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Evaluation of Cytometer Sensitivity and Stability using Automated Analysis

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INTRODUCTION

Flow cytometers are standard tools of the researcher, providing simultaneous measurement of multiple parameters for single cells. The performance of a flow cytometer may vary between instruments, or over time within the same instrument. Evaluation of cytometer performance is essential to ensure results are consistent within these parameters. Basic capabilities of each fluorescent detector can be estimated using the Q and B calculations, where Q is detection efficiency and B is background [1-4].

In this study hard-dyed multi-level, multi-dye beads were collected in three different identically configured cytometers over 11 days, in 14 different fluorescent detectors at six flow rates (12.5 µL/min, 25 µL/min, 100 µL/min, 200 µL/min, 500 µL/min, and 1000 µL/min). All data was first evaluated with flowCut to check for spurious events. An automated approach to data analysis (flowQB) was then utilized to provide unbiased evaluation of Q and B [5-6]. These were used to evaluate intra-instrument and inter-instrument performance and stability.

MATERIALS

- Three identically configured Invitrogen™ Attune™ NxT Flow Cytometers equipped with four lasers and 14 fluorescent detectors (Thermo Fisher cat # A24858) (Figure 1)
- Attune Performance Tracking Beads (PT beads) (Thermo Fisher cat # 4449754)
- Rainbow Calibration Particles, 8 peaks (Spherotech cat # RCP-30-5A)
- Attune Focusing Fluid 1X (Thermo Fisher cat # 4488621)

METHOD of ACQUISITION

- The three Attune NxT Flow Cytometers were evaluated in parallel in an identical manner. Daily Startup, Performance Tracking Testing, and Shutdown were performed each day as listed in the Attune NxT Instrument User Guide.
- Each day, three drops of PT beads were added to 2 mL Focusing Fluid in a 12 x 75 mm tube, and vortexed before running.
- Each day, three drops of Spherotech 8 peak beads were added to 2 mL Focusing Fluid in a 12 x 75 mm tube, and vortexed before running.
- The workspace used FSC (linear) vs. SSC (linear), with a region around the main population of beads for gating histogram plots (log) of each of the 14 fluorescent detectors and a time (linear) vs. BL1 (log) plot.
- Area, Height, and Width were collected for each parameter.
- Stop criteria was set at 20,000 events within the gated main bead population.
- Instrument voltages were set daily using the bright peak of the PT beads at a median of 300,000 ± 5000 as a target reference range in each of the 14 fluorescent detectors.
- Following this, daily collection of 8-peak beads used the voltages required for placement of the PT bright peak bead in the target reference range.
- Each day, 8 peak beads were collected in every fluorescent detector at six flow rates: 12.5 µL/min, 25 µL/min, 100 µL/min, 200 µL/min, 500 µL/min, and 1000 µL/min.
- Data was collected for 11 days.
- Results were analyzed in the Attune NxT Software to monitor acquisition (Figure 2).
- All 198 files were exported for automated analysis.

Figure 1. Optical Configuration of the 4 laser Attune NxT Flow Cytometer

Attune NxT instrument Cat # A24858		
Laser	**Detector**	**Emission Filter (nm)**
Blue 488 nm 50 mW	BL1	530/30
	BL2	590/40
	BL3	695/40
Yellow 561 nm 50 mW	YL1	585/16
	YL2	620/16
	YL3	695/40
	YL4	780/60
Red 638 nm 100 mW	RL1	670/14
	RL2	720/30
	RL3	780/30
Violet 405 nm 50 mW	VL1	440/50
	VL2	512/25
	VL3	603/48
	VL4	710/50

