[0234] The beam splitter 133 separates the side scattered light having high intensity from the fluorescent light having low intensity, reducing or preventing crosstalk of the side scattered light to the fluorescent light. In addition, by providing the beam splitter, it is possible to separate and transmit multiple light beams into two or more wavelength division multiplexers. The optical elements included in the beam splitter 133 and their configuration may be changed, and are not limited to the example shown and described herein.

[0235] In some examples, the first wavelength division multiplexer 131 receives the side scattered light from the beam splitter 133 via the first fiber 137, and separates from each other the optical signals of the side scattered light based on their wavelengths. For example, the optical signals associated with the light beam in the red light spectrum emitted by the first light source 111a, the optical signals associated with the light beam in the yellow light spectrum emitted by the second light source 111b, the optical signals associated with the light beam in the blue light spectrum emitted by the third light source 111c, and the optical signals associated with the light beam in the violet light spectrum emitted by the fourth light source 111d are separated in the first wavelength division multiplexer 131. In the first wavelength division multiplexer 131,

each optical signal is transmitted along an optical transmission path 510 corresponding to an optical channel of the optical signal. Thereafter, the side scattered light enters an SSC detector 515 which can include a photodiode, an avalanche photodiode (APD), or a photomultiplier tube for analyzing the side scattered light. In the example illustrated in FIG. 6, there are six separate side scatter channels.

[0236] The first wavelength division multiplexer 131 includes a first filter 511 and a second filter 512 for each optical channel. The first filter 511 and the second filter 512 are arranged at a certain distance from each other along the optical transmission path of the optical channel in a non-parallel manner. Crosstalk between side scattered light can be reduced or prevented by providing the two filters. The first and second filters 511 and 512 are not arranged in parallel so as to avoid multiple reflections of light between them and achieve a better optical density.

[0237] The second wavelength division multiplexer 132 receives a fluorescent beam from the beam splitter 133 via the second fiber 138, and divides the optical signals of the fluorescent beam having different wavelengths from each other. In the second wavelength division multiplexer 132, each optical signal is transmitted along an optical transmission path 520 corresponding to an optical channel of the optical signal. Since the fluorescent signal is weak, the second wavelength division multiplexer 132 includes a single filter 521 for each optical channel. Thereafter, the filtered fluorescent light enters a light detection element 525 (e.g., a photodiode, an avalanche photodiode (APD), a photomultiplier tube) for further processing. In the example illustrated in FIG. 6, there are six separate fluorescence channels.

[0238] Alternative suitable configurations for the wavelength division multiplexers may be used. For example, the first and second wavelength division multiplexers 131, 132 can include notch filters corresponding to the respective optical channels. The notch filters can reduce or eliminate the crosstalk of the side scattered light to the fluorescence light. In this case, the beam splitter 133 may only include the dichroic mirror 532 with no notch filter 534.

[0239] In the side collection unit 130, a diameter of the collection fiber 136 may be different from diameters of the first fiber 137 and the second fiber 138 according to the light transmission efficiency. Lenses in the beam splitter may cause aberration, and thus the output light spots may be larger than input of the beam splitter, and the fiber diameters may be selected accordingly.

[0240] The forward collection unit 152 includes an obscuration bar 155, a concave mirror 151, a filter 157, and a forward detector 159. The obscuration bar 155 blocks a large portion of the light transmitted through the flow chamber 15 to reduce background noise created by the excitation light beams transmitting directly through the flow chamber 15, and to allow collection of only forward scattered light from the particles. In some examples, the majority of the transmitted light is blocked so as not to saturate the forward detector 159.

[0241] The concave mirror 151 reflects a forward scattered beam emitted from the particles. The filter 157 allows forward scattered light with a high signal-to-noise ratio to pass, and block other light. As further shown in FIG. 2, the forward detector 159 receives the filtered forward scattered light from the filter 157, and processes and analyzes the forward scattered light.

[0242] FIG. 3 schematically illustrates an example of a method 300 of using flow cytometry performed by the flow cytometer 100 to improve centrifugation performed by the centrifuge 150. The method 300 can be performed by the system 10, as shown in FIG. 1.

[0243] The method 300 includes an operation 302 of receiving a specimen inside a rotor chamber of the centrifuge 150. Operation 302 can include receiving at least one of the container 106 that includes the specimen. In some examples, operation 302 includes receiving a plurality of containers 106 that each includes the specimen. Operation 302 includes receiving the one or more containers 106. As an illustrative example, the specimen includes a blood sample.

[0244] The method 300 includes an operation 304 performing centrifugation on the specimen received in operation 302. Operation 304 can include performing ultracentrifugation by rotating the rotor chamber at high speeds for high g-force separations. For example, operation 304 can include rotating the specimen from 100,000 xg to 1,000,000 xg to purify and/or characterize one or more target particles in the specimen. Accordingly, operation 304 includes rotating a rotator of the centrifuge 150 at a predetermined speed, duration, and temperature to isolate and/or purify target particles within the specimen such as extracellular vesicles (EVs).

[0245] The method 300 includes an operation 306 of performing a flow cytometry analysis of the specimen after centrifugation is completed in operation 304. Operation 306 can include receiving in the sample station 104 the container 106 that includes the

specimen after centrifugation is completed. The flow cytometry analysis can characterize the particles in the specimen such as by determining their size and refractive index. By characterizing the particles, the purity or yield of the target particles in the specimen can be determined. In some examples, operation 306 includes comparing the purity or yield of the target particles to a threshold for determining whether the centrifugation satisfies quality control. In some instances, operation 306 includes using the flow cytometry to sort the particles in the specimen to increase their purity.

[0246] The analysis performed in operation 306 is different from analyses typically performed during ultracentrifugation because the flow cytometry analysis in operation 306 analyzes each particle individually instead of a bulk analysis which is typically performed in ultracentrifugation. Thus, the flow cytometry analysis provides a higher level of granularity.

[0247] The method 300 includes an operation 308 of sample optimization such as adjusting one or more parameters of the centrifugation based on the characterization of the particles determined in operation 306. As described above, the particles are characterized to determine the purity or yield of one or more target particles. Operation 308 of sample optimization can include adjusting one or more the centrifugation speed, duration, and temperature to increase the purity or yield of the target particles. Thus, the method 300 includes using the flow cytometer 100 as an analytical tool that provides a direct feedback loop for improving the centrifugation performed by the centrifuge 150. This method is described in the context of using a centrifuge in conjunction with a flow cytometer, but this disclosure contemplates using other laboratory instruments rather than a centrifuge, e.g., an electron microscope ELISA, ELISPOT, or column chromatography, or any combination of centrifugation, electron microscopy, ELISA, ELISPOT, and column chromatography. The ability of a CytoFLEX nano to effectively characterize nano particles, e.g., down to a size of about 20nm, means it is convenient to use as a quick analytical tool to help confirm, discovery, and/or characterize particles in samples in conjunction with other laboratory techniques like electron microscopy, ultra centrifugation, ELISA, ELISPOT, and/or column chromatography. In embodiments, the use of a flow cytometer is used to provide feedback to conditions used for electron microscopy, ultra centrifugation,

ELISA, ELISPOT, and/or column chromatography in order to better isolate and/or characterize particles of interest.

[0248] The plurality of side scattering channels and the plurality of fluorescence channels in the flow cytometer 100 can be used to characterize and distinguish nanoparticles such as lipid nanoparticles from contaminants such as extracellular debris that have sizes less than 100 nm. In some examples, scores can be calculated based on one or more attributes in the plurality of side scattering channels and the plurality of fluorescence channels. The scores can be used as part of a radar map methodology to identify and distinguish nanoparticles. In some examples, comparing data collected from one or more side scatter channels with data collected from one or more fluorescence channels can be used to identify patterns for characterizing the nanoparticles.

[0249] As an example, multiple channels of side scatter data and multiple channels of fluorescence data can be collected from nanoparticles having a known refractive index. The data can be fed into a database to train a machine learning algorithm to detect attributes and/or patterns in the side scatter and fluorescence channels. Thereafter, the machine learning algorithm can be used to characterize or distinguish other types of nanoparticles having unknown refractive indices that exhibit similar attributes or patterns in the side scatter and fluorescence channels.

[0250] FIG. 4 schematically illustrates an example of a method 400 of characterizing nanoparticles that can be performed by the flow cytometer 100. The method 400 uses a multicolor panel to identify different types of target particles. As an illustrative example, the method 400 can identify different types of EVs in platelet-poor plasma (PPP) samples.

[0251] The method 400 includes an operation 402 of preparing samples such as an LNP or PPP sample from a blood sample collected from a subject. Operation 402 can include centrifuging the sample. As an illustrative example, a blood sample can be centrifuged for 5 min at 200xg. Operation 402 can further include removing the sample from a top of a container and filtering through a 200nm syringe. As an illustrative example, about 1mL of PPP can be removed from the container. Operation 402 can include further purifying the PPP that has been removed by using an isolation column that isolates the PPP based on size (e.g., 70 nm) by size exclusion chromatography.

[0252] Operation 402 can further include screening selected fractions of the PPP using single-color stain and multicolor stains for immunophenotyping. Operation 402 can further include staining the fractions of the PPP with antibodies. For example, the fractions of the PPP can be stained with CD81 PB, CD9 FITC, CD63 APC, CD61 PC7 and CD235a PE.

[0253] The method 400 includes an operation 404 of collecting flow cytometry data from the samples prepared in operation 402 by using the flow cytometer 100. Operation 404 can include illuminating particles in the sample that individually pass through the interrogation zone 18 with one or more excitation light beams. Operation 404 can include collecting side scatter data from the particles, which is collected by plurality of side scatter channels in the first wavelength division multiplexer 131 of the flow cytometer 100. Operation 404 can further include collecting fluorescence data from the particles, which is collected by the plurality of fluorescence channels in the second wavelength division multiplexer 132.

[0254] The method 400 includes an operation 406 of characterizing nanoparticles based on the analysis performed in operation 404. For example, based on the size distribution and fluorescence staining of the fractions collected from the PPP samples, the fractions where EVs reside can be identified. The flow cytometer 100 is able to detect and distinguish more than one fluorescence (FL) signal on single EVs particles, including PB, FITC, PE, PC7 and APC. At the proper antibody concentration and the use of proper controls, antibody aggregates and antibody background interference are avoided. Further, the flow cytometer 100 can compensate spillover between different fluorescent dyes allowing analysis of the multicolor staining simultaneously.

[0255] In embodiments, method 400 is implemented or modified before implantation to characterize EVs, LNPs or virus particles with different contents, different surface markers, different targeting ligands, or any combination thereof. In embodiments, the degree of modification or insertion of surface markets, targeting ligands, or combination thereof is characterized.

[0256] FIG. 5 schematically illustrates an example of a method 500 of characterizing lipid nanoparticles that can be performed by the flow cytometer 100. Lipid nanoparticles (LNPs) have become increasingly valuable research and therapeutic tools. LNPs consist of capsids filled with compounds with pharmaceutical properties such as modified mRNA. The method 500 utilizes the flow cytometer 100 to

characterize LNPs. The method 500 minimizes sample preparation, and provides fast results which can be correlated to functional cell transduction assays. The method 500 can be performed as part of a flow cytometry workflow utilizing the flow cytometer 100 to characterize LNPs. The method 500 can be performed as a quick measure of LNP quality.

[0257] The method 500 includes an operation 502 of preparing a first sample for analysis by the flow cytometer 100. As an illustrative example, operation 502 can include acquiring commercially available empty and full LNPs such as encapsulated green fluorescent protein (GFP) encoding mRNA. Operation 502 can further include diluting the LNPs. For example, the LNPs can be diluted 1:200 in filtered phosphate-buffered saline (PBS).

[0258] The method 500 includes an operation 504 of preparing a second sample for analysis by the flow cytometer. The second sample is a functional assay. Operation 504 can include diluting a suspension of Chinese hamster ovary (CHO) cells in an appropriate medium and seeding the diluted CHO cells in a plurality of wells. As an illustrative example, 200 μ L of the diluted CHO cells can be seeded into U-bottom 96-well plates (0.25 - 1.0 million cells/mL). Operation 504 can further include adding commercially available LNPs to the wells in a range of 1 – 10 μ L per well (100 – 30000 ng mRNA/well). After a period of time elapses (e.g., 24 – 48 hours), operation 504 can include removing the CHO cells from the wells.

[0259] The method 500 includes an operation 506 of analyzing the first and second samples prepared by operations 502, 504 to quantify a percentage of GFP positive cells. Operation 506 includes analyzing the first and second samples using the flow cytometer 100. In some examples, PBS controls are used to understand and reduce background interference. Operation 506 can further include using fluorescent microscopy to confirm presence of GFP positive cells.

[0260] The method 500 includes an operation 508 of characterizing the LNPs. For example, the empty and full LNPs are distinguishable based on a detectable increase in scatter. This increase in scatter can be quantitated for the positive/full LNPs. Accordingly, a correlation exists between the LNP scatter levels and the ability of LNPs to transduce CHO cells.

[0261] The multiple side scattering channels in the flow cytometer 100 can further extend the dynamic range for capturing side scattered light under the violet light

spectrum for identifying protein aggregates such as amyloid plaques associated with diseases such as ALS, Alzheimer's, Parkinson's diseases. For example, the dynamic range of the side scattered light under the violet light spectrum can range between 40 nm to 1000 nm. In some instances, the flow cytometer 100 incorporates aspects described in U.S. Provisional Patent Application No. 63/608,615, filed on December 11, 2023, the disclosure of which is herein incorporated by reference in its entirety.

[0262] The flow cytometer 100 has a sensitivity in the violet light spectrum for detecting side scattered light that can be used to detect protein aggregates, which can vary in size such as between 100 nm to 1 μ m. Further, once detected, the flow cytometer 100 can characterize and distinguish different types of protein aggregates. In some examples, the flow cytometer 100 uses an attenuated scatter to preferentially detect protein aggregates. In some examples, a dye can be used to specifically mark aggregates such as proteostat, and sensitivity of the fluorescence channels in the flow cytometer 100 allows detection of marked aggregates.

