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Advanced Unmixing Single Color Controls for Better Spectral Flow Cytometry Unmixing Accuracy

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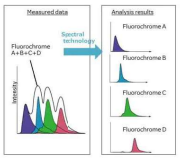
Abstract: Spectral flow cytometry has revolutionized high-dimensional immunophenotyping, enabling simultaneous detection of numerous biomarkers. Accurate unmixing of spectral data is critical for reliable analysis, and compensation beads play a pivotal role in this process. This study investigated the performance of different compensation beads in a 20-color multiplexing panel and compared their unmixing accuracy to lymphocyte cells. Commercial compensation beads from different suppliers were investigated side by side on a spectral flow cytometer. Results revealed significant variability among beads. A newly developed beads demonstrated nearly 100% unmixing similarity to cells in this specific panel, enabling precise spectral representation and optimal separation of overlapping fluorophores. In contrast, other type of beads achieved only 50% unmixing accuracy, leading to compromised data quality and increased spectral spillover. These findings highlight the importance of selecting appropriate compensation beads tailored to specific panels and fluorophore combinations. Although in most cases, using cell to perform single color control experiment is preferred, however for limited cell sources, acquiring single color control data on compensation beads is necessary. Proper bead selection not only improves unmixing fidelity but also reduces the reliance on limited cell samples for compensation controls. This is particularly advantageous in studies with rare cell populations or small sample sizes, helping ensure efficient experimental design and preserving sample integrity. This poster showed spectral unmixing bivariate plot of 20-color panel, the beads performance variation was clearly demonstrated in terms of unmixing accuracy, the newly developed compensation beads demonstrated exceptional spectral unmixing power between fluorophores with high spectral similarity, such as Alexa Fluor™ 488 and Alexa Fluor™ 532. By optimizing bead selection, researchers can facilitate panel design and enhance panel performance, improve reproducibility, and streamline workflows in spectral flow cytometry applications and improve data comparison capability across research institutions and laboratories.

Why use spectral unmixing/compensation single color control beads

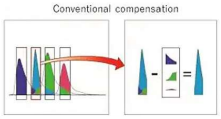
Spectral Unmixing

Accurately determine spectral **fingerprint** of each fluorophore



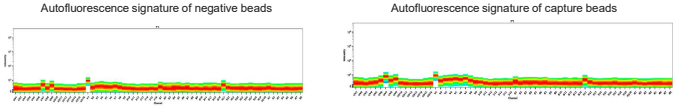
Conventional Compensation

Accurately determine spectral **overlap** of each fluorophore



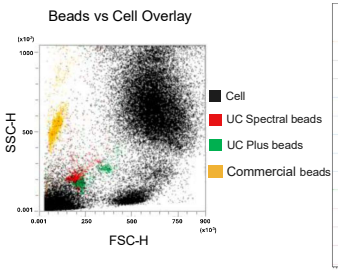
- Enables fluorescence signals are similar to cell samples
- Enables adequate separation between negative and positive populations
- Enables proper data interpretation
- Do not waste cell samples to setup up compensation
- Display minimal autofluorescence

UltraComp Spectral eBeads™ unmixing single color controls has low autofluorescence

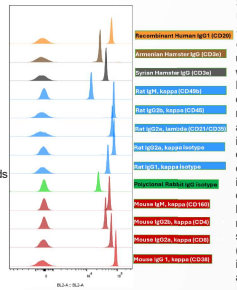


Similarity Index vs Negative Beads						
	Overall	UV	V	B	YG	R
UltraComp Spectral eBeads™ unmixing beads	99.845	99.866	99.793	99.826	99.873	99.345

UltraComp Spectral eBeads™ SCC beads on scale with human lymphocytes on a forward vs side scatter plot



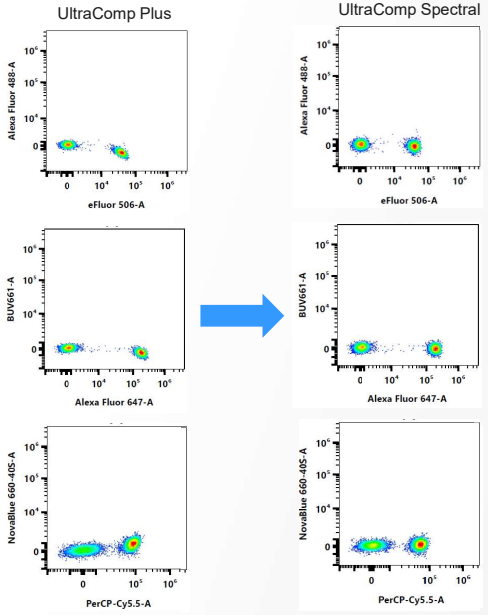
UltraComp Spectral eBeads™ SCC beads designed for all lasers and common conjugated monoclonal antibodies



Staining of UltraComp Spectral eBeads™ unmixing beads with 13 different R-PE conjugated monoclonal antibodies, including one of each subclass commonly used in flow cytometry. Each histogram represents one staining antibody (clone and isotype indicated at right)

Designed for use in compensation with all fluorochromes excited by ultraviolet (355 nm) violet (405 nm), blue (488 nm), green (532 nm), yellow-green (561 nm), and red (633-640 nm) lasers

UltraComp Spectral eBeads™ SCC had better Unmixing Accuracy



Extensive testing of UltraComp Spectral eBeads™ SSC and other commercial SCCs were carried out using the 5-Laser cytek Aurora as the spectral flow cytometry platform. Using a 33-color panel, individual dyes on cells were replaced by bead based single color controls and the panel was unmixed. Representative unmixng results of green emitting, red emