UVA EV Workshop 2024:
SMALL PARTICLE FLOW CYTOMETRY LAB

Joanne Lannigan

CEO,
Flow Cytometry
Support Services, LLC

Mike Solga

Director,
Flow Cytometry Core Facility,
UVA School of Medicine

Joshua Welsh

Staff Scientist,
Advanced Technology Group,
BD Biosciences

Director,
The Measuring Stick, Ltd

This workshop's protocols were developed based on the published protocols in: Cook S, Tang VA, Lannigan J, Jones JC, Welsh JA. ***Quantitative flow cytometry enables end-to-end optimization of cross-platform extracellular vesicle studies.*** Cell Rep Methods. 2023 Dec 18;3(12):100664. doi: 10.1016/j.crmeth.2023.100664. [pdf, website].

Lab information

Participation

Session	FCM Name	FCM Model	Participant
Morning (9:30-12:25)	Chewie	CytoFLEX	Fabian Bolte Andrew Thede
	Luke	CytoFLEX	Scott Skalak Kevin Guzmán-Nawrath
	Leia	Aurora	Casey Scott-Weathers Riham Osman Swagatama Mukherjee
	C3-PO	Aurora	Wei-Chun Kao Licia Vellucci Melina Bellotto
Afternoon (1:30-4:35)	Chewie	CytoFLEX	Chad Aldridge Jonathon Sewell
	Luke	CytoFLEX	Samuel Wachamo Kim AeRyon
	Leia	Aurora	Viktor Yurevych Andy Snipes Ying Zhang
	C3-PO	Aurora	Jyoti Asundi Gabrielle Sangiuliano Jonel Lawson

Agenda

Date	Activity	Duration	Morning Session	Afternoon Session
Thursday, 14th March	Introductions	15 mins	9:35-9:50	1:45-2:00
	Part A, Protocol 1	25 mins	9:50-10:15	2:00-2:25
	Part B, Overview	15 mins	10:15-10:30	2:25-2:40
	Part B, Protocol 1	10 mins	10:30-10:40	2:40-2:50
	Part B, Protocol 2	20 mins	10:40-11:00	2:50-3:10
	Break	15 mins	11:00-11:15	3:10-3:25
	Part B, Protocol 3	25 mins	11:15-11:40	3:25-3:50
	Part B, Protocol 4	30 mins	11:40-12:10	3:50-4:20
	Part B, Protocol 5	25 mins	12:10-12:35	4:20-4:45
	Lunch	70 mins	12:35-1:45	
Friday March 15th	Data Comparison	45mins	9:00-9:45	

Lab Materials

Table 1. Scatter bead catalogue

Diameter (nm)	Diameter CV (%)	Refractive Index (RI)	RI Wavelength (nm)	Cat No.	Manufacturer	Composition	Concentration (mL^{-1})*
80	10.1	1.59	589	3080A	Thermo Fisher Scientific	Polystyrene	3.55E+13
100	6.1	1.59	589	3100A	Thermo Fisher Scientific	Polystyrene	1.82E+13
151	3.5	1.59	589	3150A	Thermo Fisher Scientific	Polystyrene	5.18E+12
202	2.5	1.59	589	3200A	Thermo Fisher Scientific	Polystyrene	2.207E+12
240	2.0	1.59	589	3240A	Thermo Fisher Scientific	Polystyrene	1.32E+12
303	2.2	1.59	589	3300A	Thermo Fisher Scie30ntific	Polystyrene	6.54E+11
345	1.9	1.59	589	3350A	Thermo Fisher Scientific	Polystyrene	4.24E+11
401	1.3	1.59	589	3400A	Thermo Fisher Scientific	Polystyrene	2.82E+11
453	1.7	1.59	589	3450A	Thermo Fisher Scientific	Polystyrene	2.00E+11

**Estimated from % solids (https://joadwe.github.io/Percent-Solids-Calculator/)*

Table 2. Fluorescence MESF bead catalogue

Fluor	Manufacturer	Ref Values						Cat No.	Lot No.
APC (4peak)	Bangs Laboratories	*0*	*994*	*4456*	*24312*	*73490*		*823B*	*16382*
FITC (5 peak)	Bangs Laboratories	0	2928	18137	63489	122407	360246	555B	15951
PE (4 peak)	Bangs Laboratories	0	270	1886	9113	48107		827B	16480

Table 3. Multipeak Fluorescence bead catalogue

Fluorophore	Manufacturer	Cat No.	Lot No.	Populations
Rainbow beads (QbSure)	Cytek Bioscience	B7-10005	AF01	6