[0263] The flow cytometer 100 can further be used to detect and sort particles based on their secretion. This is advantageous in view of current methods unable to detect single cell secretion. For example, assays that are generated for detecting proteins typically average protein secretion of large groups of cells, and thereby ignore the microenvironment of single cells. The flow cytometer 100 provides an improvement over these methods by finding specific cells that secrete desired protein in a mixture of cells. The flow cytometer 100 can sort the particles based on detected attachment to heterofunctional particles, which are used to isolate the particles based on their secretion, as described in PCT International Patent Application No. PCT/US2022/072754, filed June 3, 2022, the disclosure of which is herein incorporated by reference in its entirety.

[0264] The flow cytometer 100 can further be used to detect and sort particles based on complete and incomplete assembly. For example, viral loading count can be detected by the flow cytometer 100. As an illustrative example, vesicular stomatitis virus (VSV) has been used to develop therapeutics. While being developed, VSV is known to suffer from incomplete assembly. For examples, as the particle grows/matures, the ratio of the inner and outer components varies. The flow cytometer 100 can be used to detect the inner and outer components of VSV for determining

particle maturation. In some examples, the flow cytometer 100 can be used to sort VSV based on particle maturation.

[0265] As another example, the flow cytometer 100 can be used to detect a degree of vector assembly for lentivirus and other retroviruses. For example, lentivirus is multi-layered particle such that it is prone to layers being stripped and/or damaged during processing. The flow cytometer 100 can detect a presence or absence of specific envelope proteins that would indicate particle maturity and integrity as it relates to functionality. It is contemplated that the flow cytometer 100 could be used to assess physical properties of the particles linked to functionality, which would be significantly faster and less costly than cell-based assays which are complex and take at least a full day to execute. The flow cytometer 100 may also be used to discriminate wildtype viruses (i.e., naturally occurring, non-mutated strain of a virus) from one another.

[0266] The flow cytometer 100 can further be used to determine an amount of protein on a surface of or within a LNP, EV, vectors for lentivirus, and the like. For example, the flow cytometer 100 can detect empty load, partial load, or fully loaded lentiviral particles, as well as that of other particles having a diameter of about 90 nm. The flow cytometer 100 can further characterize drug loading in liposomes by providing single particle label free characterization of the liposomes including characterizing receptor/ligand binding.

[0267] The flow cytometer 100 can further detect attributes from a combination of data sources such as LNPs or viral vectors, using multiple fluorescent dyes to assess encapsulation efficiency or quantify cargo packaged in a particle. The flow cytometer 100 can further detect a degree of modification or insertion of surface markers or targeting ligands such as chemical addition of lipid-targeting moieties into an LNP surface or engineering adenovirus to express and present a particular surface marker, which is useful for developing mRNA vaccines. Further, the flow cytometer 100 is sensitive to less abundant biomarkers and small sample sizes such that it could be used more effectively detect presence of biomarkers in samples acquired from subjects.

[0268] FIG. 6 schematically illustrates an example of a computing device 600 for implementing aspects of the system 10, including functions of the flow cytometer 100 and the workstation 200. Examples of the computing device 600 include a server computer, a desktop computer, a laptop computer, a tablet computer, a mobile

computing device (such as a smartphone), or other devices configured to process digital instructions.

[0269] The computing device 600 includes one or more processing devices 602. Examples of the one or more processing devices 602 include central processing units (CPUs), digital signal processors, field-programmable gate arrays, and other types of electronic computing circuits. The one or more processing devices 602 can be part of a processing circuitry having a memory for storing instructions which, when executed by the processing circuitry, cause the processing circuitry to perform the functionalities described herein.

[0270] The computing device 600 further includes a system memory 604, and a system bus 606 that couples various system components including the system memory 604 to the one or more processing devices 602. The system bus 606 is one of any number of types of bus structures including a memory bus, or a memory controller; a peripheral bus; and a local bus using any of a variety of bus architectures.

[0271] The system memory 604 can include a read only memory (ROM) 608 and a random access memory (RAM) 610. A basic input/output system (BIOS) 612 containing the basic routines that act to transfer information within computing device 600, such as during start up, can be stored in the system memory 604. The RAM 610 can be used for loading and subsequently analyzing the waveform data (e.g., stored in a raw waveform data file, which can include digitalized raw waveform data).

[0272] The computing device 600 can also include one or more secondary storage devices 614 such as a hard disk drive for storing digital data. The one or more secondary storage devices 614 are connected to the system bus 606 by a secondary storage interface 616. The one or more secondary storage devices 614 and associated computer readable media provide nonvolatile storage of computer readable instructions (including application programs and program modules), data structures, and other data for the computing device 600. Although the example described herein employs a hard disk drive as a secondary storage device, other types of computer readable storage media are used in other embodiments. Examples of these other types of computer readable storage media include the ROM 608 and/or the RAM 610. Some examples include non-transitory media. Additionally, such computer readable storage media can include local storage or cloud-based storage.

[0273] The computing device 600 typically includes at least some form of computer readable media. Computer readable media includes any available media that can be accessed by the computing device 600. By way of example, computer readable media include computer readable storage media and computer readable communication media.

[0274] Computer readable storage media includes volatile and nonvolatile, removable and non-removable media implemented in any device configured to store information such as computer readable instructions, data structures, program modules or other data. Computer readable storage media includes, but is not limited to, random access memory, read only memory, electrically erasable programmable read only memory, flash memory or other memory technology, or any other medium that can be used to store the desired information and that can be accessed by the computing device 600.

[0275] Computer readable communication media can embody computer readable instructions, data structures, program modules or other data in a modulated data signal such as a carrier wave or other transport mechanism and includes any information delivery media. The term “modulated data signal” refers to a signal that has one or more characteristics set in a manner as to encode information in the signal. For example, computer readable communication media includes wired media such as a wired network or direct-wired connection, and wireless media such as acoustic, radio frequency, infrared, and other wireless media. Combinations of any of the above are also included within the scope of computer readable media.

[0276] A number of program modules can be stored in secondary storage device 614 or the system memory 604, including an operating system 618, application programs 620, program modules 622 (such as the software engines), and program data 624. The computing device 600 can utilize any suitable operating system, such as Microsoft Windows™, Google Chrome™, Apple OS, and any other operating system suitable for a computing device.

[0277] A user provides inputs to the computing device 600 through one or more input devices 626. Examples of input devices 626 include the mouse 206, the keyboard 208, a microphone 632, and a touch sensor 634 (such as a touchpad or touch sensitive display). Additional types of the input devices 626 are contemplated. The input devices 626 are often connected to the one or more processing devices 602 through an

input/output interface 636 that is coupled to the system bus 606. These input devices 626 can be connected by any number of input/output interfaces, such as a parallel port, serial port, game port, or a universal serial bus. Wireless communication between input devices and the input/output interface 636 is possible as well, and includes infrared, BLUETOOTH® wireless technology, 802.11a/b/g/n, cellular, or other radio frequency communication systems in some possible embodiments.

[0278] The display monitor 204 can include a liquid crystal display device, a touch sensitive display device, and the like. The display monitor 204 connects to the system bus 606 via an interface, such as a video adapter 640. In addition to the display monitor 204, the computing device 600 can include various other peripheral devices, such as speakers or a printer.

[0279] When used in a local area networking environment or a wide area networking environment (such as the Internet), the computing device 600 is connected to the network 160 through a network interface 642, such as an Ethernet interface. Other possible embodiments use other communication devices. For example, some embodiments of the computing device 600 include a modem for communicating across the network 160.

[0280] The computing device 600 is an example of programmable electronics, which may include one or more such computing devices. When multiple computing devices are included, such computing devices can be coupled together with a suitable data communication network so as to collectively perform the various functions, methods, or operations disclosed herein.

[0281] The flow cytometer instrument can be employed to determine amount of therapeutic payload in a nanoparticle, amount of antibody binding to nanoparticle, and/or predict cell transduction efficiency by using methods of the disclosure. The methods can include setting instrument configuration parameters for calculations in subsequent operations, finding collection angles, finding calibration factors, and analyzing measurements.

[0282] A method of determining a flow cytometer instrument calibration factor is illustrated at FIG. 59 Step 1. In some cases, the method of flow cytometer instrument calibration shown in Step 1 includes illuminating a plurality of standard particles each with known size and refractive index 5905, collecting side scatter from all wavelengths of interest 5910, calculating Mie scattering profiles for each standard particle 5915,

integrating Mie scattering profiles over 0-90 degrees collection half angle for each standard particle size 5920, calculating goodness of fit for current half angle 5935, reporting collection half angle with highest goodness of fit 5940, and calculating average calibration factor and CV over all standard particle sizes for collection half angle from previous step 5945. Flow cytometer empirical data collection methods are illustrated in FIG. 59 Step 2. In some cases, the method of flow cytometer particle (e.g., nanoparticle) empirical data collection includes illuminating the particles 5950 at one or more wavelengths; collecting side scatter from all wavelengths of interest 5960; and identifying mean scatter intensity at each of the wavelengths of interest 5965 and 5970. In some cases, the mean side scattered light intensity at each of the wavelengths of interest 5960 is compared to the calibration factor 5945 determined in the same flow cytometer, comprising correlating the particle empirical data to theoretical data for the plurality of standard particles having known refractive indices and sizes. If the gains and the wavelengths from the empirical data are the same as the theoretical data found in the instrument calibration of Step 1, the method proceeds to a post processing step 3. If the gains and the wavelengths from the empirical data are different from the theoretical data found in the instrument calibration factor of Step 1, the method includes rerunning instrument calibration method of Step 1 with the standard particles of known size and refractive index to obtain a calibration factor of Step 2. Post processing step methods are illustrated in FIG. 59 Step 3. In some cases, the post processing step includes inputting known particle parameters 5975; inputting initial guesses for particle parameters, bounds of particle parameters, and constraints of particle parameters 5980; and calculating output parameters that minimize loss function and satisfy the bounds and constraints 5985. In some cases, the particle input parameters are selected from the group consisting of particle size, particle refractive index, shell thickness, shell refractive index, wavelength, buffer material, buffer refractive index, half collection angle from FIG. 59 Step 1, and calibration factor from Step 1 or Step 2, 5945 or 5970.

[0283] In some cases, the post processing step includes particle (e.g., nanoparticle) input and output parameters as illustrated in FIG. 60 for ten different use cases including for solid nanoparticles and core-shell nanoparticles. In some cases, the post processing step particle input parameters are selected from the group consisting of solid particle diameter, solid particle refractive index, core-shell particle shell thickness,

core-shell particle shell refractive index, core-shell particle core refractive index, and core-shell particle core diameter. In some cases, the post processing step particle output parameters are selected from the group consisting of solid particle diameter, solid particle refractive index, core-shell particle shell thickness, core-shell particle shell refractive index, core-shell particle core refractive index, and core-shell particle core diameter. In some cases, the post processing step includes an input parameter of solid particle diameter and an output of solid particle refractive index. In some cases, the post processing step includes an input parameter of solid particle refractive index and an output of solid particle diameter. In some cases, the post processing step includes input parameters of shell thickness, shell refractive index, and core refractive index and an output of core diameter. In some cases, the post processing step includes input parameters of core diameter, shell refractive index, and core refractive index and an output of shell thickness. In some cases, the post processing step includes input parameters of shell refractive index, shell thickness, and core diameter and an output of core refractive index. In some cases, the post processing step includes input parameters of core refractive index, shell thickness, and core diameter and an output of shell refractive index. In some cases, the post processing step includes input parameters of shell thickness and shell refractive index and outputs of core diameter and core refractive index. In some cases, the post processing step includes input parameters of core diameter and core refractive index and outputs of shell thickness and shell refractive index. In some cases, the post processing step includes input parameters of core refractive index and shell refractive index and outputs of core diameter and shell thickness. In some cases, the post processing step includes input parameters of core diameter and shell thickness and outputs of core refractive index and shell refractive index.

[0284] In some cases, the post processing step includes input of particle (e.g., nanoparticle) initial guesses, bounds, and constraints as shown in FIG. 61. In some cases, the initial guesses, bounds, and constraints for the solid particle refractive index are 1.4, 1.33-2.5, and none, respectively. In some cases, the post processing step includes input initial guesses, bounds, and constraints for the solid particle size of 100 nm, 10 nm-1500 nm, and none, respectively. In some cases, the post processing step includes input initial guesses, bounds, and constraints for the core-shell particle core size of 100 nm, 10 nm-1500 nm, and none, respectively. In some cases, the post

processing step includes input initial guesses, bounds, and constraints for the core-shell particle shell thickness of 10 nm, 0 nm-1500 nm, and none, respectively. In some cases, the post processing step includes input initial guesses, bounds, and constraints for the core-shell particle core refractive index of 1.4, 1.33-2.5, and none, respectively. In some cases, the post processing step includes input initial guesses, bounds, and constraints for the core-shell particle shell refractive index are 1.4, 1.33-2.5, and none, respectively. In some cases, the post processing step includes input initial guesses, bounds, and constraints for the particle core-shell nanoparticle core refractive index of 1.4, 1.33-2.5, and none, respectively; and the core diameter of 50 nm, 10 nm-1500 nm, and none, respectively. In some cases, the post processing step includes input initial guesses, bounds, and constraints for the particle core-shell nanoparticle shell refractive index of 1.4, 1.33-2.5, and none, respectively; and the shell thickness of 10 nm, 0 nm-1500 nm, and none, respectively. In some cases, the post processing step includes input initial guesses, bounds, and constraints for the particle core diameter of 50 nm, 10 nm-1500 nm, and shell thickness > core diameter, respectively; and shell thickness of 75 nm, 0 nm-1500 nm, and shell thickness > core diameter, respectively. In some cases, the post processing step includes input initial guesses, bounds, and constraints for the particle core diameter of 100 nm, 10 nm-1500 nm, and shell thickness < core diameter, respectively; and shell thickness of 10 nm, 0 nm-1500 nm, and shell thickness < core diameter, respectively. In some cases, the post processing step includes input initial guesses, bounds, and constraints for the particle core diameter of 100 nm, 10 nm-1500 nm, and none, respectively; and shell thickness of 10 nm, 0 nm-1500 nm, and none, respectively. In some cases, the post processing step includes input initial guesses, bounds, and constraints for the particle core refractive index of 1.4, 1.33-2.5, and shell RI > core RI, respectively; and the shell refractive index of 1.45, 1.33-2.5, and shell RI > core RI, respectively. In some cases, the post processing step includes input initial guesses, bounds, and constraints for the particle core refractive index of 1.45, 1.33-2.5, and shell RI < core RI, respectively; and the shell refractive index of 1.4, 1.33-2.5, and shell RI < core RI, respectively. In some cases, the post processing step includes input initial guesses, bounds, and constraints for the particle core refractive index of 1.4, 1.33-2.5, and none, respectively; and the shell refractive index of 1.45, 1.33-2.5, and none, respectively.

