Detector Linearity, Relative Efficiency, and Relative Background Calculation Using the ZE5 Cell Analyzer

Protocol

Introduction

This protocol explains how to create a template experiment in order to calculate detector linearity, relative efficiency (Qr), and relative background (Br) across all fluorescence channels and all detector/laser configurations of the Bio-Rad ZE5 Cell Analyzer. This procedure can be used to verify the linearity of a cytometer's amplifiers in response to fluorescent signal input and to determine each fluorescent detector's efficiency at converting optical signals to electronic signals. This information can help assess detector sensitivity and resolution in order to select the best detector for optimal dim-signal detection, and to compare the optical performance of detectors across instruments or over time.

Background

The Qr and Br results are a relative readout of instrument performance using a specific set of molecules of equivalent soluble fluorochrome (MESF) values. The channel assignments of the MESF values were based on those supplied by Spherotech, Inc. for a specific set of beads (AJ02). The results do not represent absolute values of minimum detectable fluorescent molecules or molecules of background in any channel. Instead, they are intended as a guide in identifying which channels have better resolving power for dim signals and provide lower background levels.

If an absolute value is required, MESF values provided by Spherotech can be assigned to the channels available on the instrument. The assignment of these values should be based on operator judgment. MESF values can be downloaded from the Spherotech website. For an in-depth discussion of the principle and practice of this method, refer to Hoffman and Wood (2007).

Required Materials

- ZE5 Cell Analyzer of any configuration. System should be in good working order and have passed quality control after the most recent startup
- Spherotech Ultra Rainbow Calibration Kit, 6 peaks, Fluorescent Particles (#URCP 38-2K or URCP 38-20K)
- PBS (calcium- and magnesium-free)
- Choice of media (sample vessel), such as 1.5 ml Eppendorf tube or 5 ml tube
- Flow Cytometry Standard (FCS) Express Software template
- FCS Express Software, version 7.16.0035 or higher
- Spherotech MESF values corresponding to the bead lot used in the protocol (found on the Spherotech website)
- Pipet and tips

Procedure

Template Setup and Data Collection

1. Using the Ultra Rainbow Calibration Kit, dilute the fluorescent particles into PBS. Add 2 drops of blank beads and 2 drops of fluorescent beads to 1 ml of PBS in a desired sample vessel type.
2. Load sample onto loader.
3. Click "New Experiment" in Everest Software.
   a. Name the experiment "linearity test."
   b. Select appropriate media that corresponds with your sample vessel.
   c. In the "Fluorophores" tab, go to "Available Detection" and click "Enable All" to enable all detectors.
   d. In the "Plate Setup" tab, select a sample position that corresponds to your loaded sample. Name the sample "URCP-38-2K Lot#" and add any other important identifying information (instrument number, experiment number, date) to distinguish samples during analysis.

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4. Set up sample run conditions as follows:
   a. Stop Volume: 100 µl
   b. Event limit: 30,000 events
   c. Wash-ON: Normal
   d. Agitation-ON: 5 seconds
   e. Return Sample: ON
   f. Rate: 0.3 µl/s
   g. Reagent: OFF
5. In the "Plots and Gates" tab, create a rectangular gate in the default FSC 488 vs. SSC 488 density plot (default name R1).
6. In the "Plots and Gates" tab, use the hammer tool to create histograms of all parameters, select Filter = R1, then select "Log and Area" for histograms display.
7. Send the experiment to the "Acquisition" tab.
8. In the "Acquisition" tab, select "Setup and Cycle" for 1.0 sec, then begin the sample acquisition.
9. Click "PMT Control" and select the 488 nm laser. Adjust the FSC 488/10 and SSC 488/10 PMTs until the singlet population of beads is in the center of the plot. Move and size R1 over the region to include only the singlet bead population (Figure 1). Peaks should now begin to appear in all histograms.

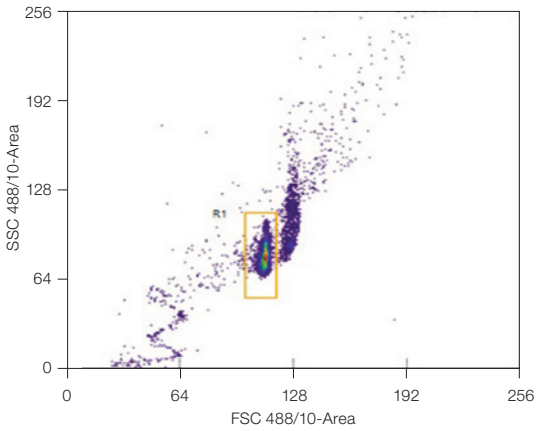

Fig. 1. Scatter plot showing gated bead population. FSC, forward scatter; SSC, side scatter.

10. Adjust the following:
    a. Adjust each fluorescent channel to ensure peaks are visible and within the linear range by selecting the first laser plot (for example, 355 nm–387/55-A) and drawing an R2 gate. The R2 region should span between channel 1 (close to the zero axis) and channel ≈30 (between 101 and 102) to ensure accurate data.
    b. Adjust the PMT voltage until all six peaks are visible in the plot. It may be necessary to increase the PMT voltage initially above the optimal setting to ensure that the blank bead peak is on-scale before fine tuning. Fine-tune by adjusting the voltage until the dimmest peak median is between channels 5–10 (Figure 2). Once adjusted, delete the R2 gate for optimal performance. Repeat this process sequentially for every fluorescent parameter in every laser tab (excluding scatter channels).

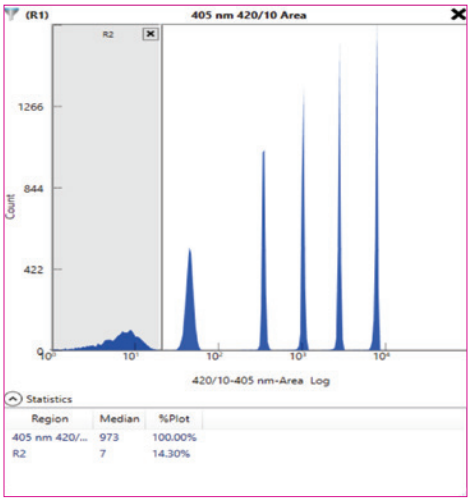

Fig. 2. Example histogram plot showing six peaks with correctly adjusted voltage setting.

Note: In some channels you may not be able to resolve all six peaks due to the optical properties of the dyes incorporated into the beads. If this occurs, adjust the PMT voltage until the dimmest merged peaks have a median of 5–10 (Figure 3). Expected peak numbers can be found at the end of this document.

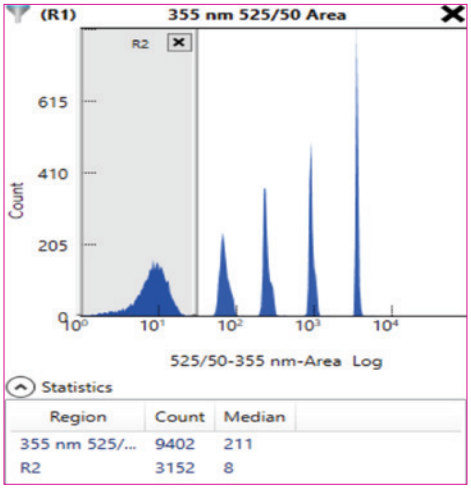

Fig. 3. Histogram showing five peaks with merged dim peaks.

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11. Once all PMTs have been adjusted appropriately, stop the setup run. Click “Acquisition” and run the experiment.

12. When finished, export the FCS file from the acquisition screen. Export area only for all scatter and fluorescent parameters, as height and width measurements are not used. If height or width measurements are exported, the template will not work as intended.

    Note: Configurations containing a 640 nm laser with the 640/20-561 detector require a separate collection with the 640 nm laser turned off. In the presence of the 640 nm laser light, this channel will appear to have an exceptionally high background (Figure 4) due to scattered 640 nm laser light entering this detector. Running the collection a second time with the 640 nm laser manually disabled will allow for an accurate reading for this parameter. See step 13 below for collection instructions.

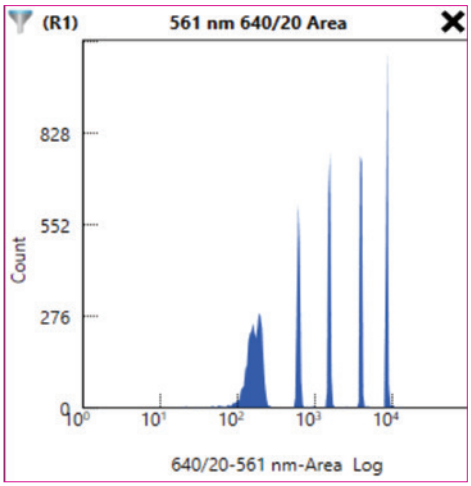

Fig. 4. Histogram showing high background signal in 640/20-561 nm channel.

13. (Optional) If the configuration includes a 640 nm laser with a 640/20-561 detector, perform an additional collection with the 640 nm laser turned off for optimal results. To perform the collection:
    a. Edit the experiment used to collect the bead sample.
    b. Rename both the experiment and the sample to include “640 nm off.”
    c. From the fluorophore selection tab, deselect the 640 nm laser (all plots associated with that laser will be deleted). This can also be achieved in the acquisition tab by closing the 640 nm laser shutter.
    d. Return to the acquisition screen and, in setup mode, begin collection of samples.
    e. Adjust the PMT voltages of the plot displaying data for the 640/20-561 detector until all six peaks are visible and the MFI of the lowest peak is between 5 and 10. The other data plots should not require voltage adjustment.
    f. Enter Acquisition mode and collect the sample. When finished, export as in step 9 above.

Using the FCS Linearity, Qr & Br Template

1. Open the “Linearity, Qr & Br Template” in FCS Express. Carefully read the instructions on the introduction page of the template before loading your data.

   Note: Before using the FCS Express template, be sure to confirm the correct data scaling for the Bio-Rad ZE5 Cell Analyzer in the FCS Express “Options” tab and reset as necessary. (File > Options > Data Loading > FCS File options > Instrument Specific settings > Bio-Rad ZE5 > Fluorescence Parameters > Division Factor > Scale by Factor > 0.0001).

2. Load your FCS file into the Data List of the Linearity Template. Data should automatically populate the plots and spreadsheets of the template.

3. Review the Scatter Gate and the plot settings per instructions on the introduction page (if needed).

4. Confirm the MESF applied values are correct for your configuration by reviewing the “MEFL” tab.

   Note: The software has already input the MESF values from URCP 38-2K Lot# AK03 and AJ02 into the “MEFL” tab in the upper region of the spreadsheet. It has also assigned channels for a standard five-laser (5-L) configuration in the lower region of the spreadsheet. If a different bead lot is used, check and update the MESF values in the spreadsheet from the values included in the Spherotech technical page, mentioned previously. Additionally, if you are using an instrument without a standard 5-L configuration, you will need to reassign the MESF values to the correct FL channels. You can determine whether you have a standard 5-L system by looking for an instrument serial ID with a number starting with 820.

   MESF assignments were made by matching the laser source and emission wavelength as closely as possible with the supplied values from Spherotech. If specific laser/emission pairs are absent from the Spherotech spreadsheet, the software selects values from the closest match within the same laser line. You can make your own assignments as you deem appropriate. However, it is critical to consistently maintain these assignments to compare the same system performance over time. See the “MEFL” tab on the FCS Express template for additional instructions and example assignments.

5. Inspect all gates and marker regions in every plot on the FL# tabs to ensure all populations and peaks are appropriately designated and adjust if necessary. In plots where the dim bead peaks are merged, it is acceptable to overlap M1 and M2 markers (see example in Figure 5).

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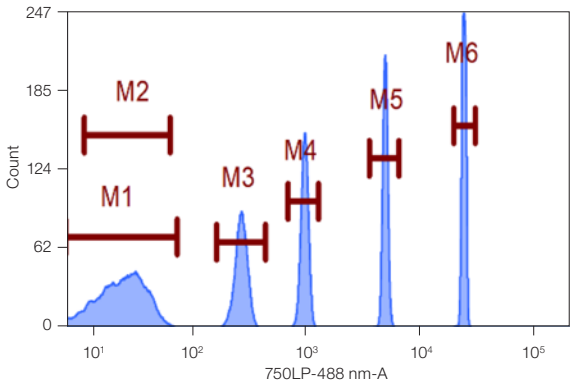

Fig. 5. Correct gating for channels with merged peaks.

6. The results will update in real time as adjustments are made to gates and markers. When finished, the linearity results will be displayed in the “Linearity” tab, and the relative Qr and Br of each parameter will be displayed in the “QB” tab of the template spreadsheet.

Subsequent Runs on the Same System

Once the experiment has been set up on the instrument and the linearity template has been adjusted for gates and markers, it is relatively easy to run the test again to check instrument performance.

1. In Everest Software, open a new session of the Linearity Experiment. All the PMT voltages and gate positions will be retained from the last session.
2. If you want to use a different type of sample media, such as a 1.5 ml Eppendorf tube, you will need to edit the current experiment and select the new media type, designate the position you want to sample from, and re-enter the run conditions for the new acquisition.
3. It should not be necessary to change any voltages or gate positions. Check that all plots look the same as the last session, then record and export the data as before.
4. Import the new FCS file into the pre-existing FCS Express template from the previous session and generate a new report for that day. Be sure to rename the FCS Express template with a unique identifier before closing the session.

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ZE5 Cell Analyzer Laser and Filter Configuration Guide

5-Laser (27 colors)

Laser, nm	Filter	Fluorophore	Expected # Peaks
355	387/11	SBUV400, BUV395	6
	447/60	SBUV445, AF350, DAPI	6
	525/50	SBUV510, BUV496	5
	670/30	SBUV665, BUV661	5
	700LP	SBUV740, SBUV795, BUV737	5
405	420/10	BV421	6
	460/22	SBV440, Pacific Blue	6
	525/50	SBV515, BV510, CFP	5
	615/24	SBV610, BV605	5
	670/30	SBV670, BV650	5
	720/60	SBV710, BV711	5
	750LP	SBV760, SBV790, BV786	5
488	488/10	Side scatter	N/A
	525/25	FITC, GFP, YFP, AF488, Kiravia520	6
	593/52	SBB580	6
	692/80	SBB675, SBB700, PerCP-Cy5.5	5
	750LP	SBB765, SBB810	5
561	577/15	SBY575, PE	6
	589/15	DsRed, tdTomato	6
	615/24	SBY605, PE-Dazzle, PE-CF594, mCherry	6
	640/20	mPlum	6
	670/30	SBY665, PE-Cy5, PE-AF647	6
	720/60	SBY720, PE-Cy5.5	6
	750LP	SBY800, PE-Cy7, PE-AF750	6
640	670/30	SBR670, APC, AF647	6
	720/60	SBR715, AF700	6
	775/50	SBR775, APC Cy7, AF750	6
	800LP	SBR815, AF700, APC/Fire810	6

5-Laser 7 Off UV Option A (27 colors)