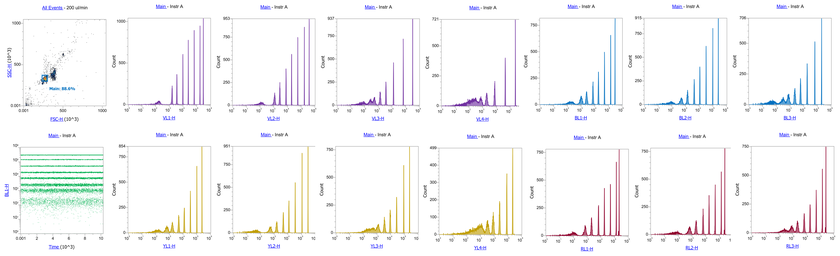

Figure 2. Example of 8-peak bead data as displayed on the Attune NxT Flow Cytometer. Gating is on the main population of beads in FSC vs. SSC, collected at 200 µL/minute flow rate, as analyzed in the Attune NxT Software in all 14 fluorescent detectors. A time vs. BL1 plot was also used to monitor fluidic stability. Data taken from Instrument A day 11.

RESULTS

Flow cytometers may have fluidic inconsistencies due to a number of issues; clogging of the flow cell, sample injection port, or tubing are among the most common. Of the 198 files analyzed, flowCut found two files that met the criteria for removal of minor outlier events (Figure 3). This indicates stable fluidics across all instruments and days in the study. The use of flowQB automated gating eliminates subjective gating (Figure 4) and identifies differences between samples (Figure 5). Q and B were largely stable across time and cytometers. Q decreased at the two highest flow rates of 500 µL/minute and 1000 µL/minute (Figure 7A-F), an expected finding as these flow rates run at double the fluidic velocity of the other flow rates tested. Instrument C has a slightly different Q value.

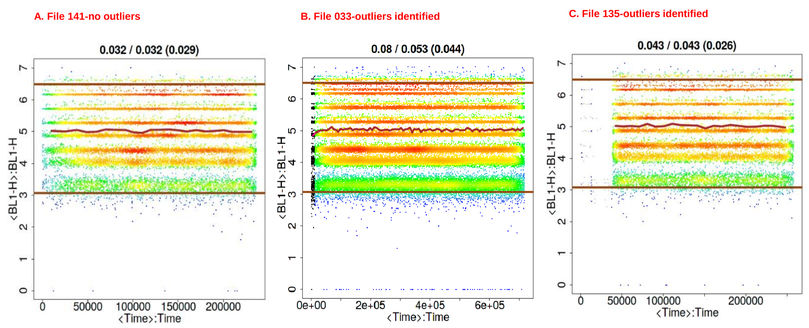

Figure 3. Example flowCut analysis, time vs. BL1 fluorescence
The flowCut analysis identified only two files out of 198 files where outlier events were identified and removed, indicating fluidic stability of instruments tested. Plots show time vs. BL1 with flowCut removed points in black or grey A) flowCut data file has no outlier events identified, Instrument C day 11 using the 12.5 µL/minute flow rate. B) flowCut data file has minimal outlier events identified and removed, Instrument A day 4 using the 12.5 µL/minute flow rate. C) flowCut data file has minimal outlier events identified and removed, Instrument C day 10 using the 12.5 µL/minute flow rate.

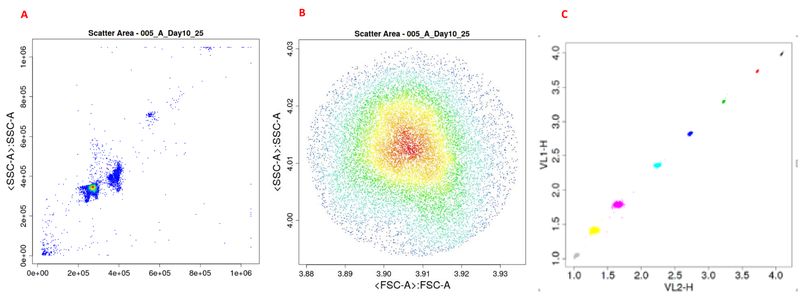

Figure 4. flowQB automated gating example
A) displays the beads in FSC vs SSC B) shows the main bead population identified and used for further analysis. C) is an example of a dual parameter plot of the multi-peak data gated on the main bead population.