[0285] Ten use case scenarios having various input and output parameters are provided as shown in FIGs. 60 and 61. These are graphically illustrated in FIGs. 62-64. FIG. 62 discloses two use case scenarios for solid particles. In use case 1: the refractive index is the known input parameter and size is unknown output parameter. In use case 2: the size is the known input parameter and the refractive index is the unknown output parameter. FIG. 63 discloses four use case scenarios for core-shell modeling. Use case 3: refractive index (RI) of the core, shell RI, shell thickness are known input parameters, core size is unknown output parameter. Use case 4: shell RI, core RI and core size are known input parameters and shell thickness is unknown output parameter. Use case 5: shell RI, shell thickness, and core size are known input parameters and core RI is unknown output parameter. Use case 6: shell thickness, core RI, core size are known input parameters and shell thickness is unknown output parameter. FIG. 64 discloses four use case scenarios for core-shell modeling. Use case 7: shell RI, shell thickness are known input parameters; core RI and core thickness are unknown output parameters. Use case 8: core RI and core size are known input parameters; core RI, core size are unknown output parameters. Use case 9: core RI and shell RI are known input parameters; shell thickness, core size are unknown output parameters. Use case 10: core size and shell RI are known input parameters; core RI, core size are unknown output parameters.

[0286] A method of instrument calibration (step 1) to define the average calibration factor based on identified half angle is illustrated in FIG. 65. Empirical data collection was obtained from a plurality of polystyrene size reference beads having known size and RI. The graphs in the top row show violet VSSC1 and VSSC2 histograms for two sets of polystyrene size reference beads: a first set of polystyrene reference beads (low vis) of nominal sizes 40 nm, 89 nm, 103 nm, and 141 nm, and a second set of polystyrene reference beads (high vis) of nominal sizes 141 nm, 304 nm, 600 nm, and 1020 nm, respectively. The tables at upper right show nominal size (nm), CV%, and RI of polystyrene beads at four illumination wavelengths of 405 nm (RI = 1.6253), 488 nm (RI = 1.6039), 561 nm (RI = 1.5931), and 633 nm (RI = 1.586). Next, the half angle was defined per each instrument for three instruments MP03, MP05 and MP07. The three graphs across middle row show confidence of fit % ("goodness of fit") as a function of scattering angle. Gains, wavelength, and particle sizes are independent variables. The half collection angle for each instrument is defined at the highest

goodness of fit. Next, the averaged calibration factors for each instrument were defined based on identified half angle. Gains, wavelength, and particle sizes are dependent variables. The table at bottom shows averaged calibration factors for three flow cytometers MP05, MP07, and MP03 over all bead sizes at different wavelengths of scatter for VSSC1 (violet), BSSC (blue), YSSC (yellow), and RSSC (red) illumination wavelengths.

[0287] A first example of empirical data collection (step 2) for a biological control for a commercially available MLV virus-like particle is shown in FIG. 66 top row. The top row shows histograms of VSSC1 (a), BSSC(b), YSSC (c), RSSC(d) and a dot plot (e) showing gating strategy for MLV (RSSC-H vs. VSSC1-H). The MLV signal is shown at arrows in histograms and encircled in the dot plot.

[0288] A second example of empirical data collection (step 2) for a biological control for a commercially available recombinant EV is shown in FIG. 66 bottom row. The bottom row shows dot plot (a) showing VSSC1 vs. B531 FL, with P2 (36.56%) gating on unstained and P1 (23.10%) for GFP positive rEVs. Dot plot (b) shows BSSC vs. B531 FL. Dot plot (c) shows YSSC vs. B531 FL. Dot plot (d) shows RSSC vs. B531 FL.

[0289] A third example of empirical data collection (step 2) for a biological control for lipid nanoparticles (LNPs) is shown in FIG. 67 top row. Top row shows histograms of VSSC1 of 20 nm for a) buffer, b) empty LNP, and c) loaded LNP.

[0290] A fourth example of empirical data collection (step 2) for a biological control is shown in FIG. 67 bottom row for LNPs along with histogram overlay of VSSC1 for 4 LNPs with polyA surrogate mRNA payloads and different amounts of mAbs on surface, as shown in the table.

[0291] A table of post processing parameters (step 3) for Use case 1 for MLV particles with known parameter RI and unknown parameter of particle diameter is shown in FIG. 68. The table identifies left to right instrument, particle type (MLV), gain (200), illumination channel (VSSC1, BSSC, YSSC, or RSSC), expected core diameter (nm) from Cryo TEM, input parameter of expected core RI, empirical data, output parameter of predicted core diameter (nm), and predicted core diameter error (predicted – expected).

[0292] A table of post processing parameters (step 3) for Use case 2 for MLV particles with known diameter and unknown RI is shown in FIG. 69. The table

identifies left to right instrument, particle type (MLV), gain, illumination channel (VSSC1, BSSC, YSSC, or RSSC), input parameter of expected core diameter (nm), expected core RI, empirical data, output parameter of predicted core RI, and predicted RI error (predicted – expected).

[0293] A table of post processing parameters (step 3) for Use case 5 for rEV particles with known shell RI, shell thickness, core size, and unknown core RI is shown in FIG. 70. The table identifies instrument, particle type (rEV), gain, channel (VSSC1, BSSC, YSSC, or RSSC), input parameters of (i) expected core diameter (nm), (ii) expected shell thickness (nm), and (iii) expected shell RI; expected core RI, empirical data; and output parameter of predicted core RI, and predicted core RI error (predicted core RI – expected core RI).

[0294] A table of post processing parameters (step 3) for Use case 10 for rEV particles with known shell size, known core size, unknown shell RI, and unknown core RI is shown in FIG. 71. The table identifies left to right instrument, particle type (rEV), gain, channel (VSSC1, BSSC, YSSC, or RSSC), input parameter of expected core diameter (nm) and input parameter of expected shell thickness (nm); expected core RI, expected shell RI, empirical data, constraint (shell RI > core RI), output parameter of predicted core RI, predicted core RI error (predicted core RI – expected core RI), output parameter of predicted shell RI, and predicted shell RI error (predicted shell RI – expected shell RI).

[0295] An example of post processing step (step 3) for Use case 2 in the algorithm is shown in FIG. 72. Step A shows an LNP nanoparticle having known diameter and unknown RI (left) at left panel. Step B shows input known parameters of size for empty LNP nanoparticle and loaded LNP nanoparticle is shown in left center panel. Step C shows calculation of output RI for empty LNPs (particle type = empty, input size 64 nm, empirical data 4962, output RI = 1.435) and loaded LNPs (particle type = loaded, input size 64 nm, empirical data 12625, output RI = 1.47) in center right panel. Step D shows calculating delta RI for loaded and empty LNPs. ($\Delta RI = RI_{loaded} - RI_{empty}$) or $1.47 - 1.435 = 0.035$. This illustrates that refractive index change from loaded to empty can be correlated to amount of mRNA payload.

[0296] An example of Step 3 post processing step for Use case 10 or Use case 6 Step A in the algorithm is shown in FIG. 73. At left panel, for Use case 10, the shell thickness and core size are known, and the shell RI and core RI are unknown (left); for

Use case 6, the shell size, core thickness, and core size are known, and shell RI is unknown. Step B shows input of known parameters of core size and shell thickness for a loaded nanoparticle without mAb on surface and for a loaded nanoparticle having mAb on surface, left middle panel. Step C shows calculation of shell RI and core RI for loaded LNP particles [instrument, particle type loaded LNP, gain 200 for all side scatter channels, channels (VSSC1, BSSC, YSSC, or RSSC), input parameter core size, shell thickness, empirical data for VSSC1 medium scatter intensity, and output parameters of shell RI and core RI] and loaded LNP particles with mAb on surface [instrument, particle type loaded LNP with mAb, gain 200 for all side scatter channels, channels (VSSC1, BSSC, YSSC, or RSSC), input parameters of core size, shell thickness, empirical data for VSSC1 medium scatter intensity, and output parameters of shell RI and core RI]. Step D shows step of comparing RI between loaded LNPs and loaded LNPs with different amount of mAb on surface where (Δ shell RI = shell RI loaded + mAb – shell RI loaded), right panel. The change of shell RI can be correlated to amount of mAb on surface of nanoparticle. The (Δ shell RI = shell RI loaded + mAb – shell RI loaded). The change of refractive index of core can be correlated to amount of mAb on surface of loaded nanoparticle.

[0297] An algorithm workflow for instrument calibration is shown in FIG. 74 including (i) calculating forward Mie scattering profile for each input bead diameter 5915; (ii) integrating Mie scattering profiles over $90 \text{ deg} \pm$ current collection half angle for each bead diameter 5920; (iii) calculating a calibration factor for each bead size 5925; (iv) calculating goodness of fit (1-CV of all calibration factors) for current collection half angle 5930; and (v) reporting collection half angle with highest goodness of fit 5935. A loop over considered collection half angles from 0 deg to 90 deg in 0.1 deg steps is performed between steps 5920, 5925, and 5930. The calibration is determined as calibration factor = measured signal/integrated calculated signal. A graph of Normalized calibration factor vs bead diameter (nm) is shown at bottom. A graph of goodness of fit vs. collection half angle showing best fit at collection half angle = 50.8 deg is shown at upper right.

[0298] A method of calculation of the forward Mie scattering profiles 5915 is shown in FIG. 75. The pySCATMECH library was used. Profiles were calculated in the range of 0-180 deg in 0.1 deg steps. The profiles do not depend on the collection half angle and so are calculated only once. Inputs include bead diameter, bead refractive

index at wavelength, medium refractive index at wavelength, wavelength, polarization of incident light, and detector polarization sensitivity. Output is Mie scattering intensity as a function of polar angle. At right is a graph of calculated Mie scattering intensity vs. angle (deg) for polystyrene beads having diameters of 44 nm (A), 80 nm (B), 141 nm (C), 304 nm (D), 600 nm (E), and 1000 nm (F).

[0299] A method of integration of the calculated scattering intensity 5920 is shown at FIG. 76. Simpson's Rule was used to numerically integrate the Mie scattering profiles over an angular range that is defined by the current proposed collection angle. The integrand needs to be weighted by $\sin \theta$. Example outputs are shown in the four graphs at right of Mie scattering intensity vs angle (deg) at collection half angles of 20 deg (upper left), 40 deg (upper right), 60 deg (lower left) and 80 deg (lower right).

[0300] A method of calculating calibration factors for each bead size 5925 is shown at FIG. 77. The calibration factor is defined as the ratio of the measured scattered intensity and the integrated forward Mie intensity that was calculated in step 2. The calibration factor is calculated for each bead diameter. The measured signal for 44 nm beads is divided by the forward Mie scattering model integrated over the entire collection angle of 44 nm beads to obtain the calibration factor for the 44 nm beads. The measured signal for 80 nm beads is divided by the forward Mie scattering model integrated over the entire collection angle of 80 nm beads to obtain the calibration factor for the 80 nm beads. The measured signal for 100 nm beads is divided by the forward Mie scattering model integrated over the entire collection angle of 100 nm beads to obtain the calibration factor for the 100 nm beads. The measured signal for 144 nm beads is divided by the forward Mie scattering model integrated over the entire collection angle of 144 nm beads to obtain the calibration factor for the 144 nm beads.

[0301] A method for calculation of goodness of fit 5930 is shown at FIG. 78. The output of step 3 is a set of calibration factors for each input bead diameter for a single proposed collection half angle. First, the Coefficient of Variation (CV) is calculated over the set of calibration factors:

$$CV = \text{standard deviation (all calibration factors)} / \text{average (all calibration factors)}.$$

Second, the "goodness of fit" for the current proposed collection half-angle is calculated: $\text{goodness of fit} = 1 - CV$. Since $CV \geq 0$, the goodness of fit is ≤ 1 .

[0302] Identification of the most probable collection angle 5935 is shown in FIG. 79. Method 5930 is repeated for each collection half-angle that is considered. The

output is a "goodness of fit" for each potential collection half-angle. The calibration factor is an instrument parameter and should not depend on the bead size, so ideally, the CV calculated over multiple bead sizes would be 0. However, there is some bead-to-bead variation due to measurement uncertainty, and so the CV is non-zero. The most probable collection half-angle is identified as the angle with the smallest CV, since this most closely aligns with the ideal case. To calculate it, the collection half-angle with the largest goodness of fit is chosen, since the definition of 1-CV means that this is the angle with the CV closest to 0. See graph of goodness of fit vs collection half angle showing best fit collection half angle at 49.5 degrees.