Table 4. Other Fluorescent beads

Product	Conc. (%)	Conc. (ml^{-1})	Manufacturer	Cat No.	Lot No.
100 nm FluoSpheres carboxylate	2	2.73×10^{13}	Thermo Fisher Scientific	F8803	2451306
200 nm FluoSpheres carboxylate	2	3.1×10^{12}	Thermo Fisher Scientific	F8811	2668598
Recombinant EV (GFP+)	NA	1×10^{10}	Sigma-Aldrich	SAE0193	0000231959

Table 5. Cytometers

Name	Model	Manufacturer
Chewie	CytoFLEX	Beckman Coulter
Luke	CytoFLEX	Beckman Coulter
Leia	Aurora	Cytek Bioscience
C3-PO	Aurora	Cytek Bioscience

PART A: Data Acquisition

Protocol 1: Optimize detection of recombinant extracellular vesicles expressing GFP (rEV-GFP)

Aim: Find the best settings to acquire your EV samples.

Steps:

1. Using the PBS and rEV-GFP samples, determine the following:
    - Threshold Trigger value for SSC (Violet - Cytoflex & Aurora, Blue - Quanteon)
    - Optimal gain for SSC (VSCC-H [CytoFLEX], SSC-H [Aurora / Quanteon])
    - Optimal gain for the detection of GFP+ EVs on FITC-A/B2-A

    Hint:
    - *Both the PBS and rEV-GFP samples should be used*
    - *Threshold Trigger is set on Violet SSC – increasing the Violet SSC gain will also increase the background noise*
    - *You may need to delay wait 30-60 sec before recording for the event rate to stabilize*

2. Once you have determined your optimal settings, record the following samples at the same detector settings on a low flow rate for 120 sec:
    - PBS
    - rEV-GFP
    - 6 peak rainbow beads
    - 100 nm NIST-traceable PS beads

3. These 4 samples will be used for **Part B: Data Analysis**

PART B: Data Analysis

Protocol 1: Getting started with FCM_{PASS} (10 mins)

Aim: In this protocol you will be cataloguing the cytometer and reference materials you will be using in the FCMPASS software for downstream use with the detector optimization and experiment calibration modules.

Steps:

1. Open the FCMPASS software
2. Click on 'Catalogue' from the startup menu
3. You will be on the 'Cytometers' tab by default.
4. Complete the 'Cytometer ID' with the nickname of your cytometer (I.e. Leia/Clanker/Chewie/Jar Jar) and the 'Manufacturer' and 'Model' fields.

ID	Chewie	Luke	Leia	C3-PO
Manufacturer	Beckman Coulter	Beckman Coulter	Cytek Biosciences	Cytek Biosciences
Model	CytoFLEX S	CytoFLEX S	Aurora	Aurora

5. Click the 'Add Cytometer' button.
6. Click 'Import reference file' and navigate to the 'Annual Flow Cytometry' folder on your desktop.
7. Select the recorded fcs files from your dataset and click 'open'.
8. The 'Manufacturer' and 'Model' fields will automatically complete.

You have now added your cytometer information that will enable you to add experiments and also perform cytometer specific rainbow bead cross-calibrations.

9. Click on the 'Light scatter' tab
10. Add the NIST size standard bead information listed in **Table 1**. The first 6 bead populations have already been added. Enter the information for the remaining scatter bead population.

You have now added the size standard bead information to a catalogue and created a set to auto-load when performing experiment light scatter calibrations. With this information added, the software can automatically adjust refractive indices for different scatter wavelengths and output standard reports for others to validate your data.

11. Now click 'Fluorescence' tab
12. Add the FITC MESF bead information listed in **Table 2**.

You have now added the FITC MESF beads that will load in your experiment and cross-calibration catalogues.

13. Now click 'Rainbow Particles' tab
14. Add the 6-peak rainbow bead information listed in Table **3**.

You have now added the 6-peak rainbows beads that will load in the detector optimization module of the software and completed cataloguing everything you will need to use for your downstream experiments.

15. Close the FCM_{PASS} Catalogue

Protocol 2: Cross-calibration of 6-peak rainbow beads (20 mins)

Aim: In this protocol you will be cross-calibrating 6-peak rainbow particles with FITC MESF beads. This will allow you to determine your fluorescence limit of detection when performing voltration analysis, it is also a more cost-effective way to calibrate single EV experiments.

Steps:

1. Refer to https://docs.fcmpass.com/cataloguing/automated-cross-calibration

Note: The cross calibration files will be found in the following path 'Desktop/Annual Flow Cytometry/Cross Calibration

Protocol 3: Fluorescence detector optimization (20 mins)

Aim: In this protocol you will be analyzing the 6-peak rainbow bead voltration files in order to determine the best settings for analyzing your small/dim particles and determine your fluorescence limit of detection.