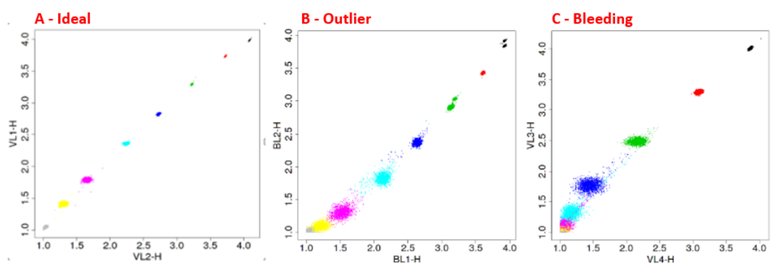

Figure 5. flowQB identifies differences
A) The expected identification of 8 distinct clusters is shown, file from Instrument A day 5 using 25 µL/min flow rate. B) There was an unusual double peak observed for peaks 6 and 8, file from Instrument C day 5 at 1000 µL/min flow rate. This is the only file removed from the data set analysis. C) The bead populations are less distinct at the lower end, and show minor bleeding of fluorescence between clusters, file from Instrument C day 7 at 1000 µL/min flow rate.

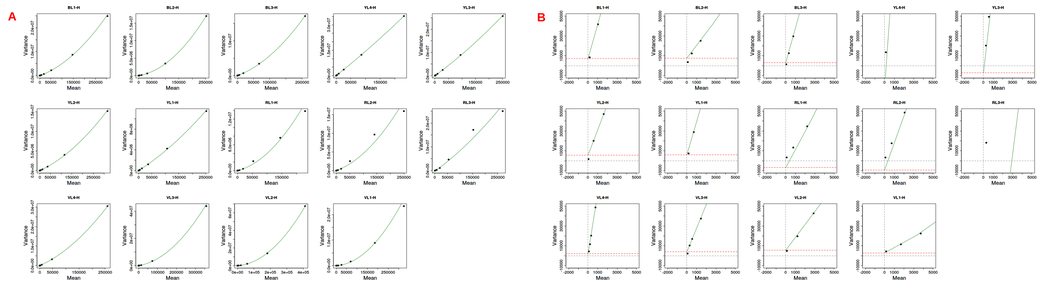

Figure 6. Curve fitting, data taken from Instrument B day 10 collected with the 200 µL/min flow rate A) Display the curve fitting results for each of the fluorescent detectors. B) zoomed-in display of the same curve fitting graphs, showing detail at the intercept.