[0303] Examples of half angle calculations in three flow cytometer instruments are shown in FIG. 80. Three graphs of confidence of fit % vs scattering angle for three flow cytometer instruments MP03, MP05, and MP07, excluding 44 nm and 103 nm bead data. The half angles at maximum confidence are 50.7 deg, 49.4 deg, and 52.1 deg, respectively, for MP03, MP07, and MP05 instruments as shown in the table. The half angle is instrument specific but gain independent.

[0304] A comparison of half angle calculations excluding 44 nm and 103 nm data (left graphs) and including 44 nm and 103 nm data (right graphs) is shown in FIG. 81 for three instruments MP03, MP05, and MP07, upper, middle, and lower graphs, respectively. By excluding the 103 nm the confidence is increased of matching empirical data with theoretical simulations.

[0305] An algorithm workflow to calculate the average calibration factor and CV over all bead sizes is shown in FIG. 82. When the correct collection angle is identified, there is little variation in the calibration factor, i.e., the calibration factor will not depend on the bead size since it is an instrument parameter. Example outputs are shown in four graphs of normalized calibration factor vs bead diameter for collection half angles of 20 deg, 40 deg, 60 deg, and 80 deg. Least variation in normalized calibration factor is exhibited at 60 deg collection half angle.

[0306] A generic algorithm for all use cases is shown in FIG. 83 with some variations between the parameters of the algorithm. All use cases use the same loss function:

Loss function = [(integrated, calculated Mie signal) x (calibration factor) – measured signal]. The calculated Mie signal is a function of the particle parameters listed in FIG. 60. In some cases, the particle parameters are selected from the group consisting of

particle diameter, particle refractive index, shell thickness, shell refractive index, core diameter, and core refractive index. The generic algorithm comprises calculating the unknown parameters that minimize the loss function and satisfy the bounds and constraint. Inputs include selecting minimization algorithm, initial guesses, bounds, and optionally constraints.

[0307] LNP1 solid modeling Mie calculations for empty and mRNA loaded LNPs is shown in FIG. 84. Histogram at left shows LNP1-empty. The histogram at right shows LNP1-eGFP. The table shows population results for LNP1-empty and LNP1-eGFP for median solid of RI values of 1.470, 1.450, 1.435, 1.430, and 1.42 (left to right), and sizes of loaded LNPs > size empty LNPs.

[0308] In Scenario #1,

- 1) Empty: RI is known -> calculate size,
- 2) If Size is not changed for loaded -> calculate RI,
- 3) Delta RI=RI (loaded)-RI(empty).

[0309] In Scenario #2,

- 1) Empty: Size is known -> calculate RI,
- 2) If RI is not changed for loaded -> calculate size,
- 3) Delta Size=Size (loaded)-Size(empty).

Scenario #3: both size and RI changed.

[0310] A flow diagram for an “example 2” using loaded LNPs and loaded LNPs with different amounts of antibodies is shown in FIG. 85. Steps include (i) illuminating with 1 or more excitation light beams, (ii) collecting light in a plurality of scatters and FL channels, (iii) extracting MFI data for loaded LNPs and loaded LNPs with different amounts of mAbs, using an algorithm with known input parameters to obtain unknown parameters, and (iv) calculating change in unknown parameter between loaded LNP and loaded LNP with mAb. In some cases, algorithm and input of size is used to calculate RI of loaded. In some cases, algorithm and input of RI is used to calculate size of loaded. In some cases, algorithm and input of loaded size, RI, shell thickness of stained sample, and RI of the shell is calculated. Calculate delta RI=RI(shell)-RI(buffer), Sensitivity of the instrument to determine protein loading.

[0311] In some cases, algorithm and input of loaded size and RI, to calculate shell thickness and shell RI. Calculate delta RI =RI(shell)-RI(buffer)- Thickness of the shell, Sensitivity of the instrument to determine protein loading.

[0312] In some cases, algorithm and input of loaded RI, RI of shell of stained sample is used to calculate shell thickness, sensitivity of instrument to determine protein loading.

[0313] In some cases, algorithm and input of loaded size and RI is used to calculate shell thickness and shell RI. Calculate $\Delta RI = RI(\text{shell}) - RI(\text{buffer})$, thickness of shell, sensitivity of instrument to determine protein loading, sensitivity of instrument to determine protein loading.

[0314] A flow diagram for an example using loaded LNPs and loaded LNPs with different amounts of antibodies is shown in FIG. 86. Steps include (i) illuminating with 1 or more excitation light beams of selected wavelength(s), (ii) collecting light in a plurality of scatters and FL channels, and (iii) extracting MFI data for unstained and stained samples. In some cases, an algorithm and input of loaded size and RI is used to calculate thickness of the shell and shell RI comprising calculating $\Delta RI = RI(\text{shell}) - RI(\text{buffer})$ and Thickness of the shell is used to determine Sensitivity of the instrument to determine protein loading. In some cases, algorithm and input of stained shell thickness and shell RI is used to calculate unstained size and RI. In some cases, algorithm and input of stained shell thickness and shell RI is used to calculate shell RI and unstained RI. In some cases, algorithm and input of stained shell thickness and shell RI is used to calculate shell thickness and unstained size.

[0315] A histogram of PBS buffer at gain 200 with identification of noise as baseline (Left panel, noise 90.68%, P2 (5.44%)), a histogram of empty LNP sample (middle panel, noise 8.42%, P2 (88.66%)), and a histogram of eGFP-encoded mRNA loaded LNP sample (right panel, noise 4.47%, P2 (94.7%)) is shown in FIG. 7.

[0316] Four histogram plots for mRNA-loaded LNP modified with no, low, medium, and high amounts of monoclonal antibody (mAb), respectively, on the LNP surface is shown in FIG 8A, from left to right. Upper right cartoon depicts models of RNA-loaded LNP (left), and RNA-loaded LNP modified with mAb (right).

[0317] Dot plots of raw data for mRNA-loaded LNPs modified with no, low, medium, and high amounts of monoclonal antibody (mAb) is shown in FIG. 8B. The 2D plots using RSSC-H versus VSSC1-H, are shown from left to right: mRNA-loaded LNP (surface naked LNP), mRNA-loaded LNP with low mAb, mRNA-loaded LNP with medium mAb, mRNA-loaded LNP with high mAb.

[0318] The various embodiments described above are provided by way of illustration only and should not be construed to be limiting in any way. Various modifications can be made to the embodiments described above without departing from the true spirit and scope of the disclosure.

EXAMPLES

Example 1: Characterization of Lipid Nanoparticles using Flow Cytometry.

[0319] The following example describes a flow cytometry workflow utilizing the Beckman Coulter CytoFLEX nano flow cytometer to characterize LNPs. In embodiments, this technique is useful for academic and biopharmaceutical laboratories, in conjunction with orthogonal techniques, as a quick measure of LNP quality.

[0320] LNP1-empty and LNP1-eGFP (LNP loaded with encapsulated mRNA encoded *E. coli* Green Fluorescence Protein (eGFP))(available from Cytiva) were diluted 1:200 in filtered phosphate buffered saline (PBS). These samples were analyzed by a CytoFLEX nano flow cytometer (available from Beckman Coulter Life Sciences). PBS only LNPs were used as a control. Data was collected and further analyzed using Kaluza software (available from Beckman Coulter Life Sciences).

[0321] The LNP1-empty and LNP1-eGFP were added to 96-well plates in a range of 1 – 10 μ L per well (100 – 30000 ng mRNA/well). Referring to FIG. 7, the LNP1-empty were analyzed using the CytoFLEX nano flow cytometer under the conditions disclosed herein. Still referring to FIG. 7, the full LNPs containing mRNA encoded eGFP were analyzed using the CytoFLEX nano flow cytometer under the conditions disclosed herein. FIG. 7 reveals that the CytoFLEX nano can quickly distinguish the differences between empty and full LNPs and mRNA loaded LNPs. The LNPs with higher scatter had higher transduction percents. The volumes of LNPs added to each well shown in FIG. 7 were 0 μ L, 3 μ L, and 10 μ L.

[0322] mRNA-loaded (LNPs) that are modified with varying amounts of monoclonal antibodies (mAbs) on their surfaces were analyzed using CytoFLEX nano flow cytometer under the conditions disclosed herein. Referring to FIG. 8A, the data is presented in histograms located in the bottom panel. Referring to FIG. 8B, the data is presented in dot plots. Both FIG. 8A and 8B depict the following samples from left to right: mRNA-loaded LNP, mRNA-loaded LNP with low amounts of mAb modification, mRNA-loaded LNP with medium amounts of mAb modification, and mRNA-loaded LNP with high amounts of mAb modification. The top panel illustrates

models of RNA-loaded LNPs and RNA-loaded LNPs modified with mAbs. The results clearly show the differences in the light scattering properties between these LNP samples modified with various amounts of mAb on the surface. Side scatter measurements using CytoFLEX nano flow cytometer, combined with the proposed models, were used to fit the data into the Mie theory model, enabling the calculation of the refractive indexes for each sample. This process was crucial for understanding the optical properties and behavior of the LNPs under different conditions, thereby aiding in the optimization of their design and functionality for therapeutic applications.

Example 2: Characterization of Events Per Second (EPS) Stabilization at Different Sample Flow Rates

[0323] The CytoFLEX nano flow cytometer allows three different sample flow rates (1 μ l/min, 3 μ l/min, and 6 μ l/min) to select from, and also provides an option to customize the flow rate in 1 μ l/min increments between the lowest (1 μ l/min) and the highest (6 μ l/min). Having adjustable sample flow rate permits the accommodation of a range of biological nanoparticle concentrations in a sample.

[0324] The characterization of the sample flow (EPS) of biological samples of different concentrations when acquired at different sample flow rates identifies a sample flow rate of 3 μ L/min for biological sample acquisition is recommended.

[0325] HEK293T Fluorescent Microvesicles and Super Folder Green Fluorescent Protein (sfGFP) samples were used for the analysis.

[0326] HEK293T Fluorescent Microvesicles were prepared as 1:250 (2.12×10^3 p/ul), 1:500 (1.06×10^3 p/ul) and 1:1000 (0.5×10^3 p/ul) dilution using 20nm filtered phosphate buffered saline (PBS). Samples were acquired on the CytoFLEX nano at three flow rates: 1ul/min, 3 ul/min and 6ul/min.

[0327] sfGFP was rehydrated in 100 μ l of 30nm W-8 water (Thermo) and diluted in 20nm filtered PBS. sfGFP was tested at concentration of 3.8×10^4 p/ul and flow rate of 3 ul/min.

[0328] The CytoFLEX nano was set to VSSC1 at Gain 100 and Threshold (TH) 360 for VSSC1-H. Data was collected by following below steps: Step 1: Run on board clean with 10 back and forward flushes. Step 2: Check baseline with baseline monitor using sample setting. Step 3: Run sample at defined concentration at the desire sample flow rate for 10min. Repeat step 1 to 3 for each combination (sample flow rate and

concentration). The number of events were gated and segmented for each minute of the 10 min acquisition from each run, for analysis.

[0329] Gate was draw at 1 min increment over the 10 min Time plot per visual evaluation. A gate for the GFP positive population was drawn per B531H vs VSSC1H plot. The GFP positive events were shown as a different color from all events within each 1 min increments. For example, FIG. 9 discloses the HEK293T Microvesicles where the number of events per minute were analyzed as the percentage to that of the first minute.

[0330] Different dilutions of the HansaBioMed Fluorescent Microvesicles were run at different sample flow rates: 1ul/min, 3ul/min and 6ul/min. Percent event recovery of each 1 min in the 10 min acquisition in comparison to the first min was calculated and the average of the 3 replicates are summarized in FIG. 10 through FIG. 17.

[0331] FIG. 10 and FIG. 11 disclose time plots of the triplicates run for HEK293T Microvesicles at 1:1000 dilution ($5.3 \times 10^5 \text{ p/mL} = 530 \text{ p/ul}$) at 1ul/min, 3ul/min and 6ul/min (columns). The bar graphs and data table are the average of the three replicates. Referring to FIG. 10 and FIG. 11, at 1uL/min, it took about 3 minutes to reach sample flow rate stability. 25% of total events and 50% of GFP positive events were lost. At 3uL/min, it took about 1 minute 20 seconds to reach stable sample flow. 15% of total events and 30% of GFP positive events were lost. At 6uL/min, the sample flow rate showed inconsistent patterns between replicates and never reached a real stable state: stable right after boost for replicate 2 and 3 while replicate 1 showed a dip first followed by a increase for the first 40 seconds before reaching stable flow rate. And all replicates showed dramatic decrease about 5 minutes 20 seconds after start. Referring to FIG. 10, grey in the time plot (top band) corresponds to all events while green (bottom band) is the GFP positive, and blue (middle band) is the noise peak. The GFP positive particles follow the same EPS decrease trend. 25% of total events and 50% of GFP positive events were lost. 3ul/min showed better events recovery and more desirable sample flow behavior.

[0332] FIG. 12 and FIG. 13 disclose time plots of the triplicates run for HEK293T Microvesicles at 1:500 dilution at 1ul/min, 3ul/min and 6ul/min. FIG. 12 and FIG. 13 are the average of the three replicates. Referring to FIG. 12 and FIG. 13, running the HEK293T Fluorescent Microvesicles at 1:500 ($1.06 \times 10^6 \text{ p/mL} = 1060 \text{ p/ul}$), resulted in similar sample flow behavior as that with 1:1000 dilution (except at 6uL/min). At 25%

of total events and 50% of GFP positive events were lost. At 1 μ L/min and 6 μ L/min about total events and 50% of GFP positive events were lost, where at 3 μ L/min about 10-13% of total events and 30% of GFP positive events were lost. 3 μ L/min showed better events recovery and more desirable sample flow behavior.

