Invitrogen Bigfoot Spectral Cell Sorter

Voltage setting based on target MFIs increases spectral resolution and simplifies spectral workflows on the Invitrogen™ Bigfoot™ Spectral Cell Sorter

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Abstract

Spectral flow cytometry is a crucial new tool useful for researchers in many different areas of science. It enables the building of higher parameter panels that identify rarer populations. Scientists can get a more complete view of a cell sample and look at many different cell subsets simultaneously. Furthermore, it saves cells and other resources because multiple samples can be combined into one tube. However, many researchers still shy away from spectral flow cytometry because they find the technology too complex. Finding solutions that make the technology less intimidating is therefore of paramount importance to drive the field forward.

One of the biggest hurdles is setting ideal voltages for each of the fluorescent detectors found in a spectral instrument and combining these settings to maximize the unique signatures that can be used at the same time. This will ensure that every fluorophore in a panel appears as bright as possible. Finding these ideal voltages consumes time and other materials. So, our group has devised a set of optimized voltages based on target MFIs and we have automated this voltage setting process. This makes instrument optimization faster and more user friendly. Furthermore, this method uses the QC beads on-board the instrument, so no additional materials need to be procured.

To arrive at these optimized target median fluorescent intensities (MFIs) the team first started with traditional voltage walks and found the most sensitive region for every detector in our system. Next, we compared four different methods of voltage setting to find the one that maximized the number of unique fluorophores peaking in distinct detectors. Finally, we fine-tuned the voltages to minimize spread in out the negative populations.

The result was a set of PMT voltages able to identify over 30 unique fluorophore signatures. High parameter panels with more than 20 colors had higher fluorophore resolution particularly for some difficult fluorophores that were previously hard to resolve. Additionally, we show that these voltage settings standardized the appearance of signatures between instruments, yielding very similar signatures on several different instruments.

We therefore added the process of setting voltages to the standard QC protocol to simplify running and sorting from high parameter spectral panels. This process enhances the spectral workflow because it makes running high parameter panels easier and more approachable for our Bigfoot™ Spectral Sorter users.

Introduction

The Bigfoot Spectral Cell Sorter is available in 5 to 9 laser configurations with up to 55 detectors used in the spectral workflow, which require optimization for peak instrument performance. In **Figure 1** we show an example detector array of a 7-laser spectral instrument. Each laser has its own set of associated detectors. Ideal settings for each detector must be found to maximize the signal of a high number of fluorophores. However, the way detector gains are balanced both within and between lasers is also crucial to maximize the signatures that can be successfully distinguished by spectral unmixing algorithms. Furthermore, the process of optimization should yield signatures that are consistent across time and look similar across different instruments.

Figure 1. Overview of the Bigfoot detectors that were optimized. All detector descriptions are in nano meters.

Laser

	UV 349	V 405	VB 445	B 488	Y 561	R 640
Dedicated Detectors	UV1 387/11	V1 420/10	VB1 465/22	B1 507/19	Y1 575/15	R1 670/30
	UV2 420/10	V2 434/17	VB2 525/36	B2 549/15	Y2 589/15	R2 700/13
	UV3 434/17	V3 455/14	VB3 583/30	B3 583/30	Y3 605/24	R3 720/24
	UV4 455/14	V4 473/15	VB4 650 LP	B4 615/24	Y4 625/15	R4 760/50
	UV5 473/15	V5 507/19		B5 670/30	Y5 661/20	R5 770/LP
	UV6 507/19	V6 549/15		B6 720/60	Y6 685/15	
	UV7 549/15	V7 575/15		B7 750 LP	Y7 700/13	
	UV8 575/15	V8 615/24			Y8 720/24	
	UV9 615/24	V9 661/20			Y9 760/50	
	UV10 670/30	V10 710/20			Y10 800/12	
	UV11 728/40	V11 748/33			Y11 832/37	
	UV12 750 LP	V12 770/LP			Y12 860 LP	

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Methods

The first step of optimization is performing voltage walks

We used the standard Bigfoot calibration beads, that are onboard the instrument for QC, and acquired them in 50-volt (V) increments ranging from 200-950 V on five instruments. The resulting FCS plots were gated, the staining index (SI) calculated and used to plot SI curves. An example of a typical SI curve can be found in **Figure 2**. The ideal target MFI of the detector was defined as the beginning of the curves plateau where the signal to noise ratio was at a maximum. Voltage walks were performed in five instruments ranging in location and configuration and the ideal voltage for each detector is plotted in **Figure 3**. Additionally, the average median fluorescence intensity (MFI) for all ten instruments was calculated and plotted. We observed some variation across instruments especially if they had different configurations. The highest variance was found in detectors of higher wavelength.