Laser, nm	Filter	Fluorophore	Expected # Peaks
355	387/11	SBUV400, BUV395	6
	509/24	SBUV510, DAPI, Zombie UV	5
	577/15	SBUV575, BUV536	5
	615/24	SBUV605, BUV615	5
	670/30	SBUV665, BUV661	5
	747/33	SBUV740, BUV737	5
	780 LP	SBUV795, BUV805	5
405	420/10	BV421	6
	460/22	SBV440, Pacific Blue	6
	525/50	SBV515, BV510, CFP	5
	615/24	SBV610, BV605	5
	670/30	SBV670, BV650	5
	720/60	SBV710, BV711	5
	750LP	SBV760, SBV790, BV786	5
488	488/10	Side scatter	N/A
	525/35	FITC, GFP, YFP, AF488, Kiravia520	6
	593/52	SBB580	6
	692/80	SBB675, SBB700, PerCP-Cy5.5	5
	750LP	SBB765, SBB810	5
561	583/30	SBY575, PE, DsRed, tdTomato, RFP	6
	615/24	SBY605, PE-Dazzle, PE-CF594, mCherry	6
	670/30	SBY665, PE-Cy5, PE-AF647	6
	720/60	SBY720, PE-Cy5.5	6
	750LP	SBY800, PE-Cy7, PE-AF750	6
640	670/30	SBR670, APC, AF647	6
	720/60	SBR715, AF700	6
	775/50	SBR775, APC Cy7, AF750	6
	800LP	SBR815, AF700, APC/Fire810	6

5-Laser 7 Off UV Option B (27 colors)

Laser, nm	Filter	Fluorophore	Expected # Peaks
355	387/11	SBUV400, BUV395	6
	460/22	SBUV445, DAPI, Zombie UV, L/D Blue	5
	509/24	SBUV510, BUV496	5
	577/15	SBUV575, BUV563	5
	670/30	SBUV665, BUV661	5
	747/33	SBUV740, BUV737	5
	780 LP	SBUV795, BUV805	5
405	420/10	BV421	6
	460/22	SBV440, Pacific Blue	6
	525/50	SBV515, BV510, CFP	5
	615/24	SBV610, BV605	5
	670/30	SBV670, BV650	5
	720/60	SBV710, BV711	5
	750LP	SBV760, SBV790, BV786	5
488	488/10	Side scatter	N/A
	525/35	FITC, GFP, YFP, AF488, Kiravia520	6
	593/52	SBB580	6
	692/80	SBB675, SBB700, PerCP-Cy5.5	5
	750LP	SBB765, SBB810	5
561	583/30	SBY575, PE, DsRed, tdTomato, RFP	6
	615/24	SBY605, PE-Dazzle, PE-CF594, mCherry	6
	670/30	SBY665, PE-Cy5, PE-AF647	6
	720/60	SBY720, PE-Cy5.5	6
	750LP	SBY800, PE-Cy7, PE-AF750	6
640	670/30	SBR670, APC, AF647	6
	720/60	SBR715, AF700	6
	775/50	SBR775, APC Cy7, AF750	6
	800LP	SBR815, AF700, APC/Fire810	6

4-Laser (24 colors)

Laser, nm	Filter	Fluorophore	Expected # Peaks
405	420/10	BV421	6
	460/22	SBV440, Pacific Blue	6
	525/50	SBV515, BV510, CFP	5
	615/24	SBV610, BV605	5
	670/30	SBV670, BV650	5
	720/60	SBV710, BV711	5
	750LP	SBV760, SBV790, BV786	5
488	488/10	Side scatter	N/A
	509/24	FITC, GFP, AF488, Kiravia520	6
	549/15	YFP	6
	583/30	SBB580, PE	6
	615/24	SBB615, PE-CF594, PE-Dazzle	6
	692/80	SBB675, SBB700, PerCP-Cy5.5, PE-Cy5	6
	750LP	SBB765, SBB810, PE-Cy7, PE-AF750	5
561	577/15	SBY575, PE	6
	589/15	DsRed, tdTomato	6
	615/24	SBY605, PE-Dazzle, PE-CF594, mCherry	6
	640/20	mPlum	6
	670/30	SBY665, PE-Cy5, PE-AF647	6
	720/60	SBY720, PE-Cy5.5	6
	750LP	SBY800, PE-Cy7, PE-AF750	6
640	670/30	SBR670, APC, AF647	6
	720/60	SBR715, AF700	6
	775/50	SBR775, APC Cy7, AF750	6
	800LP	SBR815, AF790, APC/Fire810	6

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ZE5 Cell Analyzer Laser and Filter Configuration Guide

3-Laser (17 colors)

Laser, nm	Filter	Fluorophore	Expected # Peaks
405	420/10	BV421	6
	460/22	SBV440, Pacific Blue	6
	525/50	SBV515, BV510, CFP	5
	615/24	SBV610, BV605	5
	670/30	SBV670, BV650	5
	720/60	SBV710, BV711	5
	750LP	SBV760, SBV790, BV786	5
488	488/10	Side scatter	N/A
	509/24	FITC, GFP, AF488, Kiravia520	6
	549/15	YFP	6
	583/30	SBB580, PE	6
	615/24	SBB615, PE-CF594, PE-Dazzle	6
	692/80	SBB675, SBB700, PerCP-Cy5.5, PE-Cy5	6
	750LP	SBB765, SBB810, PE-Cy7, PE-AF750	5
640	670/30	SBR670, APC, AF647	6
	720/60	SBR715, AF700	6
	775/50	SBR775, APC Cy7, AF750	6
	800LP	SBR815, AF790, APC/Fire810	6

3-Laser Option 2 (17 colors)

Laser, nm	Filter	Fluorophore	Expected # Peaks
488	488/10	Side scatter	N/A
	509/24	FITC, GFP, AF488, Kiravia520	6
	549/15	YFP	6
	583/30	SBB580, PE	6
	615/24	SBB615, PE-CF594, PE-Dazzle	6
	692/80	SBB675, SBB700, PerCP-Cy5.5, PE-Cy5	6
	750LP	SBB765, SBB810, PE-Cy7, PE-AF750	5
561	577/15	SBY575, PE	6
	589/15	DsRed, tdTomato	6
	615/24	SBY605, PE-Dazzle, PE-CF594, mCherry	6
	640/20	mPlum	6
	670/30	SBY665, PE-Cy5, PE-AF647	6
	720/60	SBY720, PE-Cy5.5	6
	750LP	SBY800, PE-Cy7, PE-AF750	6
640	670/30	SBR670, APC, AF647	6
	720/60	SBR715, AF700	6
	775/50	SBR775, APC Cy7, AF750	6
	800LP	SBR815, AF790, APC/Fire810	6

3-Laser (20 colors)

Laser, nm	Filter	Fluorophore	Expected # Peaks
405	420/10	BV421	6
	460/22	SBV440, Pacific Blue, mTurquoise	6
	525/50	SBV515, BV510, CFP, Cascade Yellow	5
	615/24	SBV610, BV605	5
	670/30	SBV670, BV650	5
	720/60	SBV710, BV711	5
	750LP	SBV760, SBV790, BV786	5
488	488/10	Side scatter	N/A
	509/24	FITC, eGFP, AF488, Kiravia520, mClover	6
	549/15	eYFP	6
	583/30	SBB580, PE	6
	615/24	SBB615, PE-CF594, PE-Dazzle	6
	692/80	SBB675, SBB700, PerCP-Cy5.5, PE-Cy5	6
	750LP	SBB765, SBB810, PE-Cy7, PE-AF750	5
561	577/15	SBY 575, PE	6
	589/15	DsRed, tdTomato, RFP	6
	615/24	SBY605, PE-Dazzle, PE-CF594, mCherry	6
	640/20	mPlum	6
	670/30	SBY665, PE-Cy5, PE-AF647	6
	720/60	SBY720, PE-Cy5.5	6
	750LP	SBY800, PE-Cy7, PE-AF750	6

AFxxx, Alexa Fluor; APC, allophycocyanin; BV, Brilliant Violet; BUV, Brilliant UltraViolet; CFP, cyan fluorescent protein; dsRed, Discosoma red fluorescent protein; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; RFP, red fluorescent protein; SBB, StarBright™ Blue; SBV, StarBright Violet; SBUV, StarBright UltraViolet; SBY StarBright Yellow; YFP, yellow fluorescent protein.

References

Hoffman RA and Wood J (2007). Characterization of flow cytometer instrument sensitivity. Curr Protoc Cytom 1, 20.

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