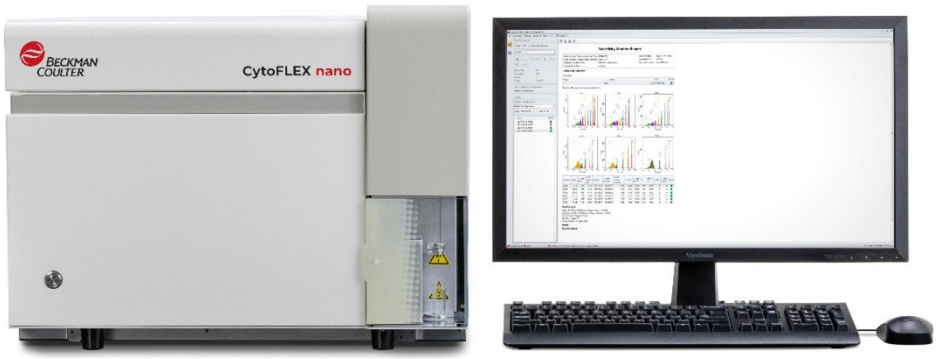
A new approach to nanoscale flow cytometry with the CytoFLEX nano analyzer



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Background

Nanoscale flow cytometry is a cutting-edge technique that combines the principles of flow cytometry with nanotechnology. It enables the analysis of particles at the nanoscale, providing valuable information about their size, composition, and surface properties. Nanoscale flow cytometry has numerous applications in various fields, including biology, medicine, and materials science. It allows researchers to analyze and characterize nanoparticles, extracellular vesicles (EVs), and other small particles with great precision and sensitivity. In addition to its research applications, nanoscale flow cytometry also holds promise in the development of diagnostic tools and targeted drug delivery systems. The ability to analyze nanoparticles based on their characteristics opens new possibilities for personalized medicine and nanomedicine.

While nanoscale flow cytometry is a powerful technique with numerous advantages, it also has some limitations, including:

Detection sensitivity: there are still limits to the smallest particles that can be detected reliably. Currently, flow cytometers are not designed to detect and characterize EVs smaller than 100 nm. The detection threshold for nanoparticles is influenced by factors such as background noise, autofluorescence, and the efficiency of labeling or detection probes.

Size resolution: Flow cytometry can characterize particles in the nanoscale range, but accurately resolving nanoparticles of similar sizes can be challenging. Distinguishing particles with very small size differences, such as distinguishing between 50 nm and 60 nm particles, may be difficult due to limitations in the detection and resolution capabilities of the instrument.

Sample preparation: Preparing samples for nanoparticle or EV detection can be complex. Sample preparation methods need to consider factors such as aggregation, stability, and potential alterations of particle properties during the process. Obtaining a representative and homogeneous sample can be crucial for accurate analysis.

Standardization: Nanoscale flow cytometry is a relatively new and evolving field, and the field is still working on standardizing protocols and reference materials. This can lead to variability in data collection and analysis across different laboratories, making it challenging to compare results and establish consistent methodologies.

Data analysis complexity: Flow cytometry generates complex and multidimensional datasets, which require sophisticated data analysis techniques. Analyzing and interpreting large datasets can be time-consuming, requiring expertise in data processing, visualization, and statistical analysis.

In this application note, we will introduce the CytoFLEX nano Flow Cytometer and its workflow. This new analyzer is the first purpose-built nanoscale flow cytometer that can detect nanoparticles, such as extracellular vesicles (EVs) at least as small as 40 nm, while simultaneously performing multiparameter fluorescent detection. Furthermore, it enables counting, characterization, and particle size definition, all within a single instrument, setting a new standard and overcoming current limitations for nanoparticle research. The CytoFLEX nano software interface, CytExpert nano, provides the sophistication to explore the unknown at the nanoscale range while providing the ease-of-use characteristics of the CytoFLEX platform. This way, getting answers to challenging research questions becomes easier than ever for EV researchers.