Figure 2: Staining Index curve showing how the staining index of a detector changes with increasing voltages. Pos MFI = the MFI of the positive bead in Bigfoot calibration beads. Neg MFI = the MFI of the negative bead in the same sample.

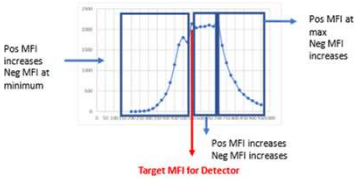


Figure 3. Ideal voltages for each detector on five representative Bigfoot instruments. Voltage walks were performed using Bigfoot calibration beads. Every color symbol represents a different instrument. The average voltage is graphed as black dots.

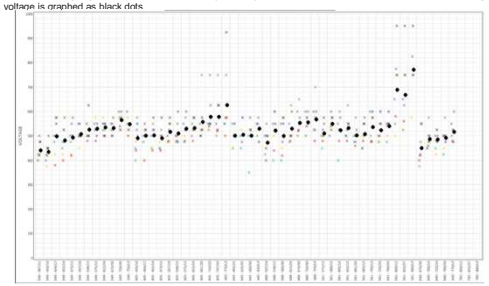
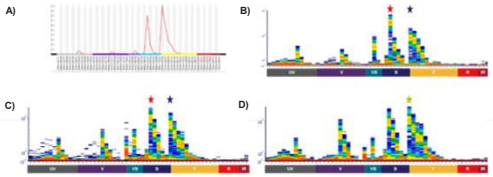


Figure 4. PE signature in four different voltage versions. A) Ideal PE signature. Shown in Invitrogen™ Sasquatch™ Software (SQS) on a 7-laser Bigfoot sorter. B) When each detector is set to optimized setting. Note highest peak in blue channel. C) When every laser's detectors are set to the laser's average target MFI. Note highest peak in blue channel. D) Using balanced laser settings. Note the peaks in this signature match image A, the expected PE signature. B,C,D were analyzed using FCS Express™ Software.



Optimizing settings maximize the number of correct fluorophore signatures

Once we compiled the data for the voltage walk, we sought to determine how fluorophores compared to theoretical signatures if we set every channel to its ideal voltage. We stained human PBMCs with anti-CD4 in 32 different fluorophores and ran the samples on a 7-laser Bigfoot sorter. We found that many fluorophores peaked in the wrong laser leading to increased spectral overlap. Additionally, many fluorophores had abnormal emission spectra (**Figure 4B**). Thus, we took further steps to optimize the settings so that the maximal number of signatures peaked in the correct detectors (**Figure 4C-D**). The efforts to fine tune each detector are summarized in **Table 1**.

Table 1. Summary of different voltage setting approaches utilized in every step of target MFI optimization.

Setting version	Brief description
1	Ideal MFI for every detector in the array
2	MFI averaged across lasers and every detector in the laser was set to that average
3	Lasers flat-topped and set relative to each other
4	Increase in MFIs in yellow laser detectors
5	Stepping down MFI the violet laser detectors
6	Reducing negative bifurcation in far red detectors
7	Reducing spread by turning down noisy detectors

Results

Testing of ideal target MFI voltages

We stained human PBMCs with anti-CD4 in 35 different fluorophores and ran the samples on four different 7- and 9-laser Bigfoot instruments using the optimized target MFIs. An example of the data collected for the is shown in **Figure 5**. The primary peak and overall signature matched for 34 of the 35 fluorophores tested. The signature peaked in the "correct" channel, according to the SQS, in 32 out of 35 fluorophores analyzed. In the three cases where the fluorophore did not peak in the expected channel there was still signature agreement across all instruments. In summary the optimized target MFI ensured cross instrument standardization and consistency.