Steps: Refer to https://docs.fcmpass.com/optimization/fluorescence/data-analysis

Note: *Step 2, parameters will be:*

a. Aurora = 'FSC-A', 'SSC-B-A', 'SSC-H';
b. CytoFLEX = 'FSC-A', 'SSC-A', 'V-SSC-H'.

Note:

- The FL Voltration files will be found in the following path 'Desktop/Annual Flow Cytometry/FL Voltration
- *Step 3, Under the FITC channel (CytoFLEX, FITC; Aurora, B2; Quanteon, FITC) and select the FITC cross calibrated rainbow bead values from the dropdown list.*

Protocol 4: Cytometer Collection Angle Determination (25 mins)

Aim: In this protocol you will be calibrating previously acquired NIST traceable size standard data in order to derive the collection angle of your instrument. Knowing the collection angle of your instrument allows you to perform light scatter calibration in future experiments on the same instrument (provided the alignment has not been changed) using only a single size standard.

Steps: Refer to hhttps://docs.fcmpass.com/calibration

Note:
The NIST Beads files will be found in the following path:

- 'Desktop/UVA-EV-LAB/NIST Beads'

Protocol 5: Calibrating your collected r-EV data (30 mins)

Aim: In this protocol you will be calibrating your acquired rEV data and utilizing your 8-peak rainbow beads and known collection angle to convert the rEV data to standard units of FITC ERF and diameter.

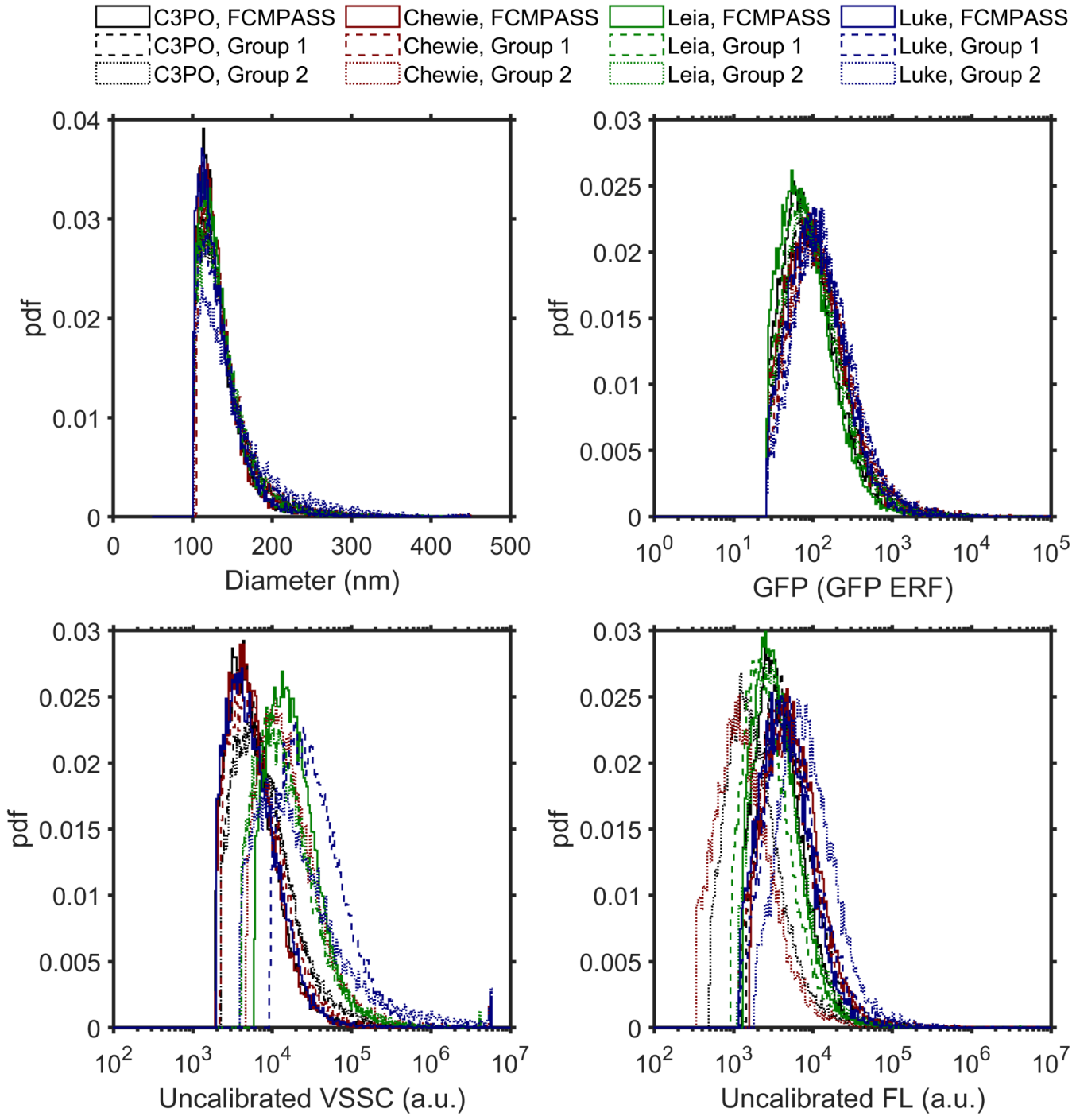
Steps: Refer to https://docs.fcmpass.com/calibration

