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Application Note

A Workflow for Spectral Compensation with SpectraComp® Synthetic Cell Controls

Summary

Flow cytometry is a versatile platform for characterizing mixed cell populations. While the introduction of spectral flow cytometry has opened a wider breadth of applications and experimental possibilities, it presents additional challenges associated with compensation and assay design. Increased reliance on more complex spectral flow experiments necessitates higher-performing compensation reagents.

In this application note, we highlight the features of SpectraComp® synthetic cell compensation controls. We demonstrate that SpectraComp®, when used in place of conventional polystyrene compensation beads, achieves superior overall performance. Using the Cytek® Northern Lights™ instrument and its SpectroFlo® software, we compared the median fluorescence intensity (MFI) of SpectraComp® beads to that achieved by commercially available alternatives. We verified binding to a range of common secondary antibodies and confirmed that SpectraComp® matches competitors by this metric of convenience. We demonstrate that SpectraComp® beads have a lower baseline autofluorescence and an overall spectral signature that matches that of genuine leukocytes. Combined, the following results illustrate that the improved user convenience of SpectraComp® is matched only by its stand-out spectral accuracy.

Introduction

Flow cytometry is a widely used cell analysis platform for characterizing cell populations based on a diversity of physical attributes and markers of interest. Applications range from profiling the activities of immune cells to evaluating cell type composition of lysed whole tissues. As a result, flow cytometry is an indispensable tool for the diagnosis of rare blood diseases and the high throughput evaluation of clinical samples.

The introduction of spectral flow cytometry has opened a greater breadth of applications and experimental possibilities. Unlike conventional flow cytometry, in which a narrow range of the emission spectrum for each fluorophore is captured, spectral flow records the full spectral profile. While this increases the complexity and power of experiments that can be run on the platform, the challenges associated with compensation and assay design increase in parallel. Rigorous experimental setup and compensation are especially vital for the use of spectral flow in a clinical setting.

Still, most flow experiments rely on plastic (polystyrene) beads to perform compensation. While fast and convenient, there are a range of performance issues associated with polystyrene beads; namely they fail to recapitulate the spectral profile of single stained cells while also exhibiting high autofluorescence. This inevitably leads to distortions that can cause under- or over-compensation, and ultimately compromise the accuracy of the data.

The ever-evolving field of flow cytometry demands a higher standard of compensation control, and to that end, SpectraComp® was developed. This application note will cover the many unique advantages of SpectraComp® and further explain its usage.

Using the Cytek® Northern Lights™ 5-laser cytometer and its accompanying software, SpectroFlo®, we analyzed the side-by-side performance of SpectraComp® synthetic leukocyte compensation controls and competitor compensation beads. We assessed the MFI of SpectraComp® and measured its ability to match the spectral profile of real cell samples stained with an array of fluorophores. Baseline autofluorescence of SpectraComp® and conventional beads was compared to that of natural cells. Together, the results presented here illustrate the superior performance of SpectraComp® over solid-core polystyrene compensation beads across several key metrics.

Results

Accounting for baseline autofluorescence is a crucial component of experiment setup in flow cytometry. Overall, high autofluorescence decreases the ratio of signal to noise, potentially drowning out biologically relevant signals. Minimizing autofluorescence is especially pertinent in experiments where the user intends to evaluate weakly positive markers.

As shown in **Figure 1**, compared to other commercially available beads, SpectraComp® demonstrated lower baseline autofluorescence, notably in the UV/violet region of the spectra. Polystyrene compensation beads exhibited an autofluorescence spectrum that deviates in the UV and infrared wavelengths. SpectraComp® performs much like real cells, demonstrating a low, even range of emission across the spectrum.

This is generally important for accurate autofluorescence removal and becomes especially key if the user intends to include dyes that emit in the far violet or infrared ends of the spectrum. SpectraComp® beads allow the researcher to access the full range of capabilities of their instrument and use fluorophores that emit at any wavelength without concern. Due to the reduced autofluorescence, in addition to the other improved performance metrics of SpectraComp®, it achieves cleaner separation when coupled with violet dyes, as shown in **Figure 2**.

Spectral unmixing relies on the full emission profile to differentiate between signals from distinct fluorophores. This allows more colors to be used in a single experiment, and further allows multiple colors with similar emission peaks to be used side-by-side. That said, for optimal spectral unmixing, single stained controls should replicate the spectra of stained cell samples.

Conventional compensation beads, while convenient, fail to do so. Mismatches in the spectra generated by compensation controls and samples can lead to over or under-compensation downstream. The quality of the data depends on the rigor and accuracy with which compensation is performed, especially as the user designs more complicated multicolor experiments.

As shown in **Figure 3**, SpectraComp® achieves spectral signatures that are nearly indistinguishable from those generated by single-stained cells, resulting in cleaner spillover calculations. In contrast, conventional compensation reagents exhibit mismatches with the spectral signature of single-stained cells. With SpectraComp®, users can trust that their spectral profiles will match sample profiles across a wide range of fluorophores.

While most compensation beads are designed to capture single species of antibodies, generally the most common: rat, mouse, and hamster. SpectraComp beads provide researchers with convenience and flexibility to capture multiple species of antibodies that allow them to be used in conjunction with many different experimental workflows. In **Figure 4**, we demonstrate the consistently high binding capacity of SpectraComp® to multiple IgG subtypes for each of the three species.

Because compensation calculations depend on the median fluorescence intensity (MFI), the MFI of compensation controls must meet or exceed the brightness of the sample. Therefore, it is important that compensation beads consistently achieve a high median fluorescence intensity (MFI) to start. By this metric, SpectraComp® outshines conventional compensation beads in multiple ways. While the performance of most beads is often limited by a smaller surface area than cells, the surface area of SpectraComp® is comparable to genuine leukocytes. Furthermore, while the chemical composition of many plastic beads interferes with the activity of tandem dyes, the unique composition of SpectraComp® avoids this issue entirely.

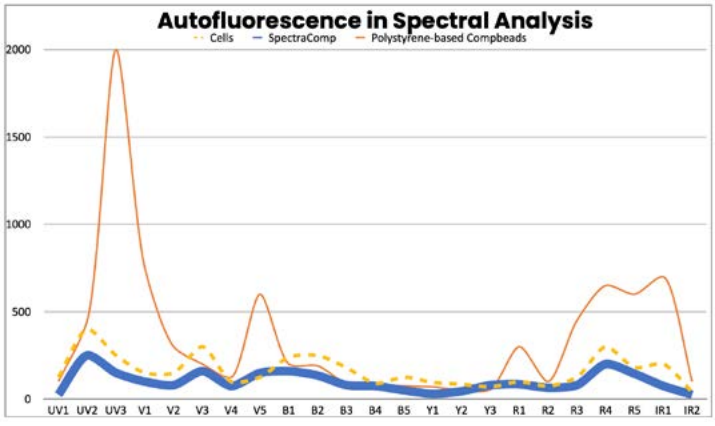

Figure 1: Autofluorescence in Spectral Analysis. Autofluorescence across a full emission spectrum was evaluated for cells, SpectraComp® and competitor polystyrene-based compensation beads.

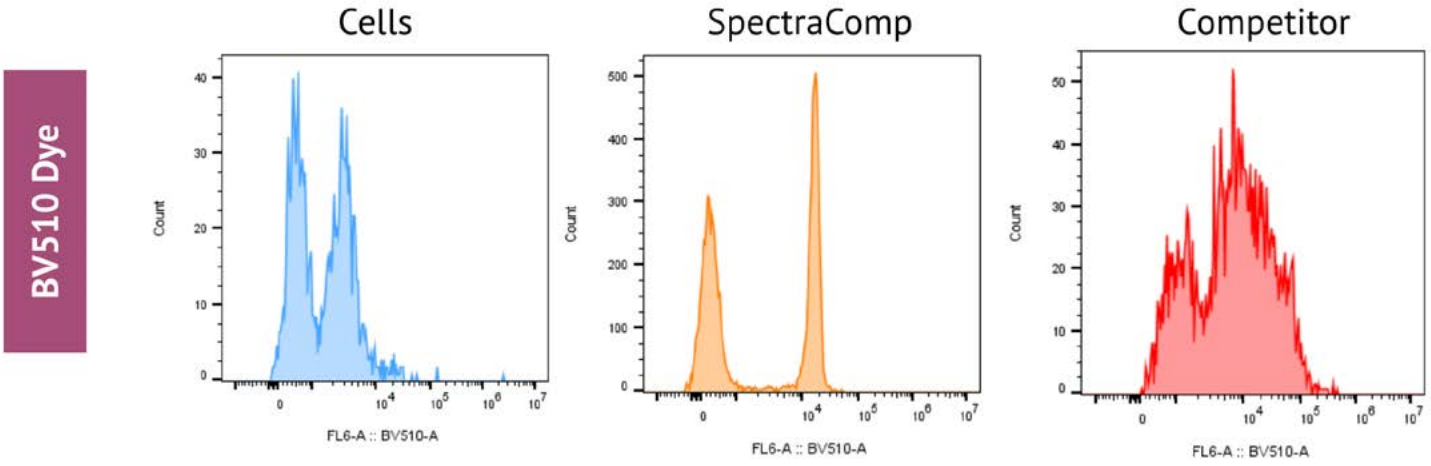

Figure 2: Violet Dye Performance. SpectraComp® beads achieve cleaner separation of positive and negative populations when used in conjunction with violet dyes such as BV510.

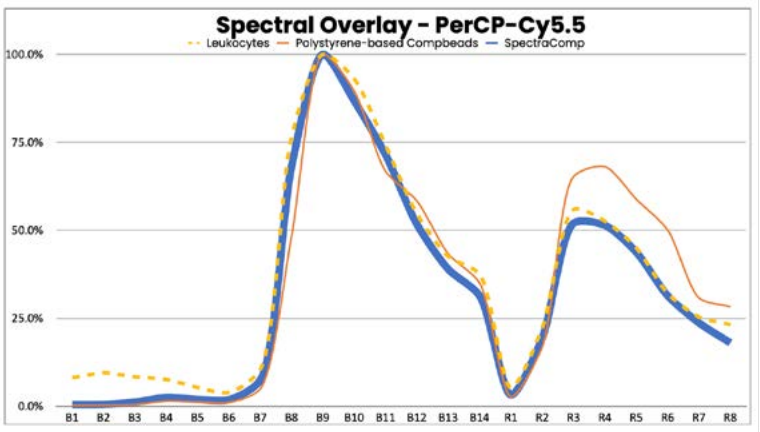

Figure 3: Complete spectral signature comparison. Spectral signatures of SpectraComp® used with the indicated fluorophores matched those of single stained leukocytes.

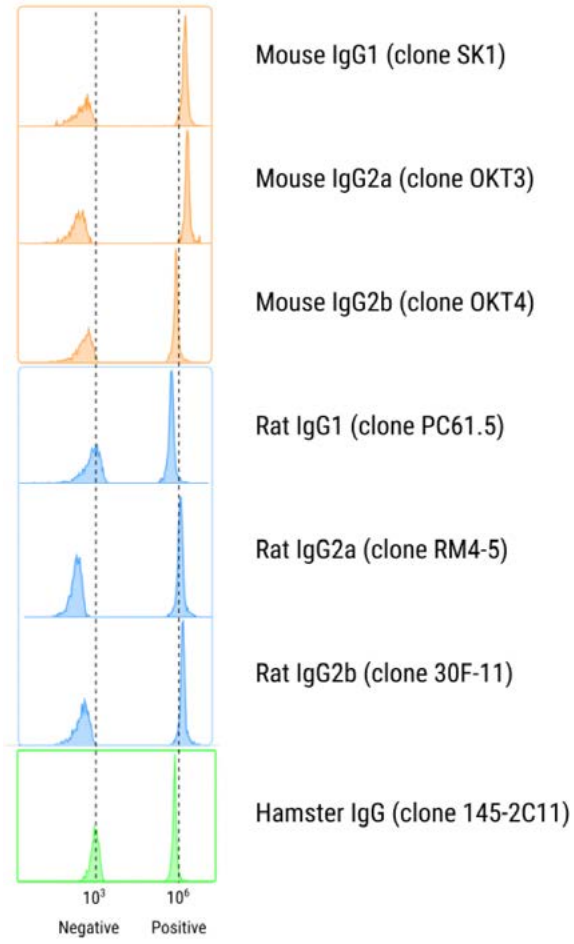

Figure 4: Ab Species Specificity. SpectraComp® beads were stained with FITC-conjugated monoclonal antibodies and histograms of positive and negative populations were generated. Each histogram shows one staining antibody for each host species, isotype, and clone (indicated in parenthesis).

Conclusion

Over the past several years, spectral flow cytometry has experienced increased use for a widening scope of applications. No matter the setting, clean and consistent compensation is a vital component of any flow experiment, spectral or conventional, and as assay design becomes increasingly complex, the need for a higher standard of compensation control only grows. In this workflow, we have shown that SpectraComp® is poised to fill this gap. We have demonstrated its superior convenience and performance across multiple important metrics. The low baseline autofluorescence, high MFI, and cell-like spectral signature of SpectraComp® controls contribute to more accurate unmixing and higher quality data, while its antibody binding capabilities create a seamless, convenient user experience.

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