



STEM CELL LABORATORY (STCL)



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Fluorescence Overlap Compensation Optimization for the BD FACSCalibur Flow Cytometer

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FLOW-GEN-032

FLUORESCENCE OVERLAP COMPENSATION OPTIMIZATION FOR THE BD FACSCALIBUR FLOW CYTOMETER

1 PURPOSE

- 1.1 The purpose of this procedure is to describe how Stem Cell Laboratory flow cytometer operators are to perform fluorescence overlap compensation optimization using an analog (non-digital) flow cytometer such as the BD FACSCalibur using BD CellQuest Pro software.

2 INTRODUCTION

- 2.1 Fluorescence overlap between emission spectra must be overcome when performing flow cytometric analysis using multicolor reagent combinations. Although some of the overlap is avoided by the use of dichroic or band-pass filters within the instrument, there is still significant overlap (spillover from one detector to another) that must be removed electronically to obtain accurate results. In order to accomplish this, antibodies conjugated to each fluorochrome used or 7AAD, if used, are added to cells as single reagents in separate tubes. During acquisition of these individual tubes, any fluorescence spillover detected by a competing fluorescent detector is removed electronically using the cytometer software as described below. This is referred to as electronic compensation. This testing must reflect all potential variables as they affect fluorescence intensity. For example if a method involves a red blood cell lyse and wash step then the compensation tube preparation should also involve a red blood cell lyse and wash step, as the intensity of fluorescence may vary from cells stained using a red blood cell lyse with no wash preparation. In other words, if the fluorescent intensity varies, then the electronic compensation requirement will vary.

3 SCOPE AND RESPONSIBILITIES

- 3.1 This process must be performed to optimize the BD FACSCalibur compensation settings as needed. It is the responsibility of the flow cytometry staff to follow this procedure if the testing method requires the use of optimized electronic compensation settings.

4 DEFINITIONS/ACRONYMS

- 4.1 CD-Cluster Designation assigned to antigen on cell
- 4.2 PBS-Phosphate Buffered Saline
- 4.3 BSA-Bovine serum albumin
- 4.4 7AAD-7-Aminoactinomycin D used for to identify dead and dying cells

5 MATERIALS

- 5.1 Fresh or stabilized (process controls) peripheral blood

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BD FACSCalibur Flow Cytometer
Stem Cell Laboratory, DUMC
Durham, NC

- 5.2 CD3-FITC, CD3-PE, CD3-PerCP, CD8-APC, 7AAD (if used), BD Biosciences
- 5.3 1% BSA/PBS for Lyse/Wash procedure methods, Invitrogen™
- 5.4 Pharm Lyse Buffer, BD Biosciences

6 EQUIPMENT

- 6.1 Adjustable manual Single Channel Pipettes, Rainin 10µl, 20µl, 200µl
- 6.2 Electronic (automatic) Single Channel pipette, Rainin 200µl – 2ml
- 6.3 Pipette tips, Rainin
- 6.4 FACSCalibur flow cytometer, Becton Dickinson
- 6.5 12mmx75mm, 5 ml polystyrene test tubes, Falcon
- 6.6 Swinging bucket Centrifuge (for lyse/wash methods), Sorval RT7 or equivalent

7 SAFETY

- 7.1 Use universal precautions when working with human blood products.
- 7.2 Review MSDS for monoclonal antibodies used in testing.
 - 7.2.1 Sodium azide warning
 - 7.2.2 Nucleic acid dye warning
- 7.3 Review MSDS for BD Biosciences 10% Lyse Buffer.

8 PROCEDURE

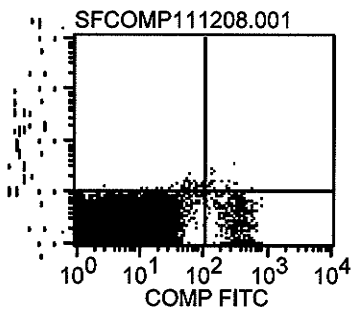
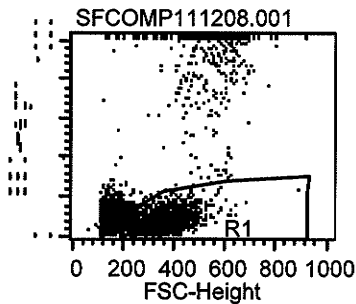
- 8.1 Make a solution of 10% BD Pharm lyse buffer by adding 1 part 10x concentrated buffer to 9 parts deionized water.
- 8.2 Label 1 test tube (C1, C2. Etc.) for each of the fluorescent conjugates or dye to be used for the day.
- 8.3 Use CD3 Fitc in C1, CD3 PE in C2, CD3 PerCP (or 7AAD if needed) in C3, CD8 APC in C4, CD3 PerCP in C5 (if 7AAD tube is needed in C3).

NOTE: If multiple staining methods are to be used during the day, prepare tubes for each methodology (i.e. lyse/wash vs lyse no/wash) using the same cell staining methodology as used in the testing procedure.

- 8.4 At the cytometer, open the Routine Test template folder and double click on the Compensation template to launch.
- 8.5 Connect to the cytometer via the Acquisition heading. In the parameter description window, assign the directory location, file name (SFCOMPmmdyy), and input the correct panel in the acquisition tube list.
- 8.6 Enter your Operator ID and for Sample ID type "CONTROL."
- 8.7 Go to the Cytometer menu heading and scroll down to INSTRUMENT SETTINGS.

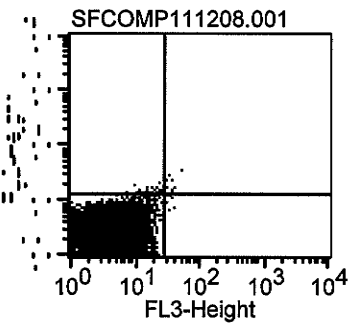
- 8.8 Click on the open button and proceed to Facstation/ BD files/ Instrument setting folder and double click on CalibFile.LNW or if setting up for wash method, select the Calib file settings.
- 8.9 In the instrument settings window, click on SET, then click on DONE.
- 8.10 Under the Cytometer menu heading, scroll to and open the Threshold window. Set the threshold for forward scatter (FS) at the level being used currently on that specific instrument. This value is posted on the side of each instrument.
- 8.11 In the Acquisition control window, click in the SETUP box.
- 8.12 Go to the Cytometer heading and open the COMPENSATION window.
- 8.13 Place each tube on the sip one at a time. Start acquisition in setup for each one.
- 8.14 Use the examples at the end of this procedure as a guide.
- 8.15 Adjust the R1 lymphocyte region (FSC/SSC plot) to exclude debris and monocytes.
- 8.16 Observe the single color population and compare it to the negative population.
- 8.17 Place the quadrant stats around the negative population, so that the negative events are in the lower left (LL) quadrant.
- 8.18 Observe the mean channel statistics (see arrows on examples) for these two populations and adjust the appropriate compensation control so that the means (either X to X mean or Y to Y mean) are within 0.50 of each other.
- 8.19 Stop the setup mode, acquire data for 10-20 seconds, click pause, then click save.
- 8.20 If optimizing compensation for 7AAD Lyse/No Wash method, save and print the settings with FS threshold and 7AAD compensation adjustments. If optimizing for a Lyse/Wash method, save and print settings accordingly with the appropriate threshold parameter saved. If optimizing for the immune reconstitution panel set the threshold at the current FL3 value, save the instrument settings as IR Panel 4-8, then print.
- 8.21 File the printed results in the appropriate binder.

Example 1: Single color compensation using FITC conjugated antibody. LL and LR Y means within 0.5 of each other. Use FL2-% FL1 to adjust as needed.



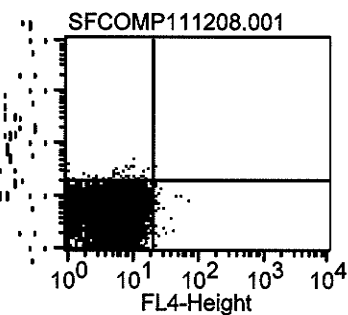
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Acquisition Date: 12-Nov-08
Gate: G1

Quad	Events	% Gated	X Mean	Y Mean
UL	63	0.44	52.34	12.97
UR	33	0.23	224.51	14.88
LL	13777	95.75	9.18	2.92
LR	515	3.58	349.00	3.00



File: SFCOMP111208.001
Acquisition Date: 12-Nov-08
Gate: G1

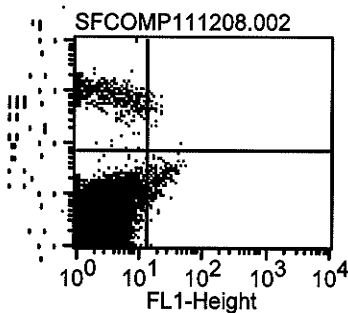
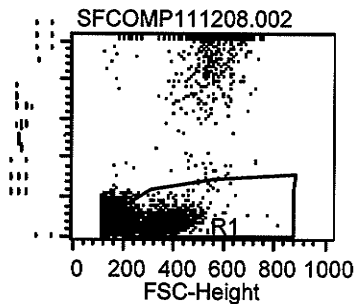
Quad	Events	% Gated	X Mean	Y Mean
UL	34	0.24	18.31	14.90
UR	10	0.07	36.39	21.14
LL	14337	99.65	6.56	2.95
LR	7	0.05	33.23	8.34



File: SFCOMP111208.001
Acquisition Date: 12-Nov-08
Gate: G1

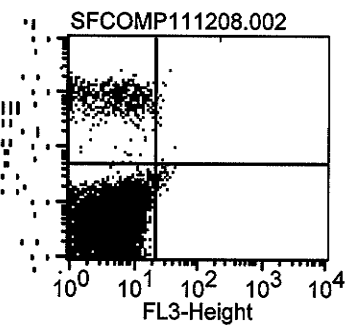
Quad	Events	% Gated	X Mean	Y Mean
UL	77	0.54	6.73	24.49
UR	2	0.01	21.67	36.24
LL	14269	99.17	5.84	6.53
LR	40	0.28	26.66	6.51

Example 2: Single color compensation using PE conjugated antibody. LL and UL X means within 0.5 of each other. Use FL1-% FL2 and FL3-%FL2 to adjust as needed.



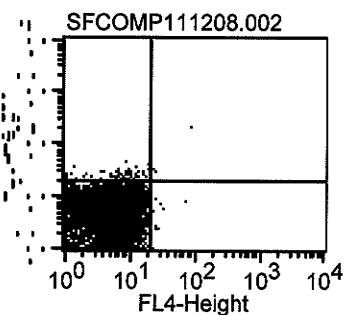
File: SFCOMP111208.002
Acquisition Date: 12-Nov-08
Gate: G1

Quad	Events	% Gated	X Mean	Y Mean
UL	567	5.89	3.49	991.28
UR	21	0.22	17.33	499.80
LL	8856	91.98	3.09	5.87
LR	184	1.91	20.92	21.21



File: SFCOMP111208.002
Acquisition Date: 12-Nov-08
Gate: G1

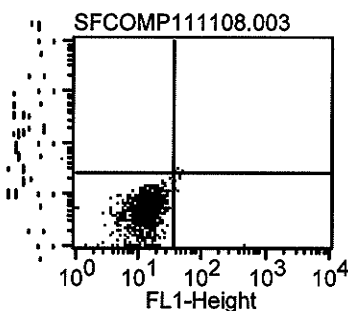
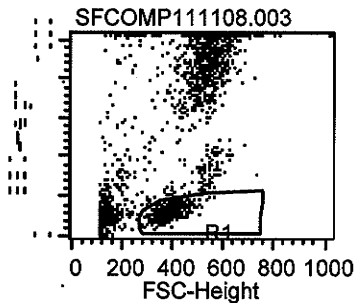
Quad	Events	% Gated	X Mean	Y Mean
UL	577	5.99	5.81	952.65
UR	17	0.18	38.75	1365.29
LL	8992	93.39	6.03	6.03
LR	42	0.44	28.07	31.35



File: SFCOMP111208.002
Acquisition Date: 12-Nov-08
Gate: G1

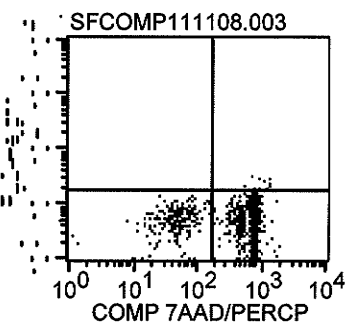
Quad	Events	% Gated	X Mean	Y Mean
UL	93	0.97	7.52	24.72
UR	3	0.03	45.23	95.02
LL	9518	98.86	5.69	5.97
LR	14	0.15	28.09	5.03

Example 3: Single color compensation using 7AAD dye. LL and LR Y means within 0.5 of each other. LL and UL X means within 0.5 of each other. Use FL2-% FL3 and FL4-% FL3 to adjust as needed.