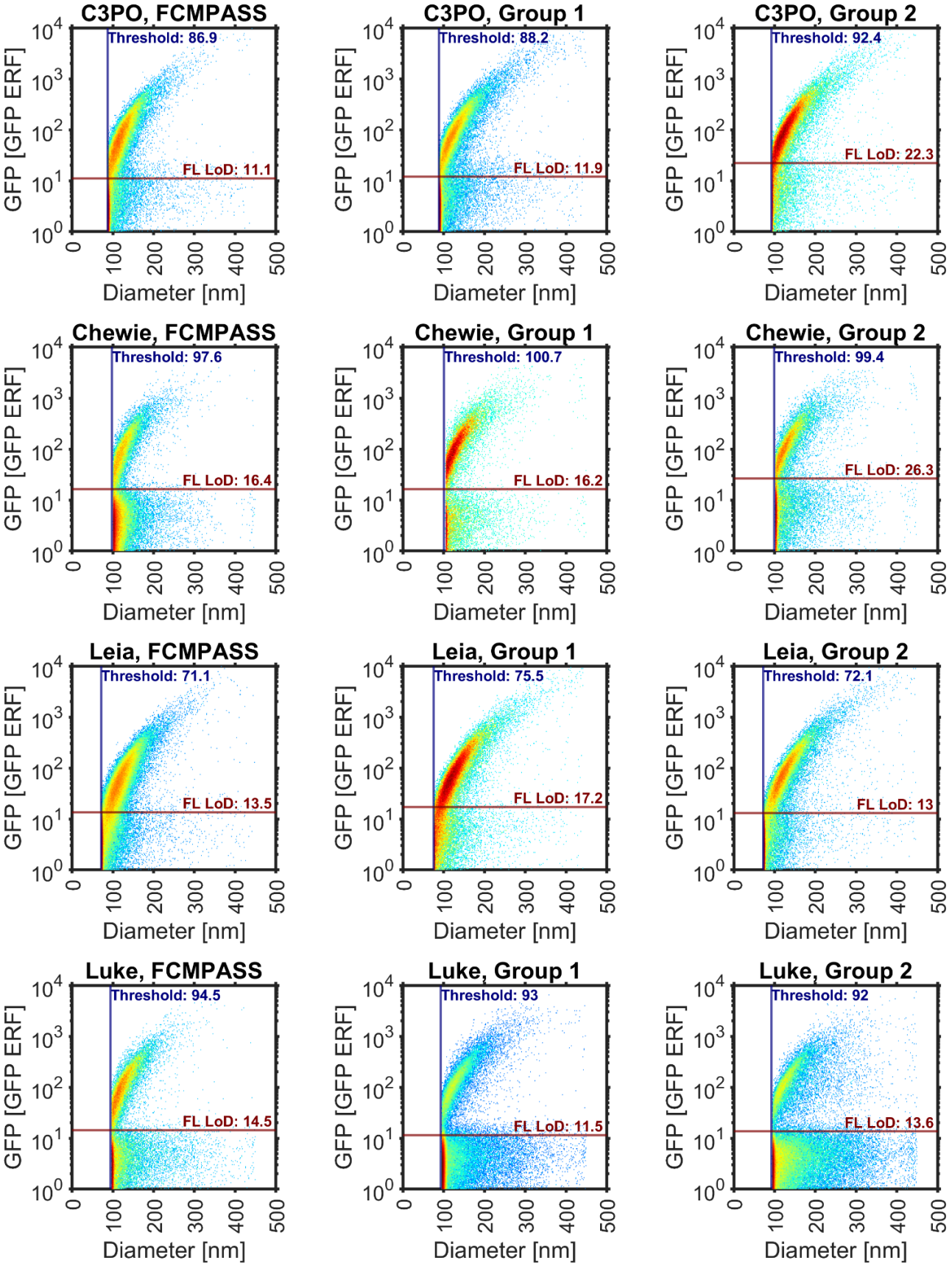
Note:
The NIST Beads files will be found in the following path depending on your session:

- 'Desktop/UVA-EV-LAB/Morning Session
- 'Desktop/UVA-EV-LAB/Afternoon Session

Part C: Comparing your results

Aim: Once your calibration is performed, Josh will take your rEV.fcs file and plot it on the projector along with the other attendee's data to see how well your instrument settings compared to FCM_{PASS} settings, and how instruments compared to one another at detecting rEVs.




