Preparing a CytoFLEX for Nanoscale Flow Cytometry

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Introduction

Built around semiconductor technology, with a number of innovations to enhance light capture, reduce noise, and prevent signal losses, the CytoFLEX is capable of detecting biological nanoparticles (NPs) as small as 80nm by light scatter, and has a linear fluorescence range that extends down into the single digits for fluorophores like FITC. However, in order to properly setup the CytoFLEX for NP analyses, a variety of considerations need to be taken into account.

In this poster, we will demonstrate how to properly setup and clean a CytoFLEX flow cytometer for NP analyses. First, we will explore the different threshold options and sensitivity ranges. Next, we will show how to setup violet side scatter (VSSC) triggering. And finally, we will discuss several important issues that affect proper sample analyses, including how to clean the instrument to reduce noise within the nanoparticle range.

Materials

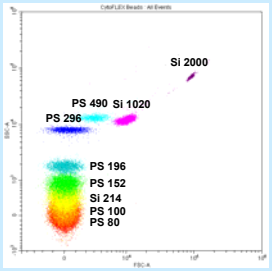
Item	Catalog #	Vendor
80nm PS NIST Size Standard	3080A	ThermoFisher
100nm PS NIST Size Standard	3100A	ThermoFisher
150nm PS NIST Size Standard	3150A	ThermoFisher
300nm PS NIST Size Standard	3300A	ThermoFisher
HPLC Water	WX0008-1	EMD Millipore
Izon qEVsingle/70nm Columns	SP2	Izon Science
0.2μm Acrodisc Syringe Filter	4612	Pall Corporation
CD61-PC7	IM3716	Beckman Coulter
FlowClean Cleaning Agent	A64669	Beckman Coulter
CytoFLEX Sheath	B51503	Beckman Coulter
CytoFLEX-S (N-V-B-R)	B78557	Beckman Coulter
CytExpert Software v2.3	N/A	Beckman Coulter
CytoFLEX Sizing Mix Prototype	N/A	Beckman Coulter

Methods

1. Upon startup, the instrument was primed, cleaned and flushed.
2. The beads were mixed together and diluted with HPLC water.
3. Extracellular Vesicles (EVs) were prepared from fresh human blood as follows:
   a) 2mL of K3-EDTA blood was first aliquotted into 12x75mm centrifuge tubes. Cells in larger volumes, further away from the max radius, do not pellet as well within a short time frame and larger EVs pellet with longer time frames, so if a larger volume is needed, increasing the number of tubes works better than a greater volume per tube.
   b) The blood was centrifuged for 5 min at 200xg to pellet the majority of cells and platelets.
   c) Roughly 1mL of platelet-poor plasma (PPP) was removed from the top, careful to minimize collection of the platelet-rich plasma near the WBC layer.
   d) The PPP was filtered through a 200nm syringe filter to remove residual large particles.
   e) Finally, the filtered PPP was further purified using Izon size-exclusion columns to remove particles smaller than 70nm.
4. All samples were acquired on a CytoFLEX-S N-V-B-R, and the data were analyzed in CytExpert v2.3.

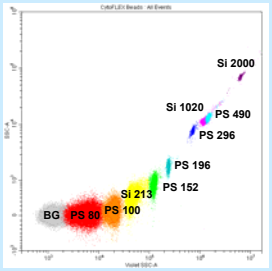
I. Light-Scatter Sensitivity

FSC-A/SSC-A



Forward Scatter (FSC) on the CytoFLEX is not small-angle scatter, as found on a typical flow cytometer. It is a digital signal-analysis method, called axial light loss detection, which is optimized for 500nm-50μm particles. It depends directly on the particle volume and is mostly independent of the refractive index.

VSSC-A/SSC-A



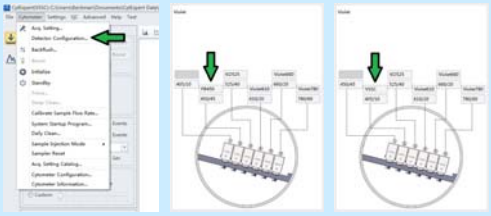
Side Scatter (SSC) is much more sensitive, but is attenuated for use with cells and other large particles. It is optimized for roughly 200nm-20μm.

Violet SSC (VSSC) is a 3rd scatter-detection mode that is available to take full advantage of the CytoFLEX sensitivity. VSSC can fully resolve 80nm PS or 100nm Si beads.

II. Setting up VSSC-H Triggering

1) Set the Configuration to VSSC

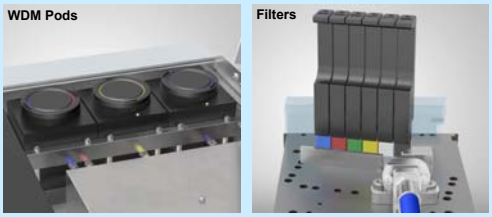
Configuration Menu | Default Configuration | V-SSC Configuration



a) If a VSSC configuration does not exist, start a new one.
b) Pick a channel and assign it as VSSC with the 405/10 filter.
c) Save the configuration and set it as Current.

2) Physically Swap the Filters

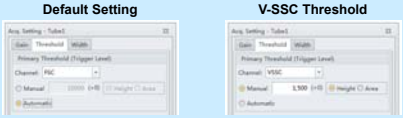
WDM Pods | Filters



a) Make sure that there is a filter in each position preceding the channels of interest or the light path will be broken.
b) Do not place an equivalent bandpass filter upstream of a channel of interest or this will also result in the loss of the desired signal.