[0333] FIG. 14 and FIG. 15 disclose time plots of the triplicates run for HEK293T Microvesicles at 1:250 (2.12×10^6 p/mL=2120 p/ul) dilution at 1 μ L/min, 3 μ L/min and 6 μ L/min. FIG. 14 and FIG. 15 are the average of the three replicates. Referring to FIG. 14 and FIG. 15, which discloses similar sample flow behavior as that with the 1:1000 and 1:500 dilutions disclosed in FIG. 10 through FIG. 13. At 1 μ L/min about 30% of sample is lost, where at 3 μ L/min about 8-10% and at 6 μ L/min about 23%. For GFP positive particles (B531-H graph and data) at 1 μ L/min about 50% of sample was lost, where at 3 μ L/min about 30%, and at 6 μ L/min about 26%. 3 μ L/min showed better events recovery and more desirable sample flow behavior.

[0334] FIG. 16, FIG. 17, and FIG. 18 disclose sfGFP viral particle testing at 1 μ L/min and 3 μ L/min, as sample control. Referring to FIG. 16 and FIG. 17, the data analysis is based on all events and GFP positive (B531-H) events. The gating strategy is disclosed in FIG. 16. Referring to FIG. 17 and FIG. 19, sfGFP 1:500 at 1 μ L/min, the sample flow stability is not achieved until almost 6 minutes after the recording started. At 3 μ L/min, the sample flow rate reached stability at about 2 minutes after the recording started. The GFP positive particles follow the same EPS decrease trend.

[0335] In comparison to the above HEK293T Microvesicles, sfGFP sample flow rate stability may take a little longer. It is recommended that user evaluate and select the appropriate sample rate for their sample needs.

[0336] In this example, flow rates were run 1 μ L/min (slow), 3 μ L/min (medium), and 6 μ L/min (fast), and the data demonstrates that the flow rate is adjustable.

[0337] Conclusion: The sample flow rates of 1 μ L/min, 3 μ L/min and 6 μ L/min were tested using biological samples at different concentrations. 3 μ L/min is the suggested sample flow rate for biological samples, based on the event recovery, EPS stabilization time, and consistency of the sample flow overtime.

[0338] The 1 μ L/min sample flow rate has the highest EPS decrease and with increasing concentration it becomes more prominent as the percentage of EPS at stability is lower than the less concentrated samples. The 3 μ L/min has the lowest EPS decrease.

Increasing concentration for this sample shows the lowest decrease with a stable and

sustained time plot. 6ul/min has EPS decrease starting after 5 min, with a similar trend between different sample concentrations.

Example 3: Volumetric Counting of Biological Particles using Flow Cytometry

[0339] The CytoFLEX nano flow cytometer can count nanoscale particles, including biological nanoscale samples. . This Test Case is to evaluate the precision performance, repeatability, and reproducibility, when testing biological nanoscale samples. The preliminary acceptance criteria were assessed and proposed based on the actual precision performance.

[0340] This example was performed first with all Aborts enabled using both V5 virus and Daudi MV samples for 5 days, then repeated using the V5 virus sample for 3 days and with 2 Aborts disabled.

[0341] The following samples were prepared fresh for an AM run (morning run) and again for a PM run (afternoon run). AM and PM runs were at least 2 hours apart. v5 Virus was stained with anti-v5 PE and diluted post staining to 1:1000 using 20nm-filtered PBS buffer. Daudi MV was stained with CD81 PE and diluted post staining to 1:500 using 20nm-filtered PBS buffer.

[0342] The CytoFLEX nano was set to the following settings. (1) for Virus v5: VSSC1 at gain50; VSSC1-H at th 250; and Y535 at gain 2000. (2) for DAUDI MV: VSSC1 at gain 100; VSSC1-H at th350; and Y535 gain 1000.

[0343] Data was collected in 2 Acquisition/Loading modes/status: Mode 1 - one load for 4x acquisitions; and Mode 2 - load and unload for each replicate, repeat 4x.

[0344] This example was designed to account for the variability from days, runs per day, instruments, and loading status and repeatability (within a run). In this example, the test was repeated for 5 days (with Aborts enabled), 2 runs per day (AM and PM with >2 hours gap in between), 4 replicates for each run on 2 instruments as illustrated as follows for each day. 1. startup-clean and QC the instrument. 2. Check baseline with baseline monitor; run 4 replicates of DAUDI MV stained with CD81 PE (with load and unload) – 10 min each. Run 4 replicates of v5 virus stained with v5 PE. These are the AM runs. 3. On board clean 1xBFF. 4. Check baseline with baseline monitor. 5. Wait at least 2 hours and run 4 replicates of DAUDI MV stained with CD81 PE (with load and unload) – 10 min each. Run 4 replicates of v5 virus stained with v5 PE. These are the PM runs. 6. On board clean 1xBFF. 7. Check baseline with baseline monitor. 8. Shutdown the instrument.

[0345] The test was repeated using v5 virus sample for 3 days (with the 2 Aborts disabled), 2 runs per day (AM and PM with >2 hours gap in between), 4 replicates for each run on 2 instruments as illustrated above for the virus.

[0346] Gating strategy and Data Analysis. V5 virus gating strategy: P1 for all non-noise particles per VSSC1-H; V5PE gated on the V5 positive virus particles as disclosed in FIG. 19. MV gating strategy: P1 for all non-noise particles per VSSC1-H; MVPE gated on the CD81-PE positive MV particles as disclosed in FIG. 20.

[0347] Data collected with Abort enabled was first analyzed: Variance components were estimated for all source of variability based on a nested structure. Source of variability were between days, between runs, between instruments, between loading status and repeatability (within run). Standard deviation (SD) and coefficient of variations (%CV) were calculated from variance component estimates.

[0348] Table 1A and Table B summarizes the variance analysis results for the virus samples stained with V5: The particle count / μ l in P1 gate. The particle count/ μ l of Peak 1. The particle count/ μ l of Peak 2. The particle count/ μ l of V5 positive virus. The repeatability of all populations was about 10%, and reproducibility (Total Variability) < 20%, met the preliminary specifications. All factors contributed to the total variability except for loading status only as to the P1 events.

Table 1A: Variance analysis results for virus samples stained with V5.

V5Virus			Repeatability		Between Runs		Between Days	
Name	N	Mean	SD	CV%	SD	CV%	SD	CV%
P1_particle_uL	80	21420.30	2302.786	10.75	2066.156	9.65	0.000	0.00
Peak_2_particle_uL	80	2080.48	195.403	9.39	136.379	6.56	57.901	2.78
Peak1_particle_uL	80	2706.16	228.274	8.44	240.839	8.90	0.000	0.00
V5_PE_uL	80	1550.19	158.570	10.23	133.046	8.58	93.991	6.06

Table 1B: Variance analysis results for virus samples stained with V5.

V5Virus			Between Instrument		Between Status		Total Variability	
Name	N	Mean	SD	CV%	SD	CV%	SD	CV%
P1_particle_uL	80	21420.30	363.377	1.70	267.720	1.25	3126.586	14.60
Peak_2_particle_uL	80	2080.48	46.289	2.22	0.000	0.00	249.553	11.99
Peak1_particle_uL	80	2706.16	0.000	0.00	0.000	0.00	331.832	12.26
V5_PE_uL	80	1550.19	39.527	2.55	0.000	0.00	230.744	14.88

[0349] Table 2A and 2B summarizes the variance analysis results for MV samples stained with CD81-PE. The particle count/ μL in P1. The particle count/ μL of CD81+ MV particles.

Table 2A: Variance analysis results for MV samples stained with CD81-PE.

MV CD81-PE			Repeatability		Between Runs		Between Days	
Name	N	Mean	SD	CV%	SD	CV%	SD	CV%
P1_particle_uL	80	19612.71	2172.823	11.08	3506.856	17.88	1424.303	7.26
MV_PE_particle_uL	80	709.14	86.714	12.23	157.449	22.20	184.197	25.97

Table 2B: Variance analysis results for MV samples stained with CD81-PE.

MV CD81-PE			Between Instrument		Between Status		Total Variability	
Name	N	Mean	SD	CV%	SD	CV%	SD	CV%
P1_particle_uL	80	19612.71	1474.910	7.52	1146.160	5.84	4747.303	24.21
MV_PE_particle_uL	80	709.14	139.071	19.61	0.000	0.00	292.538	41.25

[0350] The repeatability of both populations was $> 10\%$, and reproducibility (Total Variability) $> 20\%$, up to 40% for the positive MVs. All factors contributed to the total variability especially within run (repeatability), between days, runs and instruments. Analysis led to the following causes: 1. Low MV-CD81-PE+ particles in the sample. The lower the particle counts, the higher the variability may be. 2. This sample is not as stable as the virus sample. There was loss due to stability during sample handling and analysis, e.g., degradation over time, event loss due to absorption, dilution, etc.

[0351] A second test (re-test) was performed with the Overlap and Over length Abort functions disabled. V5 viral particle was used to exclude the impact of sample instability. Samples were stained with anti-v5 in PE by following the same protocol as above for 3 days.

[0352] FIG. 21 shows an example of different fractions obtained by size exclusion chromatography (SEC) of blood sample (after centrifugation and filtration). Data was analyzed as shown in FIG. 21 and Table 3 to visualize the distribution of the event counts at different conditions (FIG. 21) and to calculate the corresponding %CV to quantify the variability, for between days, runs, instruments, loading status and replicates (Table 3).

Table 3: Corresponding %CV.

Load Status	Instrument	Test Day	Time	V5-PE/uL (CV%)
LoadUnload	MP8006 (CytoFLEX nano)	Day 1	AM	4.55
			PM	3.99
			All	19.50
		Day 2	AM	5.37
			PM	2.40
			All	29.44
		Day 3	AM	2.51
			PM	27.58
			All	18.43
		All	All	24.34
	MP8007 (CytoFLEX nano)	Day 1	AM	7.83
			PM	6.70
			All	14.65
		Day 2	AM	7.38
			PM	4.48
			All	16.42
		Day 3	AM	4.98
			PM	17.02
			All	11.56
		All	All	20.00
	All	All	All	22.04
wo/ Unload	MP8006 (CytoFLEX nano)	Day 1	AM	14.13
			PM	15.44
			All	21.00
		Day 2	AM	16.67
			PM	17.66
			All	17.74
		Day 3	AM	11.90
			PM	14.51
			All	15.27
		All	All	20.40
	MP8007 (CytoFLEX nano)	Day 1	AM	10.64
			PM	10.31
			All	22.32
		Day 2	AM	15.25

Load Status	Instrument	Test Day	Time	V5-PE/uL (CV%)
			PM	14.70
			All	13.92
		Day 3	AM	10.86
			PM	14.57
			All	15.90
		All	All	18.87
	All	All	All	19.68
All	All	All	All	20.78

[0353] Wo/unload showed higher variability than Load/unload, like in the precedent test and it was also observed for other samples, which is the nature of the loading modes. PM tests showed higher variability than in AM in particle recovery in all days. Investigation found that the same stained samples were used for AM and PM without washing step to pause the staining after staining and the reaction was ongoing until the PM time point, resulted higher variability. Repeatability was based on the AM data, all <15%; Reproducibility of all levels were within 30%.

[0354] The final precision specifications for volumetric counting based on biological particle test data: Repeatability: 20%; Reproducibility for total variability: 30%.

Example 4: Detection and Characterization of Recombinant Extracellular Vesicles (rEVs) using Flow Cytometry

[0355] This example demonstrates the use of a CytoFLEX nano for immunophenotyping of extracellular vesicles. More specifically, the CytoFLEX nano is used to detect biological particles when stained with antibodies labeled with BV421/PB, FITC, PE, APC, AA700 or AA750, with triggering on VSSC1 or FL channels.

[0356] For Example 4 and Example 5, recombinant extracellular vesicles sigma (rEV Sigma, expressing green fluorescent protein (GFP) on their membrane surface), platelet poor plasma extracellular vesicles (PPP EVs), HansaBioMed HEK293 Exosomes, and HansaBioMed HEK293 Microvesicles were stained with the following antibodies and/or lipid dyes as disclosed in Table 4.

Table 4: Biological samples and staining combinations for use in Example 4 and/or Example 5.

Samples	Antibodies/ Lipid Dyes
rEV Sigma	Staining combos: CD81 APC 2ug/mL + GFP label CD81 PB 2ug/mL+ GFP label CD81 PE 2ug/mL + GFP label CD81 APC 2ug/ml + GFP label + vFRed 1x CD81 PB 2ug/mL + GFP label + vFRed 1x
Platelet Poor Plasma (PPP) EVs	Staining Combo: CD9 FITC (BEC) 1ug/mL CD81 PB (BEC) 1ug/mL CD63 APC (Biolegend) 1ug/mL CD61 PC7 (BEC) 1ug/mL CD235a PE (BEC) 1ug/mL
HansaBioMed HEK293 Exosomes	Staining Combo: CD81 APC 2ug/mL and vFRed lipid dye 1x (±GFP label) and CD81 PB 2ug/mL and vFRed lipid dye 1x (±GFP label)
HansaBioMed HEK293 Microvesicles	Staining Combo: CD81 APC 2ug/mL and vFRed lipid dye 1x (±GFP label) and CD81 PB 2ug/mL and vFRed lipid dye 1x (±GFP label)

[0357] In the first round of characterization, two different samples were characterized. The first sample is Sigma rEV stained with CD81 TAPA clone in three different colors (PB, PE and APC) and also stained with vFRed lipid dye alone or in combination with CD81 PB or CD81 APC. The second sample is PPP EV prepared from fresh human blood and then stained with the antibody panel published in Brittain, G.C., Chen, Y.Q., Martinez, E. et al. A Novel Semiconductor-Based Flow Cytometer with Enhanced Light-Scatter Sensitivity for the Analysis of Biological Nanoparticles. Sci Rep 9, 16039 (2019). <https://doi.org/10.1038/s41598-019-52366-4>.

