Post Acquisition Compensation of Cells and Beads Using VenturiOne®

A review of colour compensation using VenturiOne, flow cytometry data analysis software, as endorsed by Ian Dimmick and Rebecca Stewart of the Flow Cytometry Core Facility, Institute of Human Genetics, Newcastle upon Tyne University.

Introduction

Due to the increased use of flow cytometers with multiple lasers, the choice of fluorochromes available has grown considerably (1). This offers users great benefits, allowing more varied and extensive research by enabling the use of multiple lasers on individual instruments and therefore the chance to optimise fluorochrome excitation and increase the number of fluorochromes and dyes for simultaneous detection within each experiment, the overall outcome being more data with fewer cells. When using two or more fluorochromes in an experiment, the spectral overlap of the fluorochrome emission spectra needs to be taken into consideration. Collectively the emission spectra of all fluorochromes cover a broad wavelength range **(Fig. 1)**. This will lead to spectral overlap where the fluorescence spectrum of one fluorochrome spills over into the detection channel dedicated to another fluorochrome being used simultaneously. This spectral overlap causes difficulty when simultaneously trying to measure the true fluorescence of each fluorochrome, therefore causing difficulty obtaining a correct representation of the data. A correction must be applied. This correction is termed colour compensation (1). To compensate for the spectral overlap, a subtraction of the spill over of one fluorochrome from another is applied; this value can be calculated as a percentage spillover of the primary fluorochrome into a detector which is not the primary detector.

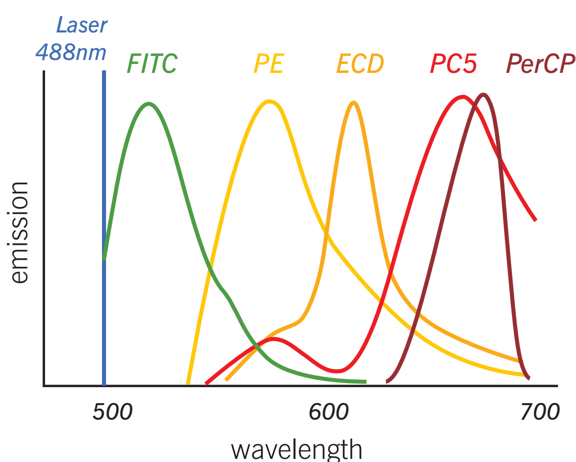

Fig. 1. Emission Profiles of Fluorochromes showing Spectral Overlap

In order to determine the amount of compensation required for each fluorochrome, individual control samples stained with each fluorochrome to be used in the experiment are used independently to establish spectral overlap, researchers can adopt a method to calculate the compensation before data is collected by the flow cytometer called pre acquisition compensation. Compensation can also be calculated after data collection within the software, called post acquisition compensation and if need be this can be used to check the integrity of the pre acquisition compensation values. The main benefit of post acquisition compensation is that it allows adjustments to be made away from the instrument environment at leisure but the basics of compensation remain the same using either pre or post acquisition compensation. Compensation can be time consuming especially when multiple fluorochromes and dyes are used therefore post acquisition is an ever increasing requirement (2).

In this review the focus is on post acquisition compensation using VenturiOne, investigating manual and automatic post acquisition compensation methods. This review also assesses the use of compensation beads and cells which are routinely used at the flow cytometry core facility, Institute of Human Genetics, Newcastle upon Tyne University, discussing both their advantages and disadvantages.

Pre Acquisition Compensation Method with a Flow Cytometer

Please refer to Instrument User Manual for full instructions

1. Set up the instrument PMT voltages using appropriate cellular baseline controls
2. Use compensation beads to obtain maximal fluorescence of each fluorochrome whilst running under the cell derived instrument settings. Compensation values are calculated. The only parameters that may have to be changed are the Forward Scatter (FS) and Side Scatter (SS) settings but these will not affect the final compensation values. Compensation beads are the preferred choice due to the advantage of giving distinct negative and positive peaks and increased signal intensity when analysed
3. Verify compensation values by re analysis of the controls to ensure correct compensation values with respect to appropriate X any Y axis negative and positive mean or median values