C3PO, FCMPASS
Threshold: 86.9
FL LoD: 11.1
C3PO, Group 1
Threshold: 88.2
FL LoD: 11.9
C3PO, Group 2
Threshold: 92.4
FL LoD: 22.3
Chewie, FCMPASS
Threshold: 97.6
FL LoD: 16.4
Chewie, Group 1
Threshold: 100.7
FL LoD: 16.2
Chewie, Group 2
Threshold: 99.4
FL LoD: 26.3
Leia, FCMPASS
Threshold: 71.1
FL LoD: 13.5
Leia, Group 1
Threshold: 75.5
FL LoD: 17.2
Leia, Group 2
Threshold: 72.1
FL LoD: 13
Luke, FCMPASS
Threshold: 94.5
FL LoD: 14.5
Luke, Group 1
Threshold: 93
FL LoD: 11.5
Luke, Group 2
Threshold: 92
FL LoD: 13.6
GFP [GFP ERF]
Diameter [nm]

Additional resources

MIFlowCyt-EV framework

Why? The MIFlowCyt-EV framework is an initiative that was developed by a flow cytometry working group of researchers from the international societies for extracellular vesicles, advancement of cytometry and thrombosis and haemostatisis (ISEV-ISAC-ISTH). The MIFlowCyt-EV framework was designed to be assay agnostic and layout a standard reporting framework for experiments using single extracellular vesicle flow cytometry analysis.

When? Every component of the MIFlowCyt-EV framework should be completed regardless of assay. While not all components may be required for a particular assay, justification should be provided for why it was not utilized. The components in the MIFlowCyt-EV framework that are seen as broadly applicable to experiment regardless of assay are highlighted with an *.

How? The MIFlowCyt-EV framework can be completed in a spreadsheet found at evflowcytometry.org/miflowcyt-ev

	Category	Components	Objective
1	Preanalytical variables & experimental design	1.1. Report preanalytical variables conforming to MISEV guidelines* 1.2. Report experimental design according to MIFlowCyt guidelines*	Reproducibility
2	Sample preparation	2.1. Sample staining* 2.2. Sample washing steps* 2.3. Sample dilution*	Reproducibility
3	Assay controls	3.1. Buffer-only* 3.2. Buffer with reagents* 3.3. Unstained controls* 3.4. Isotype controls** 3.5. Single-stained controls* 3.6. Procedural controls** 3.7. Serial dilution* 3.8. Detergent-treated EV samples	Proof of single vesicle detection
4	Instrument calibration & data acquisition	4.1. Trigger channel(s) and threshold(s)* 4.2. Flow rate & volumetric quantification (μL min^{-1} / μL)* 4.3. Fluorescence Calibration (MESF/ERF units)* 4.4. Light Scatter Calibration (nm^2)	Standardization
5	EV characterization	5.1. EV diameter/surface area/volume approximation 5.2. EV refractive index approximation 5.3. Epitope number approximation	Advanced standardization
6	FC data reporting	6.1. Complete MIFlowCyt checklist* 6.2. Calibrated channel detection range 6.3. EV number concentration 6.4. EV brightness	Reproducibility
7	FC data sharing	7.1. Share data to public repository	Reproducibility

MIFlowCyt-EV Assay controls

Buffer only

Why? Understanding the background signals and cleanliness of the buffer used to suspend samples in is an important starting point for any experiment.
How? Buffer only controls are samples of the medium being used to resuspend samples in. Use a sample of the same buffer being used to suspend samples in.

Buffer with reagent

Why? Reagents such as antibodies and dyes alone can form aggregates or micelles that can be detected as fluorescent events.
How? Reagents controls are samples containing reagent alone at the concentrations used to label experimental sample in the same buffer. Reagent controls are handled the same way experimental samples are i.e. they are analyzed at the same dilution factor and system settings.

Unstained controls

Why? In order to understand if a sample is stained positively for a reagent a reference point is needed for what the sample without the reagent looks like.
How? Unstained controls should be at the same sample concentration as stained samples but only lack the fluorescent reagent. These controls are handled and analyzed same way experimental samples are i.e. they are analyzed at the same dilution factor and system settings.