[0358] In FIG. 22 are the plots for PBS control to show baseline, and rEV-GFP unstained but with GFP label (B531). FIG. 22 discloses an embodiment of the present disclosure demonstrating the detection and characterization of recombinant

extracellular vesicles (rEVs) using flow cytometry. More specifically, FIG. 22 discloses the plots for PBS control to show baseline, and a commercially available rEV-GFP unstained but with GFP label (B531). On the left, the acquisition settings being used for gain include FSC =100, VSSC1= 50, VSSC2 =200, BSSC =100, YSSC =100, RSSC =100, V447 =1000, B531 =1000, Y595 = 1000, R670 = 1000, R710 = 1000, and R792 = 1000, Y595 = 1000, R670 = 1000, R710 =1000, and R792=1000. The first 2 rows are from PBS (sample buffer =blank). The bottom 2 rows are from rEV diluted at 1:1000, which is the dilution selected as best after titration. The rEVs are green endogenously so on the right plots for the B531 channel for both PBS and sample are shown.

[0359] rEV was successfully stained using single staining with CD81 PB, PE, APC and vFRed and detected on CytoFLEX nano instrument. In the FIG. 23, the first row is unstained rEV, the second row is rEV stained with vFRed lipid dye, and in the third row a gating where the Y595-H vs VSSC1-H plot has been drawn to show a dimmer and a brighter population for vFRed positive EVs. Second and third row shows single staining with vFRed from Cellarcus, which is a lipid dye and suppose to bind all EVs. The dimmer population consists of small EVs.

[0360] In FIG. 24, single staining for rEVs is shown. The first row is unstained rEV, so only the B531 population is present. The second row is single stained rEV with CD81 PB in pacific Blue (channel V447). The third row is single staining for CD81 PE in PE (channel Y595). The 4th row is single staining for CD81 APC in APC (channel R670). All these stainings have been compensated and show the presence of a dimmer and a brighter population positive for CD81. vFRed staining results in positive signal in the Y595 and R670 channels. CD81 PB staining results in positive signal in the V447 channel. CD81 PE staining results in positive signal in the Y595 channel. CD81 APC staining results in positive signal in the R670 channel. The three CD81 antibodies are the same clone (TAPA-1) it shows a slightly difference in sensitivity in the different channel (to consider is also the different F/P ratio and steric hindrance between them).

[0361] In FIG. 25, rEV was successfully stained using combination of the vFRed lipid dye and CD81-PB. Unstained rEV is shown in the top row. Sequential rEV staining with vFRed and CD81 PB is shown in the second row. CD81 PB staining are the positive events in the V447 channel while vFRed positivity is observed in the Y595 and R670 channels. As single color staining control, rEV stained with vFRed is shown in row 3. vFRed positivity is observed in the Y595. vFRed positive events are also

positive in the R670 channels (data not shown). As single color staining control, rEV stained with CD81 PB is shown in row 4. CD81 PB positivity is observed in the V447 channels.

[0362] In FIG. 26, rEV was successfully stained using combination of the vFRed lipid dye and CD81-APC. Unstained rEV is shown in the top row. Sequential rEV staining with vFRed and CD81 APC is shown in the second row. CD81 APC staining should be in the positive events in the R670 channel but vFRed positivity is observed in the Y595 and also in the R670 channels. The dual CD81 APC and vFRed staining does not show a distinct CD81 APC population above the vFRed positive population. This is due to the dimness of the CD81 APC positive signal that could not be resolved from the vFRed positive signal. In addition, compensation was not performed to account for the vFRed signal spillover to the R670 channel.

[0363] In FIG. 27, the antibodies were run at the same dilutions as above to evaluate the contribution of the antibody aggregates, that can affect the sample staining evaluation. The PBS, antibody (Ab) only, and unstained sample columns inform the gating strategy for the single color positivity on the Y axis, that is, V447 channel for CD81 PB, Y595 channel for CD81 PE, R670 channel for CD81 APC, and Y595 channel for vFRed. The numbers shown in each plot are the events within the gate and show that these events are not contributing greatly to the positive population in the evaluation. The highest number of antibody aggregates is CD81 PE antibody but it only contributes to less than 2% ($222/11531 = 1.9\%$) of the total positive event count. As a single color staining control, rEV stained with vFRed is shown in row 3. vFRed positivity is observed in the Y595 and R670 channels. As a single color staining control, rEV stained with CD81 APC is shown in row 4. CD81 PB positivity is observed in the R670 channels.

[0364] FIG. 28 shows antibodies background control: antibodies only were treated as sample and diluted same way to identify antibodies aggregates if present. First row is a combination of CD81 PB and vFRed and second row CD81 APC with vFRed. First two columns are the channel for those fluorochromes, third and fourth columns is the comparison with the unstained sample in those channels, last 2 columns are the sample stained and results in those channels. In FIG. 28, the antibodies and vFRed were run at the same dilutions as above to evaluate antibody aggregates and vFRed aggregates contribution to overall analysis. The 2 Ab only (PB or APC on the left column and

vFRed on the right column), and unstained sample columns inform the gating strategy for the dual color positivity on the Y axis, that is, V447 channel for CD81 PB, Y595 channel for vFRed, R670 channel for CD81 APC and vFRed. The numbers shown in each plot are the events within the gate and show that these events are not contributing greatly to the positive population in the evaluation. The highest number of aggregates is vFRed in the CD81 APC + vFRed staining. The contribution of the vFRed aggregates is about 4.2% ($515/12270 = 4.2\%$) of the total positive event count. The compensation matrix generated for the dual color staining is shown in Table 5. This compensation matrix was applied to all of the dual color staining.

Table 5: Compensation matrix for dual staining.

Compensation Matrix - rEV cd81 pb+ vFRed gain 1000							
☒ Use ☒ Show Autofluorescence							
Autofl.	Channel	-V447%	-B531%	-Y595%	-R670%	-R710%	-R792%
0.00	V447		0.00	0.00	0.00	0.00	0.00
0.00	B531	19.74		18.02	0.00	0.00	0.00
0.00	Y595	0.00	6.32		0.00	0.00	0.00
0.00	R670	0.00	0.00	0.00		0.00	0.00
0.00	R710	0.00	0.00	0.00	0.00		0.00
0.00	R792	0.00	0.00	0.00	0.00	0.00	

[0365] Conclusion for rEV characterization in Example 4. CD81 marker label a positive population in PB, PE and APC with comparable percentages for PE and APC, respectively 37.23% and 37.7%, and slightly lower in PB, as 15.94%. vFRed lipid dye shows a 54.55% positivity for this sample. Sequential staining allows combining the antibody with the lipid dye staining, even if in the APC channel there is some spillover from the dye. Looking at the single staining the contribution of the vFRed into CD81 APC staining is equal to 4.2%. Accordingly, a double staining in combination with the green label present, for a total of 3 colors, can be characterized by the CytoFLEX nano.

Example 5: Detection and Characterization of Extracellular Vesicles (EVs) from Platelet-Poor Plasma Samples using Flow Cytometry

[0366] Like Example 4, this example demonstrates the use of a CytoFLEX nano for immunophenotyping of extracellular vesicles. More specifically, the CytoFLEX nano is

used to detect biological particles when stained with antibodies labeled with BV421/PB, FITC, PE, APC, AA700 or AA750, with triggering on VSSC1 or FL channels.

[0367] Platelet-poor plasma (PPP) EVs were prepared from fresh human blood as follows: Blood was centrifuged for 5 min at 200xg. 1mL of platelet-poor plasma (PPP) was removed from the top and filtered through a 200nm syringe. PPP was further purified using Izon size-exclusion columns (qEV70nm SEC column). Selected fractions were screened and used for single-color stain and multi-color stain for immunophenotyping. PPP were then stained with the following antibodies CD81 PB, CD9 FITC, CD63 APC, CD61 PC7 and CD235a PE. Samples were acquired on the CytoFLEX nano flow cytometer and analyzed in CytExpert nano.

[0368] FIG. 29 discloses example of different fractions of PPP EVs isolated from 1 sample using a qEV70nm SEC column. Fraction 12 was used for single-color stain and multi-color stain for immunophenotyping. The instrument setting and compensation matrix used for the multi-color staining is shown in FIG. 30.

[0369] FIG. 31 and FIG. 32 disclose single staining with CD81 PB, CD9 FITC, CD63 APC, CD61 PC7 and CD235a PE. FIG. 31 shows single staining of fraction 12. Top row is unstained fraction 12. Second row is fraction 12 stained with CD81 PB (CD81 is not present in every donor, here a donor with high CD81 was selected to show positivity). Third row is fraction 12 stained with CD9 FITC. Fourth row is fraction 12 stained with CD63 APC (usually negative or very low). For each of them the plots for all the combinations are involved, as control of crosstalk and appropriate compensation. PPP EV are usually positive for CD9, negative or slightly positive for CD63, some donors are positive for CD81 (like the one in FIG. 31), and some donors completely negative. Some fractions are positive for CD61 PC7, with a decreased number as SEC fraction increases, and negative for CD235a PE if the clean-up for platelet and red blood cells was efficient. FIG. 32 shows single staining of fraction 12 continuation. Top rows show fraction 12 stained with CD61 PC7. Bottom row shows fraction 12 stained with CD235a PE (decreasing with increasing fraction #). The bivariate plots associated with the antibody dye colors and their intended detection channels are outlined in red. The data disclosed in FIG. 31 and FIG. 32 is compensated.

[0370] FIG. 33 discloses multi staining of the same fraction 12 sample with the full panel of antibodies, diluted at the same dilution in a mix. In the top row single color is

shown for the cocktail sample. The bottom row marker shows combinations of biological importance.

[0371] It shows similar results to that obtained with a single color, with minor contribution by antibody aggregates or other background.

[0372] FIG. 34 discloses the antibody background between PBS buffer, Ab only, unstained sample, and single-stained sample. This is single antibody only control. Row 1: CD9 FITC, Row 2: CD81 PB, Row 3: CD63 APC, and Row 4: CD61 PC7. First column: PBS in those channels as control, Second column: antibodies only were treated as sample and diluted same way to identify antibodies aggregates if present, Third column: sample unstained as control, and Last column: sample fully stained for comparison. The PBS, Ab only, and unstained sample inform the gating strategy for gating positive populations. The numbers in each plot are the events within the gate. The events in the PBS, Ab only, and unstained samples contribute very little to the overall event count on the fully stained EV sample.

[0373] FIG. 35 discloses the antibody background between PBS buffer, Ab only, unstained sample and multiple-stained (Ab mix) sample. The PBS, Ab only, and unstained sample inform the gating strategy for gating positive populations. The numbers in each plot are the events within the gate. The events in the PBS, Ab only, and unstained samples contribute very little to the overall event count on the fully stained EV sample. First row: same as FIG. 34 for CD235a PE so is in single color. Last three rows are from the mix of all the antibodies together in each channel of interest: V447, B531, Y595, R670 and R792 to: 1st row: define contamination in each channel by each antibody if present, 2nd row: proper compensation show no positivity in sample unstained, and 3rd row: the real sample stained and the result.

[0374] Based on the size distribution and fluorescence staining of the fractions collected from the qEV 70nm and 35 nm SEC columns, identification of fractions where EVs reside was possible. CytoFLEX nano flow cytometer was able to detect and distinguish more than one FL signal on single PPP EVs particles, including PB, FITC, PE, PC7 and APC. The data demonstrates that at the proper antibody concentration and with the use of proper controls, antibody aggregates and antibody background interference is avoided. The data also demonstrates that the CytoFLEX nano was able to compensate for the spillover between different fluorescent dyes allowing analysis of the 5-color staining simultaneously.

Example 6: Detection and Characterization of Extracellular Vesicles (EVs) from Platelet-Poor Plasma Samples using Flow Cytometry.

[0375] The above characterization of PPP EVs in Example 5 was repeated, and different trigger options were evaluated. The same protocols were followed. For Size Exclusion Chromatography (SEC) both 70nm (qEV 70) and 35nm (qEV 35) cutoff columns were evaluated.

[0376] FIG. 36 and FIG. 37 disclose the data collected from unstained and stained samples with fraction 6 selected from qEV 70nm SEC column. Shown are single color staining with CD9 FITC, CD81 PB, CD63 APC, CD61 PC7 and CD235a PE and the same sample stained with the panel of antibodies (mix). Also shown is a histogram of VSSC1-H for fraction 6 that was stained to show EV size distribution. This fraction shows small and large EVs. FIG. 38 discloses the CytoFLEX nano settings and compensation applied for the data in FIG. 36 and FIG. 37.

[0377] FIG. 39 and FIG. 40 disclose the same sample evaluated with different triggers. B531 trigger, targeting CD9 positivity and R670 trigger, targeting CD61 PC7 positive population.

[0378] FIG. 41 and FIG. 42 disclose the data obtained with fraction 6 collected from the qEV 35nm SEC column. Shown are single color staining with CD9 FITC, CD81 PB, CD63 APC, CD61 PC7 and CD235a PE and the same sample stained with the panel of antibodies (mix). Also shown is a histogram of VSSC1-H for fraction 6 that was stained to show EV size distribution. This fraction shows small and large EV. An overlay of the fractions coming from the 2 different SEC columns is shown at the bottom right of the FIG. 42 for comparison. FIG. 43 discloses the setting and compensation applied to this sample, obtained with qEV35nm.

[0379] FIG. 44 and FIG. 45 disclose the same evaluate with different triggers. B531 trigger, targeting CD9 positivity and R670 trigger, targeting CD61 PC7 positive population.

[0380] Conclusions from the characterization of PPP EVs. The CytoFLEX nano can characterize up to 5 colors without interference from antibodies aggregates by performing proper controls. With compensation, the CytoFLEX nano can manage the crosstalk brought by colinear system. Fluorescent triggering helps focus on a specific population.

[0381] As with Example 5, based on the size distribution and fluorescence staining of the fractions collected from the qEV 70nm and 35 nm SEC columns, identification of fractions where EVs reside was possible. CytoFLEX nano flow cytometer was able to detect and distinguish more than one FL signal on single PPP EVs particles, including PB, FITC, PE, PC7 and APC. The data demonstrates that at the proper antibody concentration and with the use of proper controls, antibody aggregates and antibody background interference is avoided. The data also demonstrates that the CytoFLEX nano was able to compensate for the spillover between different fluorescent dyes allowing analysis of the 5-color staining simultaneously.