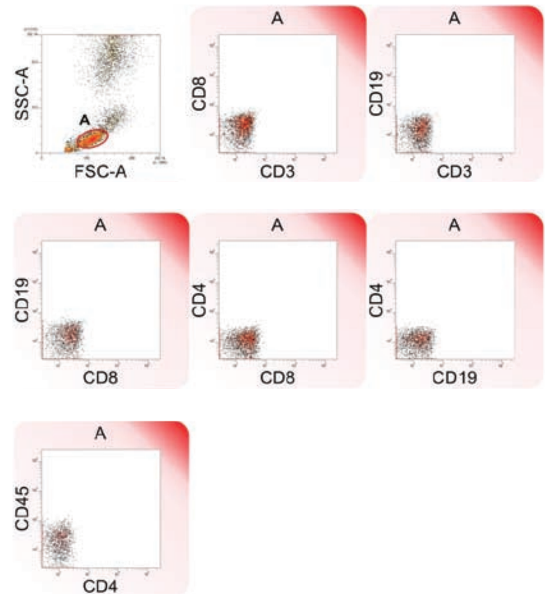

Fig. 2. Negative Control

Post Acquisition Compensation Method with VenturiOne

Please refer to VenturiOne User Manual for full instructions

1. Set the default log decade scaling to match that of the pre acquisition method
2. Open compensation files into the playlist of VenturiOne:
   - Negative control - unstained cells **(Fig. 2)**
   - Compensation cells standard - cells stained with fluorochromes
   - Compensation beads standard - beads stained with fluorochromes and negative bead population
   - Positive control - positive cells stained with fluorochromes
3. Select compensation cell file and gate on FS/SS lymph population
4. Select all the fluorescent single parameter histograms **(Fig. 3)**

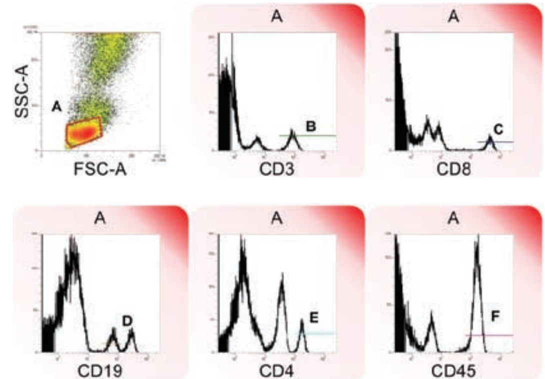

Fig. 3. Fluorescent Histograms of the Compensation Cells Uncompensated

5. Draw a linear region on the positive peaks in each histogram
6. Select each linear region in turn and click *Calculate* from the *Compensation Group* to apply automatic compensation. Automatic calculation is preferred due to the complexity of calculating multiple colour compensation alone without having to do it manually
7. Before selecting the next linear region please ensure to click *Clear Compensation*
8. Repeat steps 6 & 7
9. See **Fig. 4** for Compensation Cell Matrix

Compensation

Active Compensation | Compensation Calculations | Print... | Size to Fit

	4-488/530/30-A	4-488/585/42-A	4-488/710/50-A	1-638/670/14-A	1-638/780/60-A
4-488/530/30-A		1.20	0.60	0.25	0.33
4-488/585/42-A	8.66		0.39	0.14	0.21
4-488/710/50-A	5.28	66.12		1.26	1.20
1-638/670/14-A	1.65	0.40	7.91		22.67
1-638/780/60-A	0.31	0.06	1.11	2.39	

Fig. 4. Compensation Matrix using Compensation Cells

10. Save compensation file
11. Repeat steps 3 to 10 with the Compensation beads **(Fig. 5)**

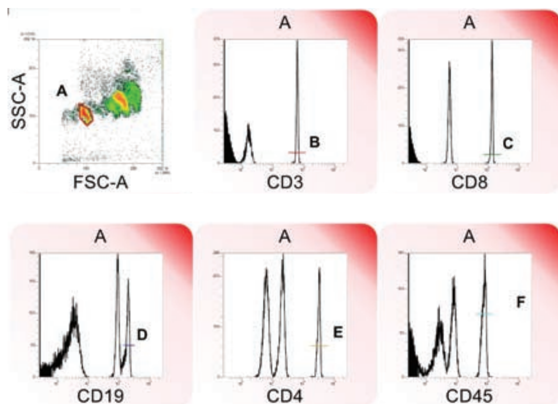

Fig. 5. Fluorescent Histograms of the Compensation Beads Uncompensated

12. See **Fig. 6** for Compensation Bead Matrix

Compensation

Active Compensation | Compensation Calculations | Print... | Size to Fit

	4-488/530/30-A	4-488/585/42-A	4-488/710/50-A	1-638/670/14-A	1-638/780/60-A
4-488/530/30-A		1.18	0.13	0.08	0.34
4-488/585/42-A	8.82		0.13	0.08	0.34
4-488/710/50-A	5.09	66.71		0.96	0.81
1-638/670/14-A	9.65	4.53	10.46		27.38
1-638/780/60-A	0.42	0.19	1.36	2.33	

Fig. 6. Compensation Matrix using Compensation Beads

13. Manual compensation can be used for minor adjustments of the compensation values
14. Verify values with positive control using compensation matrix. See **Fig. 7**. Use *V-log* to check for overcompensation

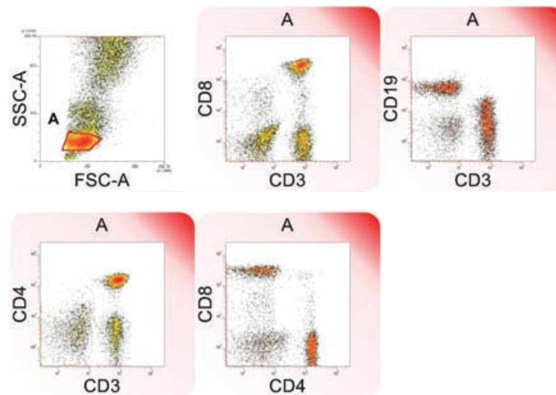

Fig. 7. Dual Parameter Plots of Compensated Positive Control using Compensation Cells Matrix

Principles of Compensation for Consideration

- Compensation beads give brighter signals than cells in the main so more accurate compensation is achieved
- Autofluorescence of positive and negative populations must be the same for all compensation controls in order to achieve correct compensation (4)
- A weak signal is produced from CD19 expression. This makes it difficult to determine positive populations from negative populations. Not just a case of the brightest peak being the positive population. See Fig. 8 where the uncompensated CD19 histogram shows two positive peaks. The brighter of these two is actually the spectral overlap of CD8 into the CD19 detector whereas the weaker positive peak is CD19 only
- CD45 expression results in a very bright signal so is a good control
- Ensure to *Clear Compensation* after calculating each fluorochromes compensation value in VenturiOne so that you are not calculating compensation on parameters that are already compensated
- Compensation values must always be recalculated if the voltages of the Photomultiplier Tubes (PMT) are altered (3)
- Due to the complexity of multiple colour compensation, use the automatic compensation feature in the software primarily and use the manual feature to make minor adjustments
- Controls for determining the compensation matrix should produce fluorescent signals that are bright as possible
- In stem cell research, stem cells may not have well expressed surface antigens therefore do not achieve bright fluorescent signals which are needed to calculate optimum compensation values. Instead compensation beads are recommended for calculating compensation as they achieve brighter fluorescent signals

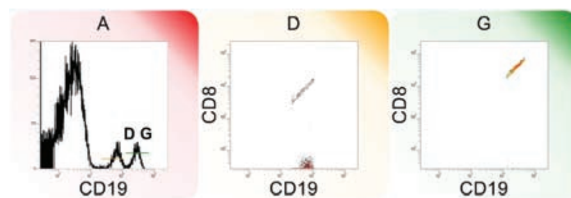

Fig. 8. CD19 Histogram Uncompensated showing the Spectral Overlap of CD8 into CD19 Detector

Summary

VenturiOne alleviates the complexity of multiple colour compensation, offering a quick and easy approach to post acquisition compensation, with the added flexibility of intuitive automatic and manual compensation options.

For further information please visit our website: www.appliedcytometry.com or send an email to info@appliedcytometry.com

References

1. **Ormerod, M** (2008). *Flow Cytometry - A Basic Introduction*
2. **Roederer, M** (2001). *Spectral Compensation for Flow Cytometry: Visualization Artifacts, Limitations and Caveats*. Cytometry **45**:194-205
3. *Setting up 2 or 3 Colour FACS Analysis*: http://users.ox.ac.uk/~path0116/tig/ccomp.html 18.12.97
4. *Compensation (An informal perspective)*: http://www.drmr.com/compensation/ May 24, 2000

Acknowledgements

With thanks to Ian Dimmick and Rebecca Stewart for dedicating the time required to collate the data for this document and for their continued support of VenturiOne.

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