Isotype controls

Why? The use of isotype controls is applicable to immunofluorescence labelling only and is one method of showing that non-specific binding of antibodies is not occurring.
How? An isotype matched control of each antibody within an assay should be used with the matching fluorophore and concentration to demonstrate non-specific binding is not occurring. Due to conjugation differences between manufacturers isotype controls should be from the same manufacturer as its matched antibodies.

Single stained controls

Why? Single-stained controls are used to identify fluorophore spillover and to enable compensation or spectral unmixing
How? Single-stained controls should be prepared and analyzed at the same concentration and settings as fully stained samples.

Procedural controls

Why? A number of extracellular vesicle staining protocols require post-staining processing methods (i.e. size exclusion columns, density gradients). It is important to verify that these post-staining protocols do not introduce artifacts into the sample sample
How? Buffer only, buffer with reagent, and unstained controls should be put through the same post-staining processing protocol as the stained samples in addition to running them without the post-staining processing protocol.

Serial dilution

Why? The use of serial dilution can demonstrate that single particles are being analyzed and there are not too high a concentration of free reagents within the sample.
How? Stained samples should be serially diluted and analyzed for a set time period or using a certain volume. Plotting the sample dilution factor against the total count and median fluorescence and light scatter signal intensity can demonstrate the concentration at which single particle detection is maximized.

Detergent treatment

Why? Detergents are used to show the lability of lipid membranes. Treatment of a lipid sample such as extracellular vesicles with detergent can verify that the stained samples of interest is a lipid structure and not an antibody aggregate or other non-labile structure.
How? Detergents (i.e. Triton X-100, Tween-20) should be added to stained samples which have already been analyzed to allow a comparison pre and post treatment.

MIFlowCyt-EV Experimental Design for EVs

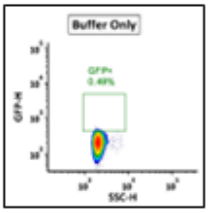
Why? Experimental Design will greatly impact on the results generated, as well as the interpretation and conclusion of the results obtained. Poor experimental design can lead to artefactual data that may go unrecognized and therefore incorrectly interpreted.
What? Well thought out experimental parameters and controls provides for a basis of interpretable results. Such parameters would include: 1) choice of antibodies 2) choice of fluorochromes/dyes 3) careful titration of reagents 4) choice of acquisition trigger and 5) rational approach to establish instrument settings. In addition to these considerations, careful control of experimental results with a set of specific controls is required for appropriate interpretation of the results achieved. These controls may include: 1) buffer only control 2) buffer plus reagents control 3) EVs only control (and/or isotype control) 4) single labeled EVs 5) EV detergent lysis control 6) procedural controls and 7) EV dilution control.
When? Initial experiments will require a careful and systematic approach to establishing conditions and parameters which are likely to generate the most reliable results. However, they are likely not necessary for every experiment going forward. Such conditions/parameters would include choice of antibodies, fluorochromes, reagent concentrations, and optimal acquisition settings. Controls, in contrast, should be run with each and every experiment to assess the validity of the data achieved.
How? With the use of appropriate controls, instrument acquisition parameters can be established, and data generated with these parameters can be evaluated with controls designed to identify potential sources of artefacts.

J. Lannigan CDW2019

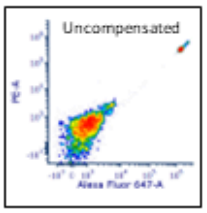
Materials

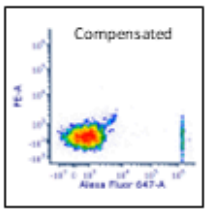
- 0.1 µm filtered PBS
- MESF beads
- Single labeled EVs/virus and/or antibody capture beads
- Unlabeled EVs/virus
- Staining reagents at experimental concentrations in 0.1 µm PBS
- 0.5% Triton X-100
- Labeled EVs/virus
- Serially diluted EVs/virus

Step 1 Using the buffer only and single labeled controls, establish the optimal gain and the minimal threshold for fluorescence and scatter that gives the best positive signal and the lowest background (noise). Do this for both scatter and fluorescence triggering. Collect the buffer only control for two minutes.

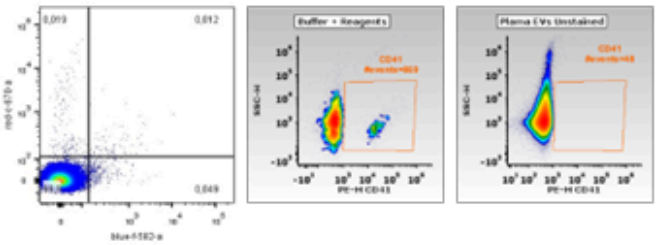


Step 2 Acquire the unlabeled and single labeled controls at the established settings. Perform spectral unmixing (compensation).