The CytoFLEX nano Flow Cytometer offers:

1. High sensitivity to detect and characterize nanoparticles at least as small as 40 nm (based on polystyrene beads).
2. High resolution to accurately distinguish particles of similar size within 10 nm difference (based on silica beads), and ability to characterize low-abundance targets in a heterogeneous population.
3. Consistency in instrument performance and data analysis, thanks to a very detailed QC process and fluorescence Sensitivity Monitor, as well as multiple options for on-board cleaning.
4. Flexibility to design experiments, thanks to 5 side scatter parameters and 6 fluorescence channels.

Introduction

Extracellular vesicles (EVs) are small membrane-bound particles released by cells into the extracellular environment. They play important roles in intercellular communication and are involved in various physiological and pathological processes. EVs are classified into different subtypes based on their biogenesis and size, including exosomes, microvesicles, and apoptotic bodies.

Exosomes or small EVs typically range in size from 30 to 150 nm. They are formed through the inward budding of multivesicular bodies (MVBs) within the cell, which then fuse with the plasma membrane, releasing the exosomes into the extracellular space. Exosomes contain various bioactive molecules, such as proteins, lipids, nucleic acids (DNA, RNA), and microRNAs, which can be transferred to target cells, influencing their function and behavior.

Microvesicles, also known as large EVs or microparticles or ectosomes, are larger than exosomes, ranging from 100 to 1000 nm in size. Unlike exosomes, microvesicles are formed by the outward budding and shedding of the plasma membrane directly. They also carry a diverse cargo of proteins, lipids, and nucleic acids, and can transfer these molecules to recipient cells.

Apoptotic bodies are the largest EVs, typically ranging from 1 to 5 µm. They are released from dying cells during the process of programmed cell death (apoptosis). Apoptotic bodies contain cellular fragments, organelles, and nuclear material, and are recognized and engulfed by phagocytic cells to facilitate their clearance.

EVs have gained significant attention in recent years due to their potential as biomarkers for disease diagnosis and prognosis, as well as their roles in cell-to-cell communication and their therapeutic applications. Researchers are studying EVs in various biological fluids, including blood, urine, and cerebrospinal fluid, to gain insights into their cargo and functions.

Technologies such as nano flow cytometry, electron microscopy, and molecular profiling techniques like RNA sequencing and proteomics are used to study and characterize EVs.

Understanding EV biology, cargo, and functions holds great promise for advancing our knowledge of cellular communication and their potential applications in diagnostics, therapeutics, and regenerative medicine.

Currently, EV analysis is critical and challenging. Isolation and purification methods can suffer from low yield, contamination from other particles, and difficulties in standardization. Heterogeneity of EV populations in terms of size, cargo, and biogenesis complicates their study. EVs can exhibit diverse biological activities and functions depending on their cellular origin and cargo. However, deciphering the specific functions and mechanisms of action of EVs in different contexts is still a challenge. The functional heterogeneity of EVs requires more comprehensive characterization and standardized functional assays.

Researchers in the field of EVs are actively working to address these limitations by developing improved isolation techniques, standardizing protocols, and advancing our understanding of EV biology. As the field progresses, overcoming these challenges will help unlock the full potential of EV research and its applications in various biomedical areas.

One of the biggest limitations is the need for multiple techniques to accurately count, characterize and determine the size of EVs, resulting in time-consuming and laborious workflows with poor repeatability.

The CytoFLEX nano Flow Cytometer combines everything in only one instrument, enabling count, characterization, and size determination, thanks to the following features:

Performance

Violet Side scatter sensitivity	VSSC1: 40 nm relative to polystyrene nanoparticles VSSC2: 80 nm relative to polystyrene nanoparticles	
Scatter Detection dynamic range	VSSC1 for small range 40-150 nm polystyrene nanoparticles VSSC2 for large range 80-1000 nm polystyrene nanoparticles	
Violet forward scatter sensitivity	300 nm relative to polystyrene nanoparticles	
Fluorescence sensitivity and resolution	Simultaneous detection of fluorescence on six fluorescence detectors, using 500 nm CytoFLEX nano Multi-intensity Fluorospheres V447: 8 peaks B531: 8 peaks Y595: 8 peaks R670: 6 peaks R710: 5 peaks R792: 4 peaks	
Fluorescence rCV	rCV <10% (using QC Fluorospheres at 1 µL/min)	
Carryover between samples	≤ 1%[a]	
Acquisition speed	Maximum electronic acquisition speed	16000 events/second with ≥95% yield
	Recommended maximum sample acquisition speed	5000 events/second to avoid possible swarming or coincidence situation
Volumetric counting accuracy	≥90%[b]	

[a] tested with polystyrene beads, [b] tested with 144nm QC Scatterspheres on 3 units under the conditions: record 3 minutes at the sample flow rates of 1 µL/min, 2 µL/min, 3 µL/min, 4 µL/min, 5 µL/min, 6 µL/min respectively, repeat 5 times, then calculated the average total events for each speed, compared the calculated the volumetric counting accuracy with the theoretical total events.

Figure 1. The CytoFLEX nano Flow Cytometer performance specifications.

Protocol

1. System Startup

After comfirming that the sheath fluid and the cleaning reagent are sufficient for the day, and waste container is empty, turn on the CytoFLEX nano Flow Cytometer and start the CytExpert nano software, using the link on the desktop. Select the System Startup procedure. The procedure will take about 6 minutes, during which the system automatically purges the sheath damper, executes debubble for the sheath filter, the sheath line, the flow cell and, the piston pump, and cleans the sample line, ensuring the fluidics system is set to start.

2. Configuration setting

The CytoFLEX nano Flow Cytometer is equipped with 2 Wavelength Division Multiplexers (WDMs), one for scatter optical filters (VioletSSC1, VioletSSC2, BlueSSC, YellowSSC and RedSSC) and one for fluorescence optical filters (V447, B531, Y595, R670, R710, R792), as shown in Figure 2 below.

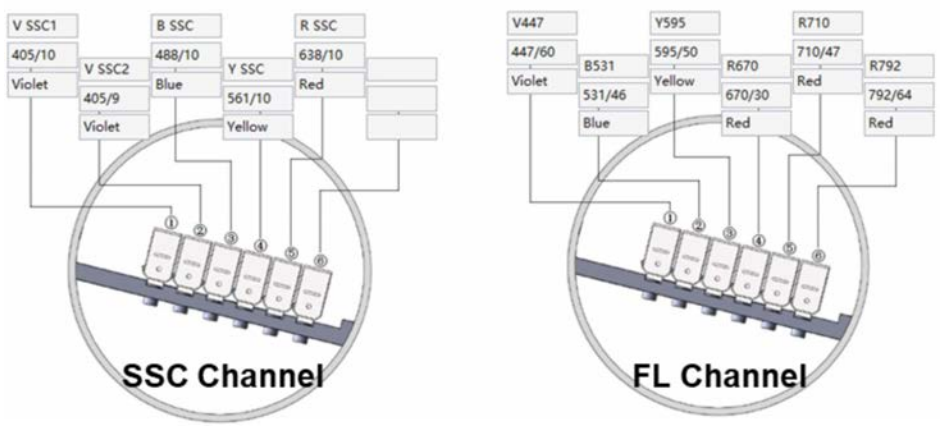

Figure 2. The CytoFLEX nano Flow Cytometer default detector configuration.

3. Perform Daily QC

Select the QC/Sensitivity menu and input the bead lots. Running QC will ensure the CytoFLEX nano Flow Cytometer will provide adequate signal strength and precision.

The QC process will start evaluating laser powers and sheath flow rate.

Next, the process is divided in three steps:

- **Instrument background assessment:** using 5 nm filtered CytoFLEX sheath the system assesses background noise in both sheath and sample line, very close to the limit of optical and electrical noise, to ensure it will achieve the lowest limit of detection.
- **Scatter performance assessment:** using CytoFLEX nano Daily QC Scatterspheres the system will monitor event rate and signal strength.
- **Fluorescence performance assessment:** using CytoFLEX nano Daily QC Fluorospheres the system will monitor event rate, laser delay and fluorescence detection performance. Between each tube the system will trigger an automatic backflush cycle with sheath fluid. The generated QC report confirms success at each step. If a failure occurs, it clearly indicates what needs attention.

4. Sensitivity Monitor

When fluorescence sensitivity is key, the sensitivity monitor will add confidence, showing the number of fluorescence peaks the CytoFLEX nano Flow Cytometer can resolve and the distance between the noise and dimmest resolvable peak. Select Sensitivity monitor in the QC/Sensitivity menu and input the lot you will be using. This process will run the Multi-fluorescent Fluorospheres, which are a mixture of multi-intensity multi-fluorescent 500 nm polystyrene beads. See Figure 3 for an example of the QC and Sensitivity Monitor Reports.

QC Report

Scatterspheres Lot No.: 2692901
Scatterspheres Expires: 2024-10-02
Fluorospheres Lot No.: BAQ03
Fluorospheres Expires: 2024-10-02
Detector Configuration: Default-Configuration
QC Date: 2024-01-16 09:44
Cytometer SN: MP80006
Cytometer Name: CytoFLEX nano
Sample Flow Rate: 1µL/min

Laser

Laser	Delay(µs)	Default Delay(µs)	Difference Delay(µs)	Power(mW)	Target Power(mW)	Result
Violet	0.00	0.00	0.00	116	108-132	✓
Blue	0.00	0.00	0.00	49	40-60	✓
Yellow	0.00	0.00	0.00	30	25-45	✓
Red	-0.48	0.00	-0.48	108	90-110	✓

Sheath Flow Rate/Background

Item	Value	Target	Unit	Result
Sheath Flow Rate	1801	1600-2000	µL/min	✓
Threshold (VSSC1-H)	398	≤600	-	✓
Sheath Noise	6	≤80	Events/Sec	✓
Sample Noise	5	≤150	Events/Sec	✓

Side Scatter Assessment

Event Rate

Value	Target	Unit	Result
197	≥ 50	Events/Sec	✓

Side Scatter Assessment Result

Channel	Gain	Target Gain	%Diff Target Gain	Median	Target Median	%Diff Target Median	FD	Target FD	Result
VSSC1	86	89	-3.54	1429937.0	1420000.0	0.70	8.50	1.00	✓
VSSC2	181	181	0.00	41014.0	40000.0	2.53	8.20	1.00	✓
BSSC	68	68	0.00	465571.3	460000.0	1.21	7.19	1.00	✓
YSSC	108	108	0.00	265961.9	260000.0	2.29	6.08	1.00	✓
RSSC	170	170	0.00	865046.6	860000.0	0.59	4.59	1.00	✓

Fluorescence Assessment

Event Rate

Value	Target	Unit	Result
187	≥ 50	Events/Sec	✓

Fluorescence Assessment Result (Single Laser)

Channel	Gain	Target Gain	%Diff Target Gain	Median	Target Median	%Diff Target Median	rCV(%)	Target rCV(%)	Result
VFSC	1241	1241	0.00	1011360.0	1000000.0	1.14	-	-	✓
V447	839	839	0.00	426396.9	410000.0	4.00	3.40	10.00	✓
B531	1123	1052	6.75	91220.6	90000.0	1.36	4.46	10.00	✓
Y595	1279	1201	6.49	167632.5	170000.0	-1.39	4.52	10.00	✓

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Sensitivity Monitor Report

Multi-intensity Fluorospheres Lot No.: BAQ04_SM
Multi-intensity Fluorospheres Expires: 2024-11-03
Sample Flow Rate: 1µL/min
Detector Configuration: Default-Configuration
Monitor Date: 2024-01-16 10:04
Cytometer SN: MP80006
Cytometer Name: CytoFLEX nano

Fluorescence Assessment

Event Rate

Value	Target	Unit	Result
1246	≥ 50	Events/Sec	✓

Fluorescence Assessment Result (All Lasers)

Channel	Gain	Target Gain	%Diff Target Gain	Median	Target Median	%Diff Target Median	rCV(%)	Target rCV(%)	FD	Peaks	Target Peaks	Result
V447	818	818	0.00	418218.7	410000.0	2.00	3.22	10.00	2.66	8	8	✓
B531	1229	1229	0.00	551101.0	560000.0	-1.59	3.34	10.00	4.57	8	8	✓
Y595	768	768	0.00	380877.8	390000.0	-2.34	3.53	10.00	2.49	8	8	✓
R670	535	535	0.00	156710.6	160000.0	-2.06	3.79	10.00	3.33	6	6	✓

