Application **Review**

AppliedCytometry
Pioneering Software Simplicity

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 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 asers 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 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 in which it is not the primary detector.

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.

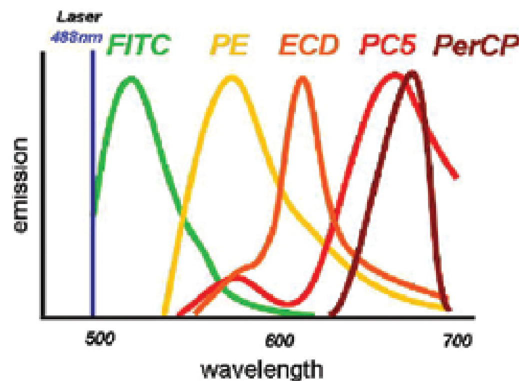

Figure 1. Emission Profiles of Fluorochromes showing Spectral Overlap

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 and Y axis negative and positive mean or median values.

Post Acquisition Compensation Method with VenturiOne®

Please refer to VenturiOne® User Guide for full instructions on Compensation Wizard

1. Set the default log decade scaling to match that of the pre acquisition method.
2. Open the compensation cells file into the playlist of VenturiOne®. Ensure there are replicate of files for each fluorochrome to be compensated.
3. Open the Compensation Wizard from the compensation tab and configure the settings for compensation (See fig 2) click *Next*

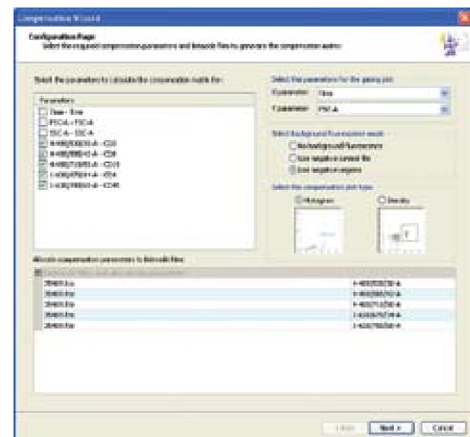

Figure 2. Configuration page of the Compensation Wizard

4. On the compensation page, adjust the gating and compensation regions to capture the populations for calculating the coefficients of the fluorochrome. See fig.3

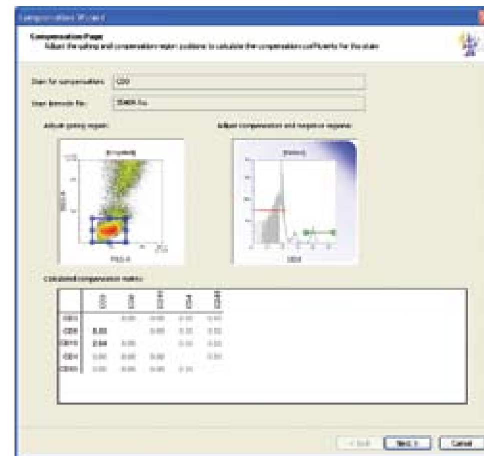

Figure 3. Compensation page of the wizard that allows adjustment of the regions for compensation.

5. Click *Next* to repeat this for every fluorochrome to be compensated
6. Then the last page of the wizard allows you to review, print or save the compensation settings file. See fig 4

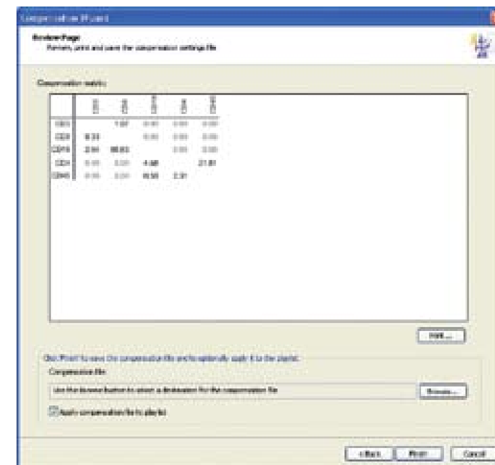

Figure 4. Review Page with Options to Save or Print the Compensation Settings

7. Save compensation file. See fig 5

Compensation

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

	CD3 4-488/530/30-A	CD8 4-499/585/42-A	CD19 4-488/710/50-A	CD4 1-638/670/14-A	CD45 1-638/780/60-A
CD3 4-488/530/30-A		1.07	0.00	0.00	0.00
CD8 4-499/585/42-A	8.33		0.00	0.00	0.00
CD19 4-488/710/50-A	2.64	65.63		0.00	0.00
CD4 1-638/670/14-A	0.00	0.00	4.68		21.81
CD45 1-638/780/60-A	0.00	0.00	0.50	2.31	

Figure 5. Compensation Matrix using Compensation Cells

8. Repeat steps 2 to 7 with the Compensation bead files.
9. See Fig 6 for Compensation Bead matrix

Compensation

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

	CD3 4-488/530/30-A	CD8 4-499/585/42-A	CD19 4-488/710/50-A	CD4 1-638/670/14-A	CD45 1-638/780/60-A
CD3 4-488/530/30-A		1.03	0.00	0.00	0.00
CD8 4-499/585/42-A	8.71		0.00	0.00	0.00
CD19 4-488/710/50-A	3.22	65.45		0.00	0.00
CD4 1-638/670/14-A	0.00	0.00	7.44		19.60
CD45 1-638/780/60-A	0.00	0.00	0.86	2.28	

Figure 6. Compensation Matrix using Compensation Beads

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

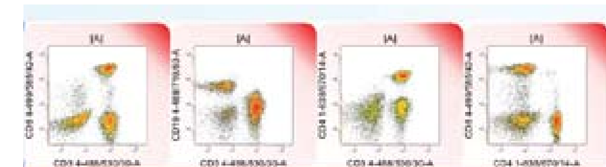

Figure 7. Dual Parameter Plots of Compensated Positive Control Using Compensation Beads Matrix

Principles of Compensation for Consideration

- Compensation beads give brighter signals than cells in the main so more accurate compensation is achieved
- Autofluoresence 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. Please note, this is note required when using the Compensation Wizard.
- 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.
- Two dimensional fluorescence plots can help determine more accurate compensation values by giving better visualization of populations requiring compensation.
- 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.

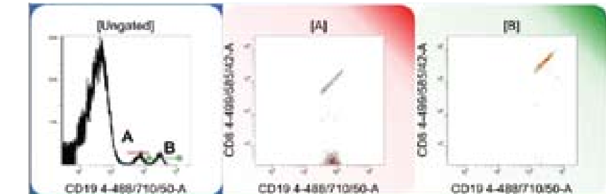

Figure 8- CD19 Histogram Uncompensated Showing the Spectral Overlap of CD8 into CD19 Detector.

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.

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Applied Cytometry
Unit 2 Brooklands Way
Brooklands Park Industrial Estate
Dinnington
Sheffield S25 2JZ
UK

www.appliedcytometry.com

T: +44 (0) 1909 566 982
F: +44 (0) 1909 561 463
E: info@appliedcytometry.com