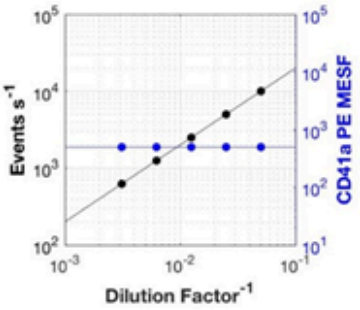




Step 3 Acquire all fully labeled samples and additional controls for two minutes at the established settings.



Serial Dilutions:
Perform serial dilutions of the experimental sample and acquire. Plot data as dilution factor against event rate and fluorescence intensity of the population of interest.



Fluorescence Calibration:
Refer to Fluorescence Calibration

Recommended Literature:

- Nolan JP, Duggan E. Analysis of individual extracellular vesicles by flow cytometry. *Methods Mol. Biol.* (2018) **1678**:79-92.
- Théry, Clotilde & Witwer, Kenneth et.al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. Journal of Extracellular Vesicles. (2018)

Useful Literature

1. Cook S, Tang VA, Lannigan J, Jones JC, Welsh JA. ***Quantitative flow cytometry enables end-to-end optimization of cross-platform extracellular vesicle studies.*** Cell Rep Methods. 2023 Dec 18;3(12):100664. doi: 10.1016/j.crmeth.2023.100664. [pdf, website]

2. Welsh JA, Arkesteijn GJA, Bremer M, Cimorelli M, Dignat-George F, Giebel B, Görgens A, Hendrix A, Kuiper M, Lacroix R, Lannigan J, van Leeuwen TG, Lozano-Andrés E, Rao S, Robert S, de Rond L, Tang VA, Tertel T, Yan X, Wauben MHM, Nolan JP, Jones JC, Nieuwland R, van der Pol E., ***A compendium of single extracellular vesicle flow cytometry***, J Extracell Vesicles. 2021 Dec;10(14):e12182. doi: 10.1002/jev2.12182., 2023, DOI: 10.1002/jev2.12299 [pdf, website]

3. Welsh JA, Van Der Pol E, Arkesteijn GJA, Bremer M, Brisson A, Coumans F, Dignat-George F, Duggan E, Ghiran I, Giebel B, Görgens A, Hendrix A, Lacroix R, Lannigan J, Libregts SFWM, Lozano-Andrés E, Morales-Kastresana A, Robert S, De Rond L, Tertel T, Tigges J, De Wever O, Yan X, Nieuwland R, Wauben MHM, Nolan JP, Jones JC., ***MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments***, J Extracell Vesicles, 2020, DOI: 10.1080/20013078.2020.1713526 [pdf, website]

4. Welsh JA, Goberdhan DCI, O'Driscoll L, Buzas EI, Blenkiron C, Bussolati B, Cai H, Di Vizio D, Driedonks TAP, Erdbrügger U, Falcon-Perez JM, Fu QL, Hill AF, Lenassi M, Lim SK, Mahoney MG, Mohanty S, Möller A, Nieuwland R, Ochiya T, Sahoo S, Torrecilhas AC, Zheng L, Zijlstra A, Abuelreich S, Bagabas R, Bergese P, Bridges EM, Brucale M, Burger D, Carney RP, Cocucci E, Crescitelli R, Hanser E, Harris AL, Haughey NJ, Hendrix A, Ivanov AR, Jovanovic-Talisman T, Kruh-Garcia NA, Ku'ulei-Lyn Faustino V, Kyburz D, Lässer C, Lennon KM, Lötvall J, Maddox AL, Martens-Uzunova ES, Mizenko RR, Newman LA, Ridolfi A, Rohde E, Rojalin T, Rowland A, Saftics A, Sandau US, Saugstad JA, Shekari F, Swift S, Ter-Ovanesyan D, Tosar JP, Useckaite Z, Valle F, Varga Z, van der Pol E, van Herwijnen MJC, Wauben MHM, Wehman AM, Williams S, Zendrini A, Zimmerman AJ; MISEV Consortium; Théry C, Witwer KW. **Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches.** J Extracell Vesicles. 2024 Feb;13(2):e12404. doi: 10.1002/jev2.12404. [pdf, website]

5. Pleet ML, Cook S, Tang VA, Stack E, Ford VJ, Lannigan J, Do N, Wenger E, Fraikin JL, Jacobson S, Jones JC, Welsh JA. **Extracellular Vesicle Refractive Index Derivation Utilizing Orthogonal Characterization.** Nano Lett. 2023 Oct 25;23(20):9195-9202. doi: 10.1021/acs.nanolett.3c00562. [pdf, website]

Additional Protocols

Preparation of rEV-GFP Serial dilutions

Aim: Serial dilutions are performed for many of the best practices recommended for small particle flow cytometry, from sample preparation to regent preparation for regent staining titrations. Below are the protocols that were used to reconstitute, prepare the serial dilutions, and acquire the data for the rEV-GFP samples.

