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Automating Software Compensation for Fluorescence Spillover

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Background

Compensation of fluorescence spillover has been a fundamental issue in flow cytometry since its beginnings. While careful panel design and instrument setup can reduce the amount of spillover, compensating for the spillover of signals into secondary detectors is almost always a requirement in cytometry applications.

Modern compensation approaches are software-based. Some use gates to identify "positive" and "negative" populations in the single-color controls, and compute spillover based on the median population intensities. Others use line-fitting routines to compute the slopes of single-stained controls. These approaches become more difficult and time consuming as the number of fluorophores increases. Gating approaches introduce errors of inclusion, exclusion, and subjectivity into the computations.

We propose an **automated, gateless approach** to fluorescence spillover compensation, requiring only that the operator identify the appropriate single-color and unstained controls.

Methods

A software method was developed to determine optimal spillover coefficients for any number of parameters using information in single-color and unstained controls. V-Comp™ (Verity Software House, USA) uses a gateless method to analyze single-color and unstained controls for each measurement to be compensated. No other user input is required to determine the spillover matrix.

This method automatically detects and eliminates outlier events as well as regions where the acquisition was unstable. A highly-accurate, iterative modeling approach to compensation is employed to determine the optimal spillover coefficients.

To evaluate the V-Comp method, compensation control files along with full-color test files were submitted from a variety of sources. Each set included a reference spillover matrix created using either FACSDiva™ (Becton Dickinson, USA), FlowJo™ (TreeStar Inc., USA), or WinList™ software (Verity Software House, USA).

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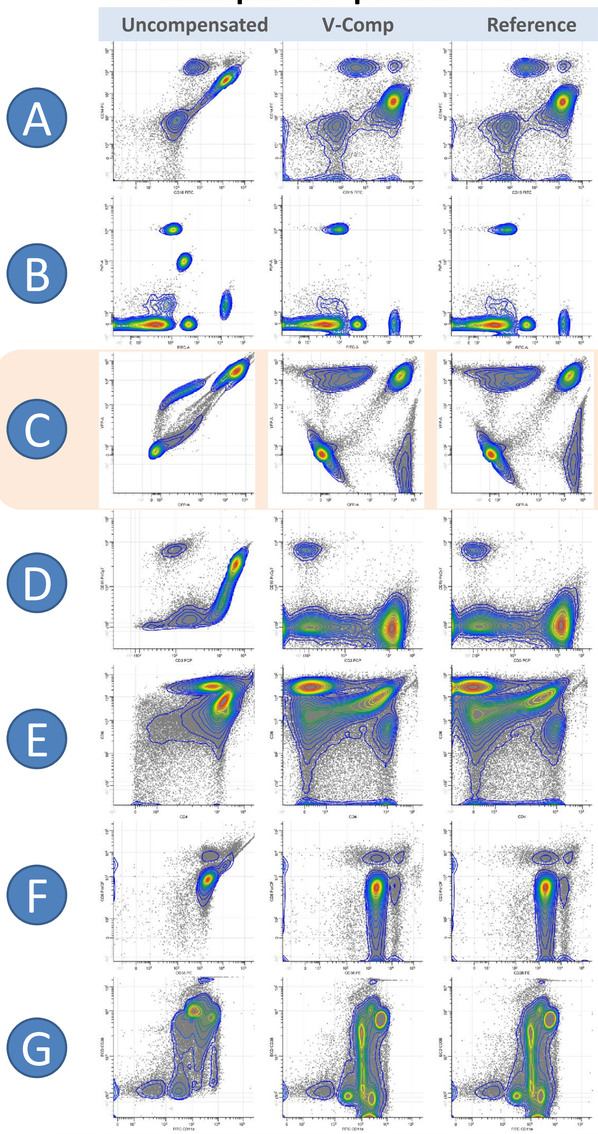
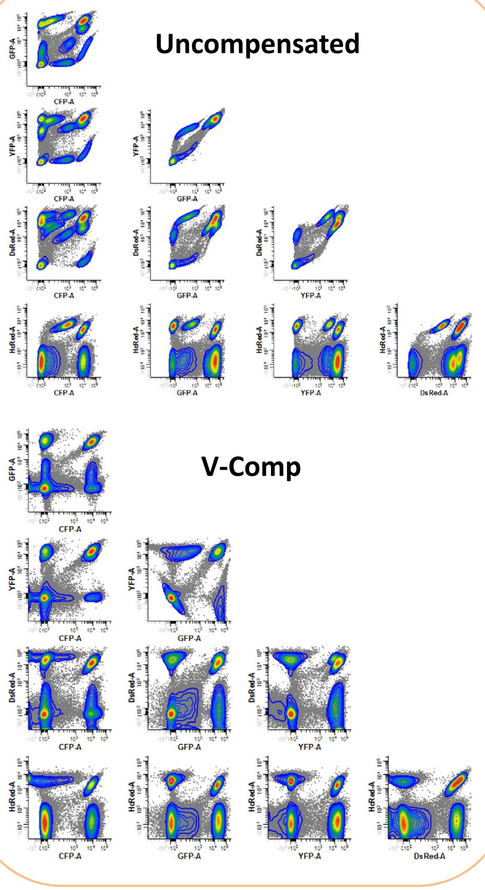
Example Comparison Plots



All Plots for Dataset C



	Assay	Instrument	Average Difference	Max Difference
A	4-color BM	FACSAria II	**0.0039**	**0.0133**
B	4-color Beads	FACSAria	**0.0013**	**0.0028**
C	5-color Fl Proteins	FACSVantage SE	**0.0006**	**0.0027**
D	6-color T-Cell	FACSCanto II	**0.0049**	**0.0463**
E	8-color T-Cell	LSR II	**0.0322**	**0.5370**
F	8-color B-Cell	FACSCanto II	**0.0084**	**0.1950**
G	14-color Lymphocyte	LSR Fortessa	**0.0021**	**0.0246**

Results

Seven assays (A through G in the graphics) were tested, ranging from 4 to 14 colors. To compare the results, the average and maximum differences were computed between the elements of each reference matrix and the associated V-Comp matrix. Bivariate plots of the data were visually compared using both matrices.

The table (center panel bottom) shows the average and maximum differences between reference and V-Comp matrices.

Populations were visually well-resolved for both the traditional and the V-Comp compensation. Example comparison plots are shown for each of the seven assays, with an uncompensated, a V-Comp compensated, and a reference compensated plot for each. The full complement of plots for assay C is also shown, both uncompensated and compensated with V-Comp.

Conclusions

The automated V-Comp method produced spillover matrices that were in all but one case comparable with the reference matrices created by FACSDiva, FlowJo, or WinList. Average differences were less than 0.9% in 6 of 7 tests and less than 3.5% in all tests.

In the one case (E) where differences were of significant magnitude, it was not clear whether V-Comp or the reference compensation from FACSDiva was the "better" compensation.

The V-Comp approach required no subjective decisions beyond the selection of control files. As a gateless method, V-Comp was not subject to errors of inclusion and exclusion that affect gate-based methods. It performed equally well with cellular and bead-based controls.

Further study is required to determine the theoretical accuracy of both V-Comp and gate-based methods. Generated "truth" datasets could be employed in a blind study where users performed both methods.