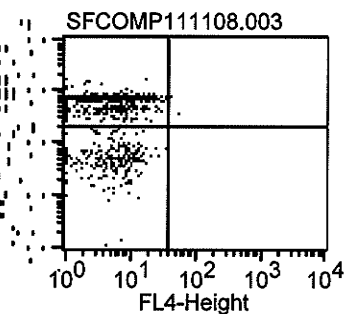
File: SFCOMP111108.003
Acquisition Date: 11-Nov-08
Gate: G1

Quad	Events	% Gated	X Mean	Y Mean
UL	0	0.00	***	***
UR	4	0.41	40.30	28.85
LL	963	99.07	13.00	5.94
LR	5	0.51	123.04	14.01



File: SFCOMP111108.003
Acquisition Date: 11-Nov-08
Gate: G1

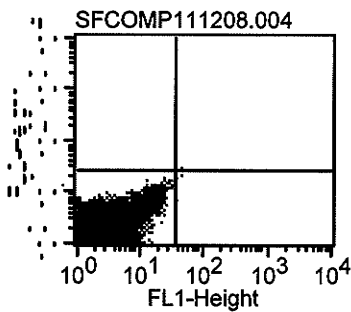
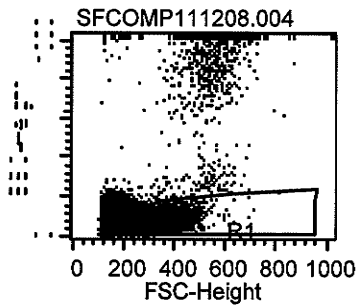
Quad	Events	% Gated	X Mean	Y Mean
UL	0	0.00	***	***
UR	11	1.13	1047.74	23.50
LL	218	22.43	55.08	5.71
LR	743	76.44	722.91	5.92



File: SFCOMP111108.003
Acquisition Date: 11-Nov-08
Gate: G1

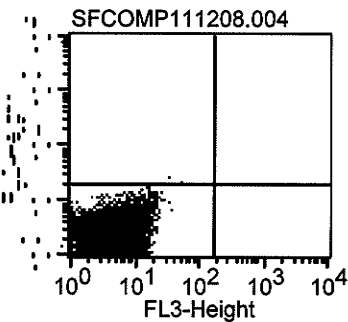
Quad	Events	% Gated	X Mean	Y Mean
UL	745	76.65	6.47	732.27
UR	3	0.31	46.16	672.98
LL	224	23.05	6.51	58.45
LR	0	0.00	***	***

Example 4: Single color compensation using APC conjugated antibody. LL and LR Y means within 0.5 of each other. Use FL3-%FL4 to adjust as needed.



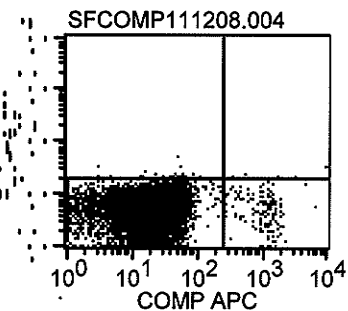
File: SFCOMP111208.004
Acquisition Date: 12-Nov-08
Gate: G1

Quad	Events	% Gated	X Mean	Y Mean
UL	0	0.00	***	***
UR	1	0.01	42.55	27.88
LL	10694	99.97	3.64	2.66
LR	2	0.02	39.56	20.09



File: SFCOMP111208.004
Acquisition Date: 12-Nov-08
Gate: G1

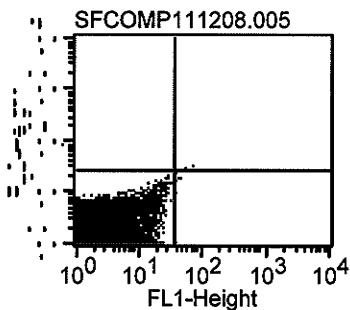
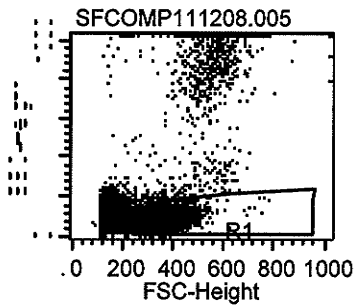
Quad	Events	% Gated	X Mean	Y Mean
UL	3	0.03	35.50	22.69
UR	0	0.00	***	***
LL	10694	99.97	6.38	2.66
LR	0	0.00	***	***



