Framework Criteria	What to report	Please complete each criterion
1.1 Preanalytical variables conforming to MISEV guidelines.	Preanalytical variables relating to EV sample including source, collection, isolation, storage, and any others relevant and available in the performed study.	See MIFlowCyt report.
1.2 Experimental design according to MIFlowCyt guidelines.	EV-FC manuscripts should provide a brief description of the experimental aim, keywords, and variables for the performed FC experiment(s) using MIFlowCyt checklist criteria: 1.1, 1.2, and 1.3, respectively. Template found at www.evflowcytometry.org.	See MIFlowCyt report.
2.1 Sample staining details	State any steps relating to the staining of samples. Along with the method used for staining, provide relevant reagent descriptions as listed in MIFlowCyt guidelines (Section 2.4 Fluorescence Reagent(s) Descriptions).	Sample staining was performed as directed in the vFC Protocol. A fresh 10x vFRed PLUS working solution was prepared from the 100x stock in Vesicle Staining Buffer. Staining reactions consisted of 5 ul diluted sample, 5 ul vFRed 10x Working Solution, and 5 uL 10x antibody added to a total volume of 50 uL in Vesicle Staining Buffer. Samples were incubated for 1 hour at ambient temperature. Following staining, sample was diluted 200-fold for analysis.
2.2 Sample washing details	State any steps relating to the washing of samples.	No washing was performed.
2.3 Sample dilution details	All methods and steps relating to sample dilution.	Samples were subjected to a pre-stain dilution (10-80-fold, as determined in preliminary experiments), and a 1000-fold post stain dilution prior to measurement.
3.1 Buffer alone controls.	State whether a buffer-only control was analyzed at the same settings and during the same experiment as the samples of interest. If utilized it is recommended that all samples be recorded for a consistent set period of time e.g. 5 minutes, rather than stopping analysis at a set recorded event count e.g. 100,000 events. This allows comparisons of total particle counts between controls and samples.	Buffer-only controls showed fewer than 500 Vesicle-gated events per 100 uL analyzed.
3.2 Buffer with reagent controls.	State whether a buffer with reagent control was analyzed at the same settings, same concentrations, and during the same experiment as the samples of interest. If used state what the results were.	Buffer plus reagent controls showed fewer than 2000 Vesicle-gated events per 100 uL analyzed.
3.3 Unstained controls.	State whether unstained control samples were analyzed at the same settings and during the same experiment as stained samples. If used, state what the results were, preferably in	Unstained controls were similar to Buffer-only controls and showed fewer than 2000 events per 100 uL analyzed.
3.4 Isotype controls.	The use of isotype controls is applicable to immunofluorescence labelling only. State whether isotype controls were analyzed at the same settings and during the same experiment as stained samples. If utilized, state which antibody they are matched to, the concentration used, and what the results were (Section 4.2, 4.3, 4.4). Due to conjugation differences between manufacturers if should be stated if the isotype controls are from the same manufacturer as the matched antibodies.	A lack of detectable Fc Receptor mediated binding was assessed on a subset of samples using an irrelvent IgG1 at a concentration of 5 nM. The median fluorescence of the isotype-stained sample was not significantly above the background of unstained sample, indicating undetectable Fc Receptor binding.
3.5 Single-stained controls.	State whether single-stained controls were included. If used state whether the single-stained controls were recorded using the same settings, dilutions, and during the same experiment as stained samples and state what the results were, preferably in standard units (Section 4.2, 4.3, 4.4).	Single stained controls were analyzed as part of optimization and validation of multicolor assays.
3.6 Procedural controls.	State whether procedural controls were included. If used, state the procedure and if the procedural controls were acquired at the same settings and during the same experiment as stained samples.	No "procedural" controls were identified.
3.7 Serial dilutions.	State whether serial dilutions were performed on samples and note the dilution range and manner of testing. The fluorescence and/or scatter signal intensity would ideally be reported in standard units (see Section 4.3, 4.4) but arbitrary units can also be used. This data is best reported by plotting the recorded number events/concentration over a set period of time at different sample dilution. The median fluorescence intensity at each of the dilutions should also ideally be plotted on the same or a separate plot.	In preliminary experiment, serial dilutions of samples were performed on selected samples to determine an optimal sample pre-stain dilution, which showed proportional decrease in events counts with minimal change in median fluorescence over a greater than >50-fold range (from ~ 1:10 – 1:640).
3.8. Detergent treated EV-samples	State whether samples were detergent treated to assess lability. If utilized, state what detergent was used, the end concentration of the detergent, and what the results were of the lysis.	Detergent lability was assessed on a sub-set of samples by treatment of stained sample with 0.1% Triton X100 prior to post-stain dilution and analysis. Greater than 90% of the marker-positive (antibody) events were eliminated by detergent treatment.
4.1 Trigger Channel(s) and Threshold(s).	The trigger channel(s) and threshold(s) used for event detection. Preferably, the fluorescence calibration (Section 4.3) and/or scatter calibration (Section 4.4) should be used in order to report the trigger channel(s) and threshold(s) in standardized units.	Data acquisition was triggered by fluorescence in the vFred channel (488-690/50) corresponding ot a diameter of ~95 nm).
4.2 Flow Rate / Volumetric quantification.	State if the flow rate was quantified/validated and if so, report the result and how they were obtained.	The sample volumetric flow rate was calibrated using counting beads (Cellarcus, nanoRainbow), and was found to be within 10% of the instrument spec.