Example 7: Detection and Characterization of Recombinant Extracellular Vesicles (rEVs) using Flow Cytometry

[0382] The above characterization of rEVs in Example 4 was repeated using the same protocols. FIG. 46 discloses unstained GFP-labeled sample and FIG. 47 discloses the CytoFLEX nano settings used in this example.

[0383] FIG. 48 discloses single staining done with CD81 antibody targeting the same epitope of the tetraspanin membrane protein in three different colors: PE (first row), APC (second row) and PB (third row) and a single stain with vFRed lipid dye (fourth row).

[0384] FIG. 49 and FIG. 50 disclose the combination of CD81APC and vFRed, together with the GFP label that were evaluated with different triggers and compared with slight MFI changes. The events below the trigger is due to compensation from other dyes into the trigger channel.

[0385] FIG. 51 and FIG. 52 discloses the combination of CD81PB and vFRed, together with the GFP label evaluated with different triggers and compared with slight MFI changes. The events below the trigger is due to compensation from other dyes into the trigger channel.

[0386] The CytoFLEX nano offers multiple trigger options with the ability to analyze samples in multiple ways and isolate specific stained populations. This permits obtaining more information from the same marker of expression. The CytoFLEX nano can detect and distinguish more than one FL signal on single particles, including PB, FITC, PE and APC, and can detect and distinguish up to 5 color staining simultaneously without interference by antibody background noise.

Example 8: Characterizing heterogeneous EVs using a Flow Cytometer or Protein Loading on the Surface of a Biological Sample

[0387] Referring to FIG. 53, a flow cytometer (e.g., CytoFLEX nano) is used to characterize unstained EVs consisting of a heterogeneous population. For example, a population of unstained EVs are loaded into a CytoFLEX nano for characterizing using multiwavelength side scatter. The CytoFLEX nano successfully differentiates among the heterogeneous population of unstained EVs, e.g., Population #1 (64-99nm) and Population #2 (59-205nm).

Example 9: Characterizing Protein Loading on the Surface of a Biological Sample

[0388] Referring to FIG. 54, a flow cytometer (e.g., CytoFLEX nano) is used to characterize a heterogenous population EVs based on the different amount of protein on the surface of EVs within the population. For example, a heterogenous population of EVs are loaded into a CytoFLEX nano for characterizing using multiwavelength side scatter. The CytoFLEX nano successfully differentiates among the heterogeneous population of EVs based on different amount of protein on the surface of each EV.

Example 10: Characterize Drug Loading in a Liposome using a Flow Cytometer

[0389] Referring to FIG. 55, a flow cytometer (e.g., CytoFLEX nano) is used to characterize liposomes based on the amount of drug loaded into each liposome. For example, the flow cytometer characterizes liposomes based on the loaded biomolecules in each liposome because of the different refractive index.

Example 11: Characterize Expression Levels of a Biomolecule using a Flow Cytometer

[0390] Referring to FIG. 56, a flow cytometer (e.g., CytoFLEX nano) is used to monitor biomolecule expression levels on the surface of biological particles, e.g., antigen expression levels in vaccine development applications.

Example 12: Characterize Single Particle Surface Marker Interaction using a Flow Cytometer

[0391] Referring to FIG. 57, a flow cytometer (e.g., CytoFLEX nano) is used to characterize single particle surface marker interaction with its corresponding ligands: receptor–ligand, protein–DNA binding, etc.

Example 13: Characterize Less Abundant Biomarkers

[0392] Referring to FIG. 58, a flow cytometer (e.g., CytoFLEX nano) is used to identify less abundant biomarkers, e.g., for early detection of cancer, as illustrated in FIG. 58.

Example 14: Isolation of Single Cells Based on their Secretion using Heterofunctional Particles

[0393] A flow cytometer (e.g., CytoFLEX nano) is used in a vaccine response study to isolate H1 Influenza Virus (H1V) antibody secreting cells. The blood of a patient who has been vaccinated is collected. The B cells from the patient's blood are enriched and stained with anti-IgD, anti-CD3, anti-CD19, anti-CD38, anti-CD14. The antibody secreting cells are captured and separated from other B cells using beads. The isolated single H1V antibody secreting cells are separated from other antibody secreting cells using a flow cytometer (e.g., CytoFLEX nano) via FACS. The sorted H1V antibody secreting cells are incubated and then secretion validation is performed with ELISA.

Example 15: Isolation of Single Cells Based on their Secretion using Heterofunctional Particles

[0394] A flow cytometer (e.g., CytoFLEX nano) is used for the quantification of secreted proteins. Secreted protein from targeted cells is captured on beads using Janus particles, e.g., Janus particles are used to target Jurkat cells via anti CD3/38. The targeting of the Jurkat cells via anti-CD3/38 on the Janus particles triggers cell activation of the Jurkat cells resulting in secretion of IL-2 from the Jurkat cells. The secreted IL-2 from the Jurkat cells bind to the anti-IL-2 on the surface of the Janus particles, which is then bound by fluorescent anti-IL-2. The secreting cells are isolated between various levels of secretion, e.g., low secretor, medium secretors, and high secretors, using a flow cytometer (e.g., CytoFLEX nano) via FACS. The isolated populations of cells are expanded and secretion validation is confirmed via ELISA.

[0395] In another embodiment of Example 15, both IL-2 and VEGF secretion rates are used to isolated Jurkat cells.

[0396] Table 6 lists information for biological materials used in the Examples unless otherwise stated herein.

Table 6: Biological materials.

Name	Description	MFG	Cat #	Lot	Stock concentration and specs	Expiry
rEV	GFP recombinant EV obtained by HEK293T cells conditioned media	Sigma	SAE0193	0000232513	$\times 10^9$ p/vial and 70% fluorescence	May 2025
DAUDI MV	Microvesicles obtained by DAUDI cells conditioned media	HansaBioMed	HBM-MVDAUDI-50	250923	3.9×10^{10} p/vial	Sept 2026
PPP EVs	Platelet poor plasma EVs obtained from fresh blood (EDTA tubes)	Blood Service BEC	-	-	-	-
v5 virus	V5 tagged viral particles	ViroFLOW	-	V1001	6.8×10^8 p/vial	1 year post receiving if kept lyophilized
sfGFP virus	Super folder GFP viral particles	ViroFLOW	-	1002A	1.7×10^9 p/mL	1 year post receiving if kept lyophilized
HEK293T FL MV	GFP labeled Microvesicles obtained by HEK293T cells conditioned media	HansaBioMed	HBM-MVF-HEK	240123-2	5.3×10^9 p/ml	July 2023

[0397] The following staining protocols were used in the Examples unless otherwise noted.

[0398] V5 Virus staining:

- 6.8×10^8 p/vial
- Resuspend in 100ul W8 water = 6.8×10^6 p/ul
- Staining with anti-v5 PE (5ul of virus+5ul PBS)

- Rb pAb ab72480 Lot GR3304043-4
- 1ul Stock 0.1mg/mL +9ul PBS = 10ug/mL
- 4ul of 10ug/mL +6ul PBS= final Ab concentration 2ug/mL
- Mix ab and sample (sample final concentration: 1.7×10^6 p/ul)
- 1h RT and dilute 1:1000 (1:5000 final)

[0399] MV DAUDI MV staining:

- 3.9×10^{10} p/mL = 3.9×10^7 p/ul
- Titration tested 1:100 (3.9×10^5 p/ul), 1:1000 (3.9×10^4 p/ul), 1:5000 (7.8×10^3 p/ul) and 1:10000 (3.9×10^3 p/ul). And chose 1:5000
- 1:5 dilution to start with = 2ul+8ul PBS=10ul sample
- 1:2 with CD81 PE (at 2ug/mL)= 10ul sample+9ul PBS+1ul CD81PE at 20ug/ml
- CD81 PE is at 120ug/mL so 1.7ul of Ab+ 8.3ul PBS=CD81 PE at 20ug/mL
- Stain for 1h at RT in the dark
- Dilute 1:500 to read

[0400] rEV preparation and staining:

- Reconstitute one vial of rEVs with 100uL of ice-cold W-8 water.
- Pipet up and down to mix (no vortex)
- Aliquot 10uL into low binding Eppendorf tubes and store at -80C
- For the double staining:
 - Prepare the rEVs at 1:1000 (fix with concentration once I have the lot#)
 - Prepare vFRed: the stock is 100x in DMSO. Dilute 1ul in total of 10ul with vFRed diluent buffer, resulting into 10x working solution
 - Prepare 2x vFRed solution: 2ul of 10x vFRed working solution + 8ul 20nmfiltered PBS
 - Mix 2x vFRed with 10ul of rEVs
 - Stain for 1hour at room temperature in the dark
 - Add CD81 APC or CD81 PB at 2ug/mL final concentration

[0401] • For single staining:

- Prepare the rEVs at 1:1000 (fix with concentration once I have the lot#)

- Prepare CD81 APC, CD81 PB and CD81 PE in single tubes at 4ug/mL in a total of 10µl in PBS 20nm filtered
- Mix each tube with 10ul of rEVs
- Stain for 1hour at room temperature in the dark
- Dilute at concentration and run
- Run at 3ul/min for 2 min

[0402] PPP EVs staining:

- Spin the amount of antibody need (+2) in a microfuge at max speed for 10min at RT

- Prep antibodies at 2ug/ml in a total of 25ul PBS 20nm filtered
- Incubate with sample: 25ul PPP EV and 25ul antibody
- Incubate all samples 1h at RT
- Dilute 1:500 (if your final is 1:1000) in PBS 20nm filtered
- Run until time plot is table and then record 1 minute

[0403] The following PPP EV preparation protocol was used with fresh blood unless otherwise noted.

- Centrifuge whole blood (Human whole blood into K3-EDTA vacutainer) at 160g for 5 min RT

- Collect supernatant and label as “PPP”; avoid collection of platelet-rich plasma near the WBC layer

- Filter PPP fraction with 0.2µm filter using a 5ml syringe, to eliminate residual platelets and large particles

- Pass filtered PPP through Izon qEV single SEC columns following manufacture IFU to cut off to eliminate abundance of proteins and lipoproteins below 70nm

- Columns were flushed with 2mL of 1X PBS
- The 150-200µl of PPP was applied
- 200µl fractions were collected for subsequent analysis
- Test fraction 4-9 by running the fractions on the CytoFLEX nano; this step will decide the fraction to stain, and which will be the final dilution to run at the instrument

- The fraction is selected based on:

- A good amount of EVs. Perform a minimum dilution of 1:200 after staining to minimize antibody background noise and minimize swarming; a good dilution post staining is between 1:200 to 1:1000
- Representation of different EV sizes, including small and large EVs.

Example 15: Flow Cytometer instrument calibration to define Calibration Factor

[0404] A method of instrument calibration to define calibration factor based on identified half angle is illustrated in FIG. 65. Empirical data collection is obtained from a plurality of polystyrene size reference beads having known size and RI. The graphs in the top row show violet VSSC1 and VSSC2 histograms for two sets of polystyrene size reference beads including a low size set of nominal sizes 40 nm, 80 nm, 103 nm, and 141 nm sizes; and a high size set of nominal sizes 141 nm, 304 nm, 600 nm, and 1020 nm. (nanoViS Nanoscale sizing standards, D03231, Beckman Coulter, Inc.) The Tables at top right show nominal reference bead size, CV% and RI at four wavelengths 405, 488, 561, and 633 nm. Next, the half angle is defined per each instrument for three instruments MP03, MP05 and MP07. The gains are independent, the wavelength is independent, and the particle size is independent variable. The graphs in middle row show confidence of fit % (goodness of fit) as a function of scattering angle; the half collection angle is defined at the highest goodness of fit. Next, the calibration factor is defined based on identified half angle. Gains are dependent, wavelength is dependent, and particle size is dependent. The table at bottom shows averaged calibration factors over all bead sizes at different wavelengths of scatter for VSSC1 (violet), BSSC (blue), YSSC (yellow), and RSSC (red) for three flow cytometers MP05, MP07, and MP03, Table 7.

[0405] Table 7: Calibration Factors for three Flow Cytometer at four wavelengths of scatter

Instrument	BSSC	RSSC	VSSC1	YSSC
MP05	7.45E+09	1.31E+10	9.6E+09	3.97E+09
MP07	5.84E+09	7.68E+09	7.23E+09	4.39E+09
MP03	5.56E+09	6.66E+09	3.79E+09	3.94E+09

[0406] All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While

the compositions and methods of this disclosure have been described in terms of the foregoing illustrative embodiments, it will be apparent to those of skill in the art that variations, changes, modifications, and alterations may be applied to the composition, methods, and in the steps or in the sequence of steps of the methods described herein, without departing from the true concept, spirit, and scope of the disclosure. More specifically, it will be apparent that certain agents, additives, and ingredients that are similar according to their physical, chemical, physiological, and/or gustative properties may be substituted for the agents, additives and ingredients described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope, and concept of the disclosure as defined by the hereinafter appended claims.

[0407] The following numbered clauses define further example aspects and features of the present disclosure:

[0408] Clause 1. A method of characterizing particles in a flow cytometer, the method comprising: illuminating the particles with one or more excitation light beams at one or more wavelengths as the particles individually pass through an interrogation zone; collecting side scattered light in a plurality of channels at a plurality of wavelengths from the particles passing through the interrogation zone; identifying the mean side scattered light intensity in each of the plurality of channels; comparing the mean side scattered light intensity in each of the plurality of channels to an average calibration factor determined in the same flow cytometer from a plurality of standard particles having known refractive indices and sizes; characterizing the particles based on the side scattered light collected in the plurality of channels; inputting one or more known parameters; inputting particle initial guess parameters and parameter bounds, and optionally parameter constraints; and calculating unknown parameters that minimize loss function and satisfy the bounds and constraints.