Reconstitution of rEV-GFP

1. Briefly centrifuge vial before opening
2. Add 100uL of ice cold MilliQ Type II water (ultrapure)
3. Pipet up and down to mix (DO NOT VORTEX)
4. Aliquot into a low binding Eppendorf tube
5. Store at 2-8^{0}C for up to one day (up to two weeks at -80^{o}C) in low protein-binding tubes

Dilution of rEV-GFP

1. Label 10 low binding tubes;, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, and 1:25600

Tube	1	2	3	4	5	6	7	8	9	10
Label	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600

2. Add 980uL of PBS to the tube labeled 1:50
3. Add 500uL of PBS to the remaining tubes
4. Add 20uL of the stock reconstituted rEVs to the tube labeled 1:50, pipet up and down to mix (DO NOT VORTEX)
5. Pipette 500uL of the 1:50 dilution tube into the 1:100 tube, pipet up and down to mix (DO NOT VORTEX)
6. Repeat the above step for each of the remaining dilution tubes until the serial dilution has been completed for all dilutions.
7. Keep on ice during acquisition

Acquisition

1. Use the instrument settings that were established from SSC and FL Optimization
2. Collect a buffer only sample (PBS that was used to dilute rEVs) for one minute on low
3. Collect all rEVs dilutions for one minute on low, starting with the highest dilution and working down (to avoid carryover)
4. (Optional) Run Buffer only sample to make sure there are no carryover events; there should be no events in the FITC-A/B2-A channel. Run the 100 nm silica beads (diluted in MilliQ ultrapure water to a concentration of 1x10^7/ml (stock=1x10^{10}/ml) at the same scatter settings as the scatter calibration
5. Acquire 8-peak rainbow calibration beads (or FITC MESF beads). Record 10,000 events in the identified bead gate.

EV Lysis Protocol

Aim: There is a large range of biological particles in the small particle size range. Not all detergents will optimally lyse particles and so it is advised that further optimization be performed to choose the type of detergent, concentration, and duration of lysis. It is important to remember that detergents will contribute to events in a sample and should be significantly diluted prior to acquisition. Below is a protocol for lysis of the rEV-GFP used in this lab with 0.1% Triton X-100 lysis.

Steps:

1. In a new tube, add 10 µL of 0.2% Triton X-100 into 10 µL of undiluted stock rEV-GFP. Incubate at room temperature for 15 min to lyse rEV-GFP.
2. Take 1 µL of the lysed rEV-GFP and add 1 mL of PBS.
3. Take 1 µL of PBS and add 1 mL of 0.1% Triton X-100 (Triton X-100 only control).
4. Acquire and record the lysed EV sample and the corresponding Triton X-100 only control for 1 min on low.

Cleaning Protocols

Instruments used for small particle analysis will require thorough cleaning prior and post sample acquisition. Sometimes you may even need to clean between samples due to the presence of sample carry-over.

Cleaning to prepare the instrument for analysis:

- For the Aurora – “Clean Flow Cell” can be used to prepare the instrument for small particle sample acquisition. Use 3% Contrad and leave for 5-15 min before continuing with the DI water flush.
- For the CytoFLEX – “Deep Clean and Prime” followed by “Daily Clean” using 10% Contrad or 2% Micro90 (10 mins) followed by DI water (5 min) can be used to prepare the instrument for small particle sample acquisition. Repeat the “Daily Clean” if necessary.

Cleaning between samples:

Assess for the presence of sample carry-over by acquiring a sample of clean PBS or Water. If fluorescent events continue to be detected, or the event rates are above that of the previous buffer alone, even post Backflush/SIT Flush, try the following cleaning solutions for 1-2 min on high. Always run a sample of water for 1-2min on high before proceeding with samples. Extend the cleaning time as needed.

- 1-3% Contrad70 (CytoFLEX /Aurora)
- 1-2% Micro90 (CytoFLEX /Aurora)
- FACSClean (CytoFLEX /Aurora)
- CoulterClenz (CytoFLEX)