4.3 Fluorescence Calibration	State whether fluorescence calibration was implemented, and if so, report the materials and methods used, catalogue numbers, lot numbers, and supplied reference units for the standards. Fluorescence parameters may be reported in standardized units of MESF, ERF, or ABC beads. The type of regression used, and the resulting scatter plot of arbitrary data vs standard data for the reference particles should be supplied.	Fluorescence response was calibrated in units of ABV (antibodies bound per vesciel) using hard-dyed nanoRainbow calibration particles (Cellarcus Biosciences, #CBS6) that had been cross-calibrated against antibody capture beads (nanoCal, Cellarcus #CBS7-MS) and PE (QuantiBrite PE beads, BD Biosciences #340495) and MESF standards (Quantum FITC, Bangs labs on the same instrument.
4.4 Light Scatter Calibration	State whether and how light scatter calibration was implemented. Light scatter parameters may be reported in standardized units of nm2, along with information required to reproduce the model.	No light scatter calibration was performed.
5.1 EV diameter/surface area/volume approximation	State whether and how EV diameter, surface area, and/or volume has been calculated using FC measurements.	EV size was estimated from the vFRed™ intensity using the linear relationship between the population surface area and vFRed fluorescence distributions. Synthetic lipid vesicles with a uniform size distribution (estimated by NTA) and a lipid composition similar to a mammalian cell plasma membrane (Lipo100™, Cellarcus Biosciences, # CBS-1), were stained with vFRed™ and measured by flow cytometry. The Lipo100™ population diameter distribution was calculated from the NTA diameter distribution assuming a spherical geometry, and linear regression performed against the vFRed™ fluorescence distribution to
5.2 EV refractive index approximation	State whether the EV refractive index has been approximated and how this was done.	No EV refractive index approximation was performed.
5.3 EV epitope number approximation	State whether EV epitope number has been approximated, and if so, how it was approximated.	Immunofluorescence intensities are presented in units of ABV (antibodies bound per vesicle), which might be considered to represent the epitope abundance to within a factor of 2, given the bivalent nature of the IgGs used.
6.1 Completion of MIFlowCyt checklist	Complete MIFlowCyt checklist criteria 1 to 4 using the MIFlowCyt guidelines. Template found at www.evflowcytometry.org.	Included in Supporting Information.
6.2 Calibrated channel detection range	If fluorescence or scatter calibration has been carried out, authors should state whether the upper and lower limits of a calibrated detection channel were calculated in standardized units. This can be done by converting the arbitrary unit scale to a calibrated scaled, as discussed in Section 4.3 and 4.4, and providing the highest unit on this scale and the lowest detectable unit above the unstained population. The lowest unit at which a population is deemed ‘positive’ can be determined a variety of ways, including reporting the 99th percentile measurement unit of the unstained population for fluorescence. The chosen method for determining at what unit an event was deemed positive should be clearly outlined.	In general, fluorescence channels had a calibrated range of ~0-10,000 MESF/ABVs, with limits of detection (LOD, background +3SD) ranging from 10-200 MESF/ABVs, depending on the channel/fluorochrome.
6.3 EV number/concentration.	State whether EV number/concentration has been reported. If calculated, it is preferable to report EV number/concentration in a standardized manner, stating the number/concentration between a set detection range.	EV concentrations are reported in EVs/mL, which is calculated from the number of events detected in the Vesicles gate and accounting for the volume analyzed and all pre- and post-stain dilutions. Marker-positive events we calculated from the number of events exceeding an arbitrary gate set at the ~99.5 percentile of the negative population.
6.4 EV brightness.	When applicable, state the method by which the brightness of EVs is reported in standardized units of scatter and/or fluorescence.	EV brightness is reported in MESF/ABV units where possible and appropriate.
7.1. Sharing of data to a public repository.	Provide a link to the experimental data in a public data repository.	Data have been uploaded to the ISAC Flow Repository.