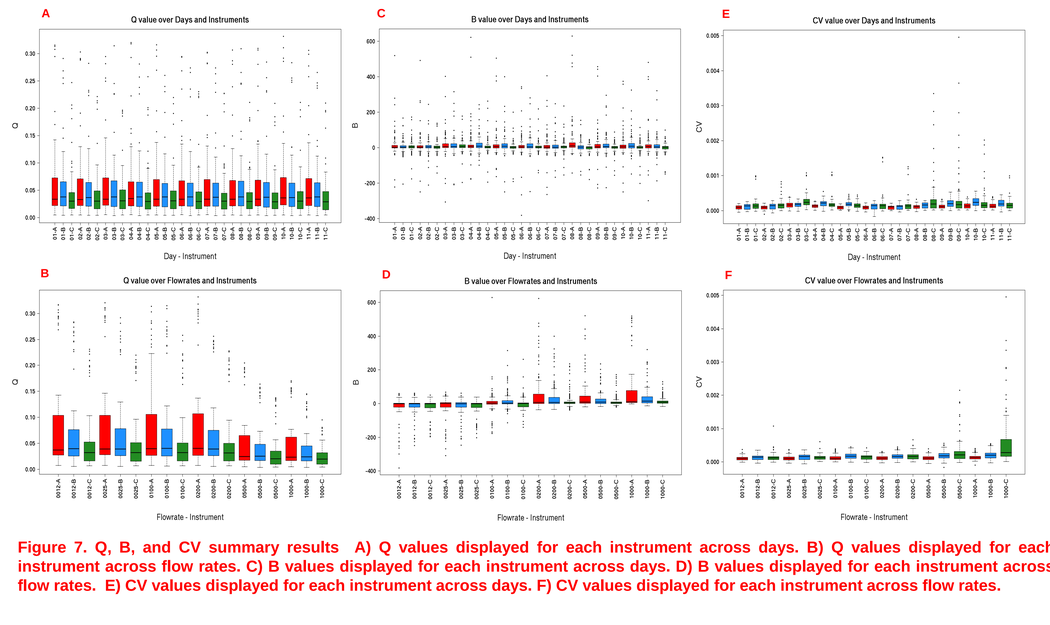

Figure 7. Q, B, and CV summary results A) Q values displayed for each instrument across days. B) Q values displayed for each instrument across flow rates. C) B values displayed for each instrument across days. D) B values displayed for each instrument across flow rates. E) CV values displayed for each instrument across days. F) CV values displayed for each instrument across flow rates.

DISCUSSION

In this study, a quadratic curve fit was applied to the bead clusters for curve fitting, the amount of curvature the quadratic curve fit has determines the CV of each sample. The closer the curve is to linear, the closer the CV value is to zero. In some cases the y-intercept is negative, making the B value negative and complicating the interpretation of the B value. These curve fitting plots show a limitation of the multi-peak bead method; by including the brightest beads in the analysis, the fit of the curve extends over a very broad dynamic range and can easily skew results at the lower end. Another limitation is from the bead set itself, as each individual bead population has a specific spectral output that is slightly different due to different ratios of dyes used. The use of multi-intensity beads with a single fluorophore may overcome this particular limitation.

The data analyzed in the study shows the Q values for each instrument are consistent across the length of the study (7A) and consistent across the lower four flow rates, with an expected slight decrease at the highest two flow rates due to fluidic velocity (7B). The Q values are similar for instruments A and B, while Instrument C has a slightly different Q value. The data shows the B values for are consistent across days and instruments (7C) and across flow rates (7D). The data shows the CV values are consistent over days and instruments (7E) and across flow rates, however instrument C has a slightly higher CV at the 1000 µL/min flow rate (7F).

CONCLUSIONS

- The paucity of event removal with flowCut indicates remarkable fluidic stability across all instruments and days in the study, and contributes to the overall system performance of the Attune NxT Flow Cytometers evaluated in this study.
- Consistency of performance across all instruments is demonstrated, over time and across flow rates within the same sheath velocity.
- Automated analysis can be used to monitor intra-instrument and inter-instrument differences, daily and over time, increasing confidence in data collected.
- This type of automated analysis allows for easy identification of outlier parameters.
- Tracking of Q and B can provide valuable information as part of an instrument QC program.
- Robust automated analysis can be used to automatically calculate the detector efficiency (Q), optical background (B) and CV of the bead set measurement, eliminating operator subjectivity and streamlining effort.

REFERENCES

1. Chase and Hoffman. *Resolution of dimly fluorescent particles: a practical measure of fluorescence sensitivity*. Cytometry, 33(2):267–279, 1998
2. Hoffman and Wood. *Characterization of flow cytometer instrument sensitivity*. Curr Protoc Cytom, Unit 1.20, 2007
3. Perfetto, et.al. *Q and B Values are Critical Measurements Required for Inter-Instrument Standardization and Development of Multicolor Flow Cytometry Staining Panels* Cytometry Part A, 85A: 1037-1048, 2014
4. Wang and Hoffman. *Standardization, calibration, and control in flow cytometry.* Curr. Protoc. Cytom. 79:1.3.1-1.3.27 2017
5. Parks, et. al. *A Methodology for evaluating and comparing flow cytometers: A multisite study of 23 instruments.* Cytometry A, 93A: 1087-1091, 2018
6. Parks, et. al. *Evaluating flow cytometer performance with weighted quadratic least squares analysis of LED and multi-level bead data.* Cytometry A; 91A: 232– 249, 2017

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