[0409] Clause 2. The method of clause 1, wherein the average calibration factor is determined comprising illuminating at one or more wavelengths calibration beads having known size and refractive index; collecting side scattered light from all wavelengths of interest; calculating forward Mie scattering profiles for each input calibration bead diameter; integrating Mie scattering profiles for each calibration bead diameter; calculating calibration factor for each calibration bead size; calculating goodness of fit for current half angle; reporting collection half angle with highest

goodness of fit; calculating Mie scattering profile at half angle identified in previous step; and calculating the average calibration factor and CV over all calibration bead sizes for half collection angle from previous step.

[0410] Clause 3. The method of clause 2, wherein the integrating Mie scattering profiles is performed over $90 \text{ deg} \pm \text{current collection half angle}$ or from 0 deg to 90 deg in 0.1 deg steps.

[0411] Clause 4. The method of clause 2 or 3, wherein the calculating of forward Mie scattering profile comprises input of a parameter selected from the group consisting of bead diameter, bead refractive index at wavelength, medium refractive index, wavelength, polarization of incident light, and detector polarization sensitivity; calculating profiles in the range of 0 deg - 180 deg , optionally in 0.1 deg steps; and output of Mie scattering intensity as a function of polar angle; and numerically integrating the forward Mie scattering intensity over an angular range defined by current proposed collection half angle.

[0412] Clause 5. The method of clause 4, further comprising calculating calibration factor comprising determining the ratio of measured scattering intensity and the integrated forward Mie intensity to obtain the calibration factor.

[0413] Clause 6. The method according to any one of clauses 2 to 5, wherein the goodness of fit is calculated as $1 - \text{CV}$ of all calibration factors, and the CV is calculated over the set of calibration factors: $\text{CV} = \text{standard deviation (all calibration factors)} / \text{average (all calibration factors)}$.

[0414] Clause 7. The method according to any one of clauses 1 to 6, wherein the calculating the unknown parameters comprises employing a minimization algorithm and initial guess parameters, parameter bounds, and optionally parameter constraints.

[0415] Clause 8. The method according to clause 7, wherein the minimization algorithm is Powell's algorithm or constrained trust region minimization algorithm.

[0416] Clause 9. The method according to any one of clauses 6 to 8, wherein the loss function is: $\text{loss function} = [(\text{integrated, calculated Mie signal}) \times (\text{calibration factor}) - \text{measured signal}]$.

[0417] Clause 10. The method according to any one of clauses 6 to 9, wherein the calculated Mie signal is a function of the particle parameters listed in FIG. 60.

[0418] Clause 11. The method according to any one of clauses 1 to 10, wherein the particles, optionally nanoparticles, are characterized as extracellular vesicles, lipid nanoparticles, virus particles, virus-like particles, or protein aggregates.

[0419] Clause 12. The method according to clause 11, where in the particles are characterized as lipid nanoparticles.

[0420] Clause 13. The method according to any one of clauses 1 to 12, wherein the particles, optionally nanoparticles, are characterized based on an amount of protein loading on a surface of the particles or an amount of therapeutic payload within the particles.

[0421] Clause 14. The method according to any one of clauses 1 to 13, wherein the particles, optionally nanoparticles, are characterized based on detection of an empty therapeutic payload, a partial therapeutic payload, or a full therapeutic payload.

[0422] Clause 15. The method according to any one of clauses 1 to 14, wherein the plurality of channels includes a plurality of side scatter channels and optionally a plurality of fluorescence channels.

[0423] Clause 16. The method according to any one of clauses 1 to 15, wherein the particles are characterized based on one or more attributes identified in the plurality of side scatter channels and optionally the plurality of fluorescence channels.

[0424] Clause 17. The method according to any one of clauses 1 to 16, further comprising: sorting the particles based on their characterization, optionally wherein the particles are sorted to increase a purity or yield of the particles following centrifugation, optionally wherein the particles are sorted based on their secretion.

[0425] Clause 18. The method according to any one of clauses 8 to 17, wherein the empty and full particles are distinguishable based on a detectable increase in scatter.

[0426] Clause 19. The method according to any one of clauses 1-18, wherein the excitation light is selected from one or more of, two of, or two or more of VSSC1, VSSC2, BSSC, YSSC, and RSSC.

[0427] Clause 20. The method of clause 18 or 19, wherein the increase in the scatter is directly correlated to the ability of the particle to transduce cells.

[0428] Clause 21. The method of clause 20, wherein the cells are selected from the group consisting of a patient cells and a production cell line, optionally wherein the production cell line is a CHO cell line.

[0429] Clause 22. The method according to any one of clauses 1 to 21, wherein the unknown parameters selected from the group consisting of solid particle size, solid particle refractive index, core shell particle shell thickness, core shell particle core diameter, core shell particle shell refractive index, and/or core shell particle core refractive index of the particles.

[0430] Clause 23. The method according to any one of clauses 1 to 22, further comprising using a flow cytometry method to improve a particle sample preparation prior to the particle characterization, the method comprising: performing a sample preparation technique on a biological specimen; performing a flow cytometry analysis of the specimen after sample preparation; and adjusting one or more parameters of the sample preparation technique based on the flow cytometry analysis.

[0431] Clause 24. The method according to clause 23, wherein the sample preparation technique is selected from the group consisting of centrifugation, membrane filtration, precipitation, and chromatographic purification.

[0432] Clause 25. The method according to clause 23 or 24, wherein one or more parameters of the centrifugation is selected from the group consisting of centrifugation speed, duration, and temperature, optionally to increase the purity or yield of one or more target particles in the specimen.

[0433] Clause 26. The method according to clause 23 or 24, wherein the membrane filtration is selected from the group consisting of ultrafiltration, tangential flow filtration, dialysis, and Tangential Flow Filtration (TFF), and

[0434] Clause 27. The method according to clause 23 or 24, wherein the chromatographic purification is selected from the group consisting of size exclusion chromatography (SEC), immunoaffinity capture, affinity chromatography, and ion exchange chromatography.

[0435] Clause 28. The method according to any one of clauses 1 to 27, wherein the particle is a nanoparticle.

[0436] Clause 29. The method according to any one of clauses 1 to 28, wherein the particle or nanoparticle is a biological particle or biological nanoparticle, respectively.

[0437] Clause 30. The method according to any one of clauses 1 to 29, wherein the known parameters are selected from the group consisting of solid particle size, solid particle refractive index, core shell particle shell thickness, core shell particle core

diameter, core shell particle shell refractive index, and/or core shell particle core refractive index of the nanoparticles.

[0438] Clause 31. The method according to any one of clauses 1 to 30, wherein the unknown parameters are selected from the group consisting of solid particle size, solid particle refractive index, core shell particle shell thickness, core shell particle core diameter, core shell particle shell refractive index, and/or core shell particle core refractive index of the nanoparticles.

[0439] Clause 32. The method according to any one of clauses 1 to 31, wherein the known parameters and the unknown parameters are different.

[0440] Clause 33. A system for performing a method of characterizing particles in a flow cytometer, the method comprising:

illuminating the particles with one or more excitation light beams at one or more wavelengths as the particles individually pass through an interrogation zone; collecting side scattered light in a plurality of channels at a plurality of wavelengths from the particles passing through the interrogation zone; identifying the mean side scattered light intensity in each of the plurality of channels; comparing the mean side scattered light intensity in each of the plurality of channels to an average calibration factor determined in the same flow cytometer from a plurality of standard particles having known refractive indices and sizes; characterizing the particles based on the side scattered light collected in the plurality of channels; inputting one or more known parameters;

inputting particle initial guess parameters and parameter bounds, and optionally parameter constraints; and calculating unknown parameters that minimize loss function and satisfy the bounds and constraints.

Clause 34. The system according to clause 33, the method comprising the method of according to any one of clauses 1 to 32.

CLAIMS

What is claimed is:

1. A method of characterizing particles in a flow cytometer, the method comprising:
 - illuminating the particles with one or more excitation light beams at one or more wavelengths as the particles individually pass through an interrogation zone;
 - collecting side scattered light in a plurality of channels at a plurality of wavelengths from the particles passing through the interrogation zone;
 - identifying the mean side scattered light intensity in each of the plurality of channels;
 - calculating an average calibration factor determined in the same flow cytometer from a plurality of standard particles having known refractive indices and sizes;
 - characterizing the particles based on the side scattered light collected in the plurality of channels;
 - inputting one or more known parameters;
 - inputting initial guess parameters and parameter bounds, and optionally parameter constraints, for the particles; and
 - calculating unknown parameters that minimize loss function and satisfy the bounds and constraints, optionally
 - wherein the known parameters are selected from the group consisting of solid particle size, solid particle refractive index, core shell particle shell thickness, core shell particle core diameter, core shell particle shell refractive index, and/or core shell particle core refractive index of the nanoparticles.
2. The method of claim 1, wherein the average calibration factor is determined comprising
 - illuminating at one or more wavelengths calibration beads having known size and refractive index;
 - collecting side scattered light from all wavelengths of interest;
 - calculating forward Mie scattering profiles for each input calibration bead diameter;
 - integrating Mie scattering profiles for each calibration bead diameter;
 - calculating calibration factor for each calibration bead size;

calculating goodness of fit for current half angle;

reporting collection half angle with highest goodness of fit;

calculating Mie scattering profile at half angle identified in previous step; and

calculating the average calibration factor and CV over all calibration bead sizes for half collection angle from previous step.

3. The method of claim 2, wherein the integrating Mie scattering profiles is performed over $90 \text{ deg} \pm$ current collection half angle or from 0 deg to 90 deg in 0.1 deg steps.

4. The method of claim 2 or 3, wherein the calculating of forward Mie scattering profile comprises

input of a parameter selected from the group consisting of bead diameter, bead refractive index at wavelength, medium refractive index, wavelength, polarization of incident light, and detector polarization sensitivity;

calculating profiles in the range of 0 deg -180 deg, optionally in 0.1 deg steps;

and

output of Mie scattering intensity as a function of polar angle; and

numerically integrating the forward Mie scattering intensity over an angular range defined by current proposed collection half angle,

optionally further comprising calculating calibration factor comprising

determining the ratio of measured scattering intensity and the integrated forward Mie intensity to obtain the calibration factor.

5. The method according to any one of claims 2 to 4, wherein

the goodness of fit is calculated as $1 - \text{CV}$ of all calibration factors, and

the CV is calculated over the set of calibration factors:

$\text{CV} = \text{standard deviation (all calibration factors)} / \text{average (all calibration factors)}$.

6. The method according to any one of claims 1 to 5, wherein the calculating the unknown parameters comprises

employing a minimization algorithm and initial guess parameters, parameter bounds, and optionally parameter constraints,

optionally wherein the minimization algorithm is Powell's algorithm or constrained trust region minimization algorithm, and

further optionally wherein the loss function is:

loss function = [(integrated, calculated Mie signal) x (calibration factor) – measured signal], optionally wherein the calculated Mie signal is a function of the particle parameters listed in FIG. 60.

7. The method according to any one of claims 1 to 6, wherein the particles, optionally nanoparticles, are characterized as extracellular vesicles, lipid nanoparticles, virus particles, virus-like particles, or protein aggregates,

optionally wherein the particles are characterized based on an amount of protein loading on a surface of the nanoparticles or an amount of therapeutic payload within the nanoparticles.

8. The method according to any one of claims 1 to 7, wherein the particles, optionally nanoparticles, are characterized based on detection of an empty therapeutic payload, a partial therapeutic payload, or a full therapeutic payload.

9. The method according to any one of claims 1 to 8, wherein the plurality of channels includes a plurality of side scatter channels and a plurality of fluorescence channels, optionally wherein the particles are characterized based on one or more attributes identified in the plurality of side scatter channels and optionally the plurality of fluorescence channels.

10. The method according to any one of claims 1 to 9, further comprising:

sorting the particles based on their characterization, optionally wherein the particles are sorted to increase a purity or yield of the particles following a centrifugation, optionally wherein the particles are sorted based on their secretion.

11. The method according to any one of claims 1 to 10, wherein the particles are distinguishable based on a detectable increase in scatter,

optionally wherein the excitation light is selected from one or more of, two of, or two or more of VSSC1, VSSC2, BSSC, YSSC, and RSSC,

optionally wherein the increase the scatter is directly correlated to the ability of the particle to transduce cells,

optionally wherein the cells are selected from the group consisting of a patient cells and a production cell line, optionally wherein the production cell line is a CHO cell line.

12. The method according to any one of claims 1 to 11, wherein the unknown parameters are selected from the group consisting of solid particle size, solid particle refractive index, core shell particle shell thickness, core shell particle core diameter, core shell particle shell refractive index, and/or core shell particle core refractive index of the nanoparticles.

13. The method according to any one of claims 1 to 12, further comprising using a flow cytometry method to improve a particle sample preparation prior to the particle characterization, the method comprising:

performing a sample preparation technique on a biological specimen;

performing a flow cytometry analysis of the specimen after sample preparation;

and

adjusting one or more parameters of the sample preparation technique based on the flow cytometry analysis, optionally wherein the sample preparation technique is selected from the group consisting of centrifugation, membrane filtration, precipitation, and chromatographic purification,

optionally wherein one or more parameters of the centrifugation is selected from the group consisting of centrifugation speed, duration, and temperature,

optionally to increase the purity or yield of one or more target particles in the specimen, further optionally wherein the membrane filtration is selected from the group consisting of ultrafiltration, tangential flow filtration, dialysis, and Tangential Flow Filtration (TFF), and

further optionally wherein the chromatographic purification is selected from the group consisting of size exclusion chromatography (SEC), immunoaffinity capture, affinity chromatography, and ion exchange chromatography.

14. The method according to any one of claims 1 to 13, wherein the particle is a nanoparticle, optionally wherein the nanoparticle is a biological nanoparticle.

15. A system for performing the method according to any one of claims 1 to 14.