Cytometry Part A

Author Checklist: MIFlowCyt-Compliant Items

Requirement	**Please Include Requested Information**
1.1. Purpose	To measure the concentration, size, and antigen expression on EVs.
1.2. Keywords	extracellular vesice, EV, vesicle flow cytometery, vFC, CD9, CD63, CD81
1.3. Experiment variables	Concentration, antigen expression
1.4. Organization name and address	Scintillon Institute, 6868 Nancy Ridge Dr, San Diego, CA 92121
1.5. Primary contact name and email address	John Nolan, jnolan@scintillon
1.6. Date or time period of experiment	March-October, 2022
1.7. Conclusions	
1.8. Quality control measures	Instrument performance was characterized using a combination of multi-intensity single fluorophore beads (Quantum FITC, Bangs Labs Quantibrite PE, BD Biosciences) whose intensity had been calibrated in units of MESF, multi-intensity multifluorophore beads (vCal nanoRainbow, Cellarcus), and antibody capture beads (vCal nanoCal antibody capture beads, Cellarcus) calibrated to report results in units of antibodies bound per vesicle (ABV). EV analysis by vesicle flow cytometry (VFC) was conducted and reported as suggested by the MIFlowCyt-EV guidelines (See attached checklist).
2.1.1.1. (2.1.2.1., 2.1.3.1.) Sample description	Media from washed, ionophore-treated cells, centrifuged (2x 1.5kxg, 15') to pellet cells.
2.1.1.2. Biological sample source description	Cell culture media.
2.1.1.3. Biological sample source organism description	
2.1.2.2. Environmental sample location	
2.3. Sample treatment description	
2.4. Fluorescence reagent(s) description	See above and Antibody Database record.
3.1. Instrument manufacturer	Beckman Coulter
3.2. Instrument model	CytoFlex
3.3. Instrument configuration and settings	The CytoFlex flow cytometer with stock filters (see table below) was configured to measure violet side scatter (VSSC) as described in the CytoFLEX Instructions for Use (https://www.beckman.com/techdocs/B49006AP/wsr-168786). Briefly, the Violet 405nm filter is placed in position 2, the Violet 450nm filter in position 3, and an unused filter in position 1. The gain on all scatter channels was set to 100, the gain on all fluorescence channels was set to 1000.

Beckman Coulter
CytoFlex

Detector	Parameter Name ($PnN)	EX-EM/BP	Stain Name ($PnS)
FSC	FSC	488-	FSC
V-1		405-780/60	
V-2	Violet SSC	405-405/10	VSSC
V-3	FL6	405-450/45	V450
V-4	FL7	405-525/40	V525
V-5	FL8	405-610/20	V610
V-6		405-660/20	V660
R-1	FL3	640-660/20	APC
R-2	FL4	640-712/25	APC700
R-3	FL5	640-780/60	APC780
B-1	SSC	488-488/8	SSC
B-2	FL1	488-525/40	FITC
B-3	FL2	488-690/50	vFRed
Y-1		561-561/10	
Y-2	FL9	561-610/20	PE610
Y-3	FL10	561-585/42	PE
Y-4	FL11	561-690/50	PE690
Y-5	FL12	561-780/60	PE780

4.1. List-mode data files	*We recommend all authors to submit their data files to http://flowrepository.org and to make them available for the peer-review process. If you have done so, please let us know by inserting the following codes (replace the red text): 1) The link for peer-review process: http://flowrepository.org/id/ This link will only be shared with reviewers of your manuscript.
4.2. Compensation description	Single color spectral reference samples were prepared using antibody capture beads (nanoCal, Cellarcus), and run to determine spectra/spectral spillover of each for spectral unmixing/compensation.
4.3. Data transformation details	
4.4.1. Gate description	Data were analyzed using FCS Express (De Novo Software). The first 20 seconds of data were discarded via a Time gate to exclue a consistent but unexplained background event anomaly observed on several different CytoFlex instruments. The remaining 100 seconds of data, corresponding to 100 uL of measured sample, and a plot of vFRed-A vs cFRed-H used to set a gate excluding certain background events that could be identified by their lower signal pulse area and widths. These events were further gated to include events with membrane fluorescence and light scatter intensity characteristic of EVs, and to exclude high light scatter intensity background events that have been noted in certain samples.
4.4.2. Gate statistics	EV immunofluorescence data was analyzed to calculate the median fluorescence intensity (MFI) of the entire EV population, the number of EVs with immunofluorescence positive above gate position at the upper threshold of an unstained sample (<0.5% “positive”), and the (MFI) of these positive EVs.
4.4.3. Gate boundaries	EV immunofluorescence data was analyzed to calculate the median fluorescence intensity of the entire EV population, the number of EVs with immunofluorescence positive above gate position at the upper threshold of an unstained sample (<0.5% “positive”), and the (MFI) of these positive EVs.