To determine the impact of these settings on a high parameter spectral panel, we evaluated a 24-color mouse bone marrow hematopoiesis panel (data not shown) and compared population resolution to pre-existing settings (**Figure 6**). After optimization, greater and more granular separation of several populations was achieved.

Figure 5. Human PBMCs stained with anti human CD4 in Super Bright 436 (A) and PE (B) and ran on four different 7- or 9-laser Bigfoot instruments using voltages set by optimized target MFIs.

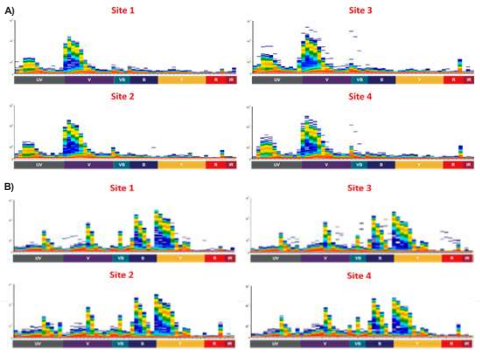
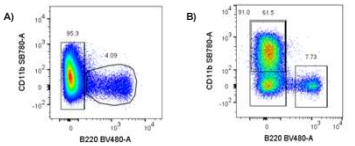


Figure 6. Comparing fluorophore performance pre and post voltage optimization. Mouse bone marrow was stained with a 24-color panel to assess hematopoiesis and acquired on a 7-laser Bigfoot sorter pre- (A) and post- (B) voltage optimization. CD11b vs. B220 plots are shown as an example of populations that show increased resolution. Analysis was performed using FlowJo™ Software..



Automation

After testing the optimized target voltage internally and receiving positive feedback from beta testers and customers, the voltage setting was automated. Starting with SQS version 19.2., each detector's voltage is optimized to meet the target MFIs detailed in **Figure 7** as part of our automated one-button QC process. The automation ensures that the performance of the instrument is standardized across days. It is additionally designed to save reagents and time.

Figure 7. Summary of the optimized target MFIs automatically set by QC.

UV 349	Target	V 405	Target	VB 445	Target	B 488	Target	Y 561	Target	R 640	Target
UV1 387/11	20000	V1 420/10	9000	VB1 465/22	1200	B1 507/19	10000	Y1 575/15	8000	R1 670/30	7500
UV2 420/10	20000	V2 434/17	9000	VB2 525/36	5000	B2 549/15	10000	Y2 589/15	8000	R2 700/13	8500
UV3 434/17	20000	V3 455/14	9000	VB3 583/30	5000	B3 583/30	10000	Y3 605/24	8000	R3 720/24	8500
UV4 455/14	20000	V4 473/15	9000	VB4 650 LP	4000	B4 615/24	10000	Y4 625/15	8000	R4 760/50	7500
UV5 473/15	20000	V5 507/19	9000			B5 670/30	10000	Y5 661/20	8000	R5 770/LP	7500
UV6 507/19	20000	V6 549/15	8000			B6 720/60	10000	Y6 685/15	3500		
UV7 549/15	20000	V7 575/15	8000			B7 750 LP	3000	Y7 700/13	2000		
UV8 575/15	20000	V8 615/24	8000					Y8 720/24	8000		
UV9 615/24	20000	V9 661/20	3000					Y9 760/50	8000		
UV10 670/30	20000	V10 710/20	7000					Y10 800/12	200		
UV11 728/40	20000	V11 748/33	5500					Y11 832/37	350		
UV12 750 LP	20000	V12 770/LP	1250					Y12 860 LP			

Conclusions

Optimized voltages based on target MFIs simplify spectral experiments on the Bigfoot Spectral Cell Sorter by:

- Maximizing the resolution of over 30 fluorophores
- Minimizing negative spread for cleaner unmixing
- Standardizing instrument performance across instruments
- Using onboard reagents
- Reducing setup time and reagent cost through automation

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