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QC Report

Scatterspheres Lot No.: 2692901
Scatterspheres Expires: 2024-10-02
Fluorospheres Lot No.: BAQ03
Fluorospheres Expires: 2024-10-02
Detector Configuration: Default-Configuration
QC Date: 2024-01-16 09:44
Cytometer SN: MP80006
Cytometer Name: CytoFLEX nano
Sample Flow Rate: 1µL/min

Channel	Gain	Target Gain	%Diff Target Gain	Median	Target Median	%Diff Target Median	rCV(%)	Target rCV(%)	Result
R670	473	514	-7.98	80352.4	80000.0	0.44	4.17	10.00	✓
R710	1105	1223	-9.68	560552.2	560000.0	0.10	4.27	10.00	✓
R792	758	856	-11.48	201150.1	200000.0	0.58	4.18	10.00	✓

Specifications

Delay: -4.00µs ≤ Difference Delay ≤ 4.00µs.
Gain: -20.00% ≤ %Difference Target Gain ≤ 20.00%.
Median: -5.00% ≤ %Difference Target Median ≤ 5.00%.
rCV: rCV(%) ≤ Target rCV(%).
FD: FD ≥ Target FD.

Result

QC Passed.

Sensitivity Monitor Report

Multi-intensity Fluorospheres Lot No.: BAQ04_SM
Multi-intensity Fluorospheres Expires: 2024-11-03
Sample Flow Rate: 1µL/min
Detector Configuration: Default-Configuration
Monitor Date: 2024-01-16 10:04
Cytometer SN: MP80006
Cytometer Name: CytoFLEX nano

Channel	Gain	Target Gain	%Diff Target Gain	Median	Target Median	%Diff Target Median	rCV(%)	Target rCV(%)	FD	Peaks	Target Peaks	Result
R710	983	983	0.00	493619.2	510000.0	-3.21	4.06	10.00	2.27	6	5	✓
R792	850	850	0.00	211389.6	220000.0	-3.91	4.21	10.00	10.63	4	4	✓

Specifications

Gain: -20.00% ≤ %Difference Target Gain ≤ 20.00%.
Median: -5.00% ≤ %Difference Target Median ≤ 5.00%.
rCV: rCV(%) ≤ Target rCV(%).
Peaks: Peaks ≥ Target Peaks.

Result

Monitor Passed.

Figure 3. Example of QC and Sensitivity Monitor Reports.

5. The CytoFLEX nano Flow Cytometer on-board clean processes

The CytoFLEX nano Flow Cytometer offers multiple automated cleaning options to meet different sample and workflow needs. Each selected workflow ensures that researchers can accurately and reproducibly characterize EVs and other biological nanoparticles, confirming that the background noise does not impact EV/nanoparticle evaluations.

Workflows available:

- Backflush is automatically integrated into Unload process, and it will run after QC and Sensitivity Monitor. It can also be triggered by clicking on Backflush in the Acquisition Control Panel. In the Cytometer menu, select Cytometer Configuration and select the number of backflushes the instrument will run when Backflush will be initiated.
- On-board Clean has 3 pre-set options in the Cytometer Configuration menu and a customized number of back and forth (BFF) with CytoFLEX cleaner at the end:
  - **Option 1:** 1 BFF (cycle time: 7min 19s)
  - **Option 2:** 5 BFF (cycle time: 10min 26s)
  - **Option 3:** 10 BFF (cycle time: 14min 18s)
  - **Option 4:** Custom - Select Back & Forth Cycles, BFF from 1 to 10. Corresponding time will appear.
- Manual Clean is a cleaning process similar to what the CytoFLEX instruments Daily Clean offers. When the time of Manual Clean is set similar to On-Board Clean, the results are comparable. Selecting Manual Clean from the Cytometer menu, the following steps are to define the time with cleaner and time with water or sample buffer.
- Shutdown Clean
  - **Option 1:** is to perform the selected On-board Clean and shutdown automatically afterwards.
  - Overnight (recommended 8 hours) soaking of the sample line is expected to completely clear the line. To extend flexibility, soaking can be stopped at any time.
- Flow Cell Clean is necessary only in extreme cases, which a service representative can help define. The process run with freshly prepared 10% Contrad 70 solution, placed in its onboard bottle, in the front of the CytoFLEX nano Flow Cytometer.
  - Select Flow Cell Clean on the Cytometer Menu. The system rinses and soaks the Flow Cell with 10% Contrad 70 solution. The soaking is suggested to be at least 30min. Following that the instrument proceeds to rinse out Contrad 70 with multiple Flow Cell Primes.
  - Flow Cell Prime (in the Cytometer menu) can be selected and used outside of the Flow Cell Clean, and it is useful when detergents are used.

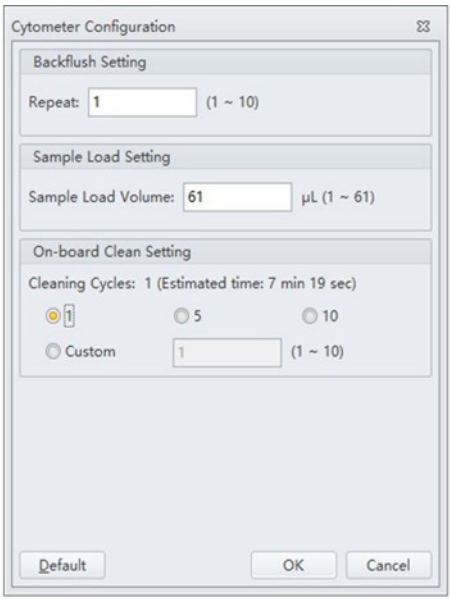

Figure 4. Backflush and On-Board Cleaning Setting.

6. Experiment Setting

- Select File and open a new experiment.
- On Cytometer Menu, select Cytometer configuration and input the volume of sample that will be collected by the sample pump from the sample tube. There is 32 µL of dead volume to consider, and the minimum volume in the sample tube is 100 µL.
- On Cytometer Menu, select Settings and Options. Sample volume is enabled by default, so the system will monitor sample consumption and stop automatically when there is less than the define volume to collect. The sample volume monitor can be disabled by unchecking that box.
- On the Acquisition Control Panel on the left, select:
  - How many events to display and the stopping rule for recording. It can be by a number of events in a specific gate, by time or by volume. If multiple options are selected the instrument will stop whichever will come first.
  - Sample flow rate. 1 µL/min is suggested for beads acquisition and 3 µL/min is suggested for biological samples. Other options available are 6 µL/min and customized between 1 and 6 µL/min.
- Use the icons on icon top bar to generate the type of histograms and plots needed for the experiment. On that menu, there are also statistics, hierarchical gating, manual and automated gates, scaling, gain and compensation tools.

7. Sizing calibration

To obtain reliable and accurate measurements, sizing calibration is needed. NanoVis product is a multi-size mix of polystyrene beads, including 44 nm, 80 nm, 100 nm 144 nm, 300 nm, 600 nm, and 1 µm. Thresholding in VSSC1, the mix results assessed by a reference calibration method will enable you to generate calibrated data with the CytoFLEX nano Flow Cytometer.

8. Sample Buffer and Baseline Monitor Setting

- Select a new tube, using the icon in the Acquisition Control Panel on the left, and load a tube of sample buffer. Click run and proceed to Acquisition Setting. From this menu, adjust threshold and gain of all the interested channels for the ongoing experiment. This step will create a baseline for the future samples that will be run.
- The sample buffer tube can be used to generate baseline monitor Settings. Select a new Blank, using the icon in the Acquisition Control Panel on the left. Right click on the tube and select Baseline Monitor. In the menu select:
  - **Blank sample type:** sample tube will require a sample buffer tube in the sample holder every time baseline monitor will be run, where sample line will trigger a pull in of 5 nm sheath in the sample line.
  - **Acceptance criteria:** events/sec or a number of events in all events or a specific gate drawn. The gate can be drawn at the right of the noise in the most sensitive scatter channel, VSSC1. This specific gate will monitor any residue in the sample line, other than the noise coming from the sample buffer, allowing a measure of the instrument readiness for the following sample.
  - **Cleaning Cycle Settings:** if the acceptance criteria are not met, define how many cycles of backflush and how many backflushes per cycle will be run. Checking On-Board Clean box will include a cycle of selected On-Board Clean. Between each cleaning cycle the instrument will reassess baseline and if the acceptance criteria are met. If yes, the monitor will stop; if not the following defined cleaning cycle will be run.
  - **Baseline monitor will be saved as fcs file:** All Cycles Data will merge all the baseline monitor run in one fcs file, where Last Cycle Data will save only the last run.
  - Once set, Baseline Monitor, with these settings, can be initiated during the experiment by generating a new Blank tube and clicking Run. Setting can be modified by right clicking on the blank tube and selecting Baseline Monitor.

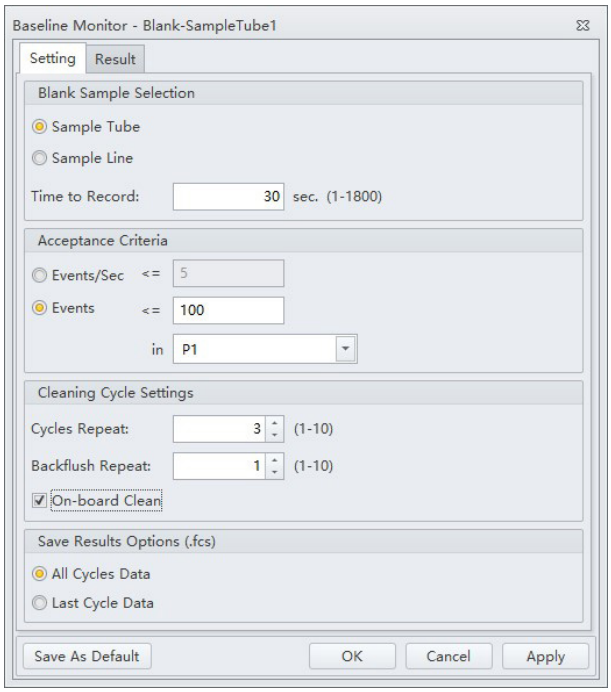

Figure 5. Baseline Monitor Setting.

9. Sample acquisition

- Using the icon in the Acquisition Control Panel on the left, create a tube with the same setting of the sample buffer, and load a sample tube.
- When working with a new sample type, start with a sample titration, running from the lowest to the highest concentration. A slight modification of gain and threshold can be applied to best resolve the sample. If that is the case, we suggest repeating the sample buffer run and changing the Baseline Monitor Setting.
- Coincidence and swarming, as cellular flow cytometry, depend on sample concentration but for the CytoFLEX nano Flow Cytometer, it is also dependent on particle size. For samples with a size higher than 100-150 nm, the order of 104 particles/µL can be considered a good concentration and a limit before swarming. The more representation of small particles (and/or higher the small EV/large EV ratio) allows a higher concentration to be run. If swarming conditions are too high the Events Processed % in the Acquisition Control Panel will be lower than 100%.
- Once the best sample dilution is selected, based on the number of events per second or in a specific gate, and sample resolution and representation in the plots of interest, start running single-color stained samples and adjust fluorescence gains in the acquisition setting menu.
- When a panel of color will be run, compensation can help correct for fluorescence spillover, removing undesired signal. Compensation can be done using the Compensation Matrix, selecting the icon in the Acquisition control panel, or done manually using the icon in the top bar.
- CytoFLEX nano Flow Cytometer offers fluorescent triggering options that can help when focusing on specific stained/labeled populations.
- Baseline Monitor is suggested between different sample types, or when a lower concentration will be run, or when an unstained sample will be run after a stained one.
- Always run all controls as suggested by MIFlowCyt-EV position paper (ref #1). If a detergent-treated sample will be run, we suggest a detergent titration first, starting from the lowest concentration. This will evaluate the detergent background on CytoFLEX nano Flow Cytometer. When multiple tubes of detergent or a high concentration will be run, perform debubble options and Prime to eliminate possible nano- and micro-bubble formation.

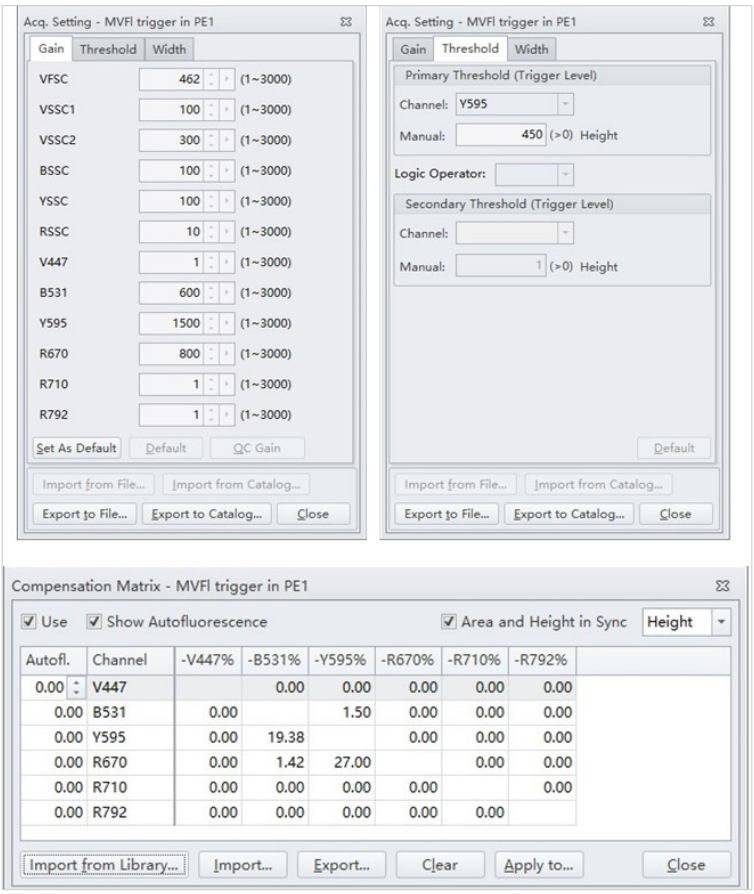

Figure 6. Acquisition setting and Compensation Matrix.

10. Cleaning and System Shutdown Program

- Between different experiments, an On-Board Clean is suggested. Select Cytometer in the menu bar, followed by On-Board Clean.
- At the end of the day, select Cytometer in the menu bar, followed by System Shutdown program. The program provides three options for different needs:
  - Clean the sample line with cleaner with the selected On-Board clean cycle, prior to the System Shutdown.
  - Running long-term soaking prior to the System Shutdown.
  - System Shutdown only.

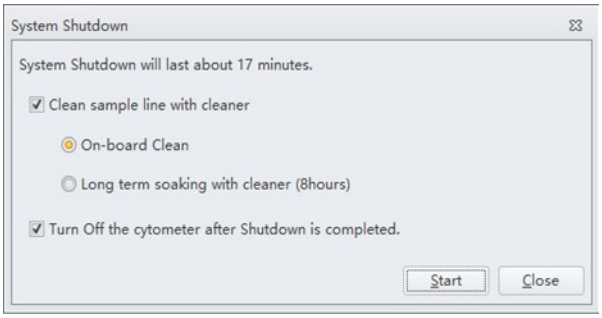

Figure 7. System Shutdown.

Conclusions

The CytoFLEX nano Flow Cytometer is the first flow cytometer that can clearly demonstrate detection of extracellular vesicles down to a size of 40 nm (measured with polystyrene beads). Its high sensitivity and resolution for small particles, combined with automated cleaning, extensive QC process and Fluorescence Sensitivity monitor, will undoubtedly propel the field of research forward. The capabilities and features of this instrument will enable researchers to explore previously uncharted territories and obtain more comprehensive and accurate data. With this cutting-edge tool, we anticipate significant advancements in research findings and a deeper understanding of extracellular vesicles, and their applications.

References

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