File: SFCOMP111208.004
Acquisition Date: 12-Nov-08
Gate: G1

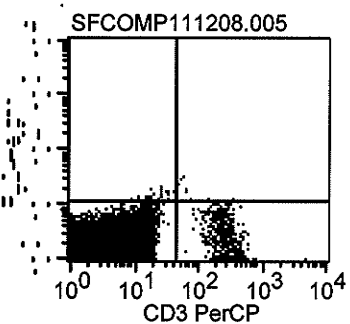
Quad	Events	% Gated	X Mean	Y Mean
UL	25	0.23	39.93	24.64
UR	7	0.07	2416.17	23.84
LL	10517	98.32	26.23	6.33
LR	148	1.38	1203.12	6.39

Example 5: Single color compensation using PerCP conjugated antibody. LL and LR Y means within 0.5 of each other. LL and UL X means within 0.5 of each other. Use FL2-% FL3 and FL4-% FL3 to adjust as needed.



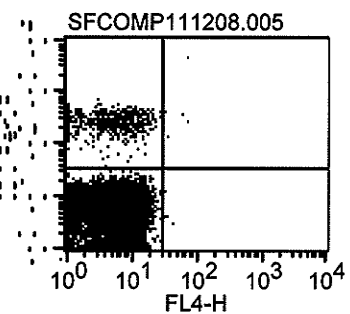
File: SFCOMP111208.005
Acquisition Date: 12-Nov-08
Gate: G1

Quad	Events	% Gated	X Mean	Y Mean
UL	0	0.00	***	***
UR	2	0.02	55.95	31.39
LL	12975	99.95	3.55	2.58
LR	4	0.03	38.08	19.67



File: SFCOMP111208.005
Acquisition Date: 12-Nov-08
Gate: G1

Quad	Events	% Gated	X Mean	Y Mean
UL	35	0.27	21.43	14.20
UR	9	0.07	143.65	19.18
LL	12356	95.19	7.10	2.54
LR	581	4.48	307.47	2.72



File: SFCOMP111208.005
Acquisition Date: 12-Nov-08
Gate: G1

Quad	Events	% Gated	X Mean	Y Mean
UL	585	4.51	6.18	296.45
UR	6	0.05	50.38	1091.13
LL	12386	95.42	6.22	7.14
LR	4	0.03	36.52	5.93

9 RELATED DOCUMENTS/FORMS

9.1 NA

10 REFERENCES

- 10.1 Roederer, Mario. Spectral Compensation for flow cytometry: Visualization artifacts, limitations, and caveats. 2001. Cytometry 45:194-205.

11 REVISION HISTORY

Revision No.	Author	Description of Change(s)
02	Melissa Reese	<ul style="list-style-type: none">• Removed reference to archived Bone Marrow procedure and replaced terminology with Lyse/Wash method.• Added revision history section• Edited/Corrected format and grammatical errors.

Signature Manifest**Document Number:** FLOW-GEN-032**Revision:** 02**Title:** Fluorescence Overlap Compensation Optimization for the BD FACSCalibur Flow Cytometer**FLOW-GEN-032 Fluorescence Overlap Compensation Optimization for the BD FACSCalibur Flow Cytometer****Author Approval**

Name/Signature	Title	Date	Meaning/Reason
Melissa Reese (MGREESE)		09 May 2013, 03:44:29 PM	Approved

Manager Approval

Name/Signature	Title	Date	Meaning/Reason
Barbara Waters-Pick (WATE02)		10 May 2013, 04:52:42 PM	Approved

Medical Director Approval

Name/Signature	Title	Date	Meaning/Reason
Joanne Kurtzberg (KURTZ001)		10 May 2013, 07:35:50 PM	Approved

QA Approval

Name/Signature	Title	Date	Meaning/Reason
Linda Sledge (SLEDG006)		13 May 2013, 07:55:05 AM	Approved

Document Release

Name/Signature	Title	Date	Meaning/Reason
Sandy Mulligan (MULLI026)		17 May 2013, 06:28:18 PM	Approved

Notification

Name/Signature	Title	Date	Meaning/Reason
Sharon Hartis (SH259)		17 May 2013, 06:28:18 PM	Email Sent