3) Set the Threshold to VSSC-H

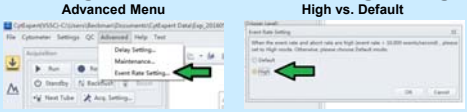
Default Setting | V-SSC Threshold



a) VSSC-Height is the most sensitive trigger on the CytoFLEX for light-scatter detection. VSSC triggering requires 1, 2, and 3 together.
b) The specific threshold level will depend on the VSSC Gain, and will change proportionally to any adjustments to the gain.
c) The instrument always triggers in Height. Area back-calculates a relative Height setting, so it is more precise to set the Height directly.

4) Change the Event Rate Setting to High

Advanced Menu | High vs. Default

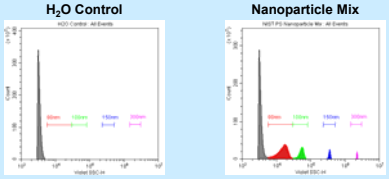


a) If the Event Rate Setting is in Default, change it to High.
b) The Default setting broadens the pulse window in order to help with setting up laser alignments and delays if they are out of line. The broader window will also increase the optical signal noise sampled.
c) The High setting reduces background and improves event processing. The instrument should always be set on High for sample acquisition.

III. Important Considerations for Effective Nanoscale-Flow-Cytometry Experimentation

1) Identify the Optimal Threshold

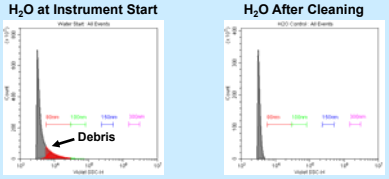
H_2O Control | Nanoparticle Mix



a) Use appropriate reference particles to identify the optimal threshold. Using buffer alone will not properly identify the detection boundaries.
b) On the CytoFLEX, the appropriate threshold level is generally around 10-fold higher than the gain. Above, the Gain = 400, Threshold = 3K.

2) Clean the Sample Probe

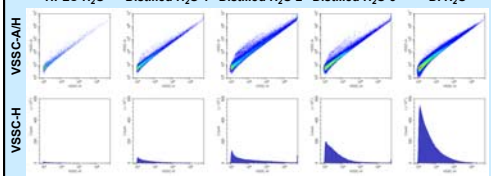
H_2O at Instrument Start | H_2O After Cleaning



a) Debris from samples and buffers builds up in the probe, mostly visible below the 100nm PS range. Carryover can even occur between reads.
b) Clean the probe by running a panel of Bleach, FlowClean, and then 2x Water to flush, for 1-2 min each at the max rate. Repeat if needed.

3) Use Clean Buffers

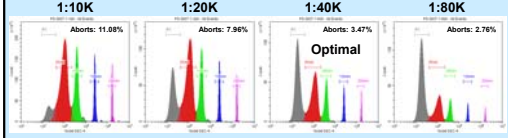
HPLC H_2O | Distilled H_2O 1 | Distilled H_2O 2 | Distilled H_2O 3 | DI H_2O



a) Any particulate in the sample buffers will also be detected as events.
b) Filtering dirty buffers is generally insufficient because the particulate is often smaller than the filter. Discard and use fresh clean buffers.

4) Optimize the Sample Concentration

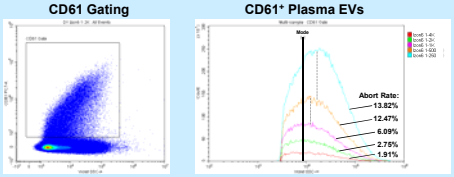
1:10K | 1:20K | 1:40K | 1:80K



a) Flow cytometers have an optimal rate, inversely proportional to size. If the sample concentration is too high, swarming will occur.
b) The concentration needs to be titrated to find the optimal range.
c) At the appropriate concentration range, different dilutions will result in a shift up or down on the Counts axis, without shifting the intensity.
d) The Abort Rate is also a swarming indicator: <5% is best.

5) The Abort Rate Can Help Optimize

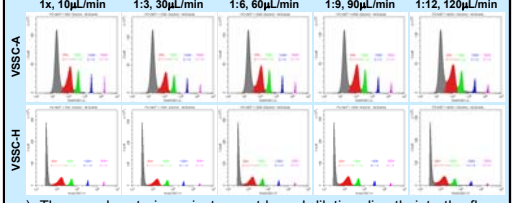
CD61 Gating | CD61+ Plasma EVs



a) The Abort Rate is particularly helpful with optimizing the dilutions of complex samples, e.g., without a tight monomodal distribution.
b) When under 10%, the population medians should be consistent between dilutions. <5% is best.

6) Balance the Concentration and Rate

1x, 10μL/min | 1:3, 30μL/min | 1:6, 60μL/min | 1:9, 90μL/min | 1:12, 120μL/min



a) The sample rate is an instrument-based dilution directly into the flow cell. Faster rates proportionally increase the effective concentration.
b) Faster rates also broaden the core stream and can increase CVs.

Discussion

Ultimately, the CytoFLEX is one of the most sensitive flow cytometers on the market. However, with such great power comes great responsibility to properly prepare the instrument and samples for effective nanoscale-flow-cytometry experiments. Nanoscale flow cytometry introduces a number of additional variables that are largely extraordinary to conventional flow cytometry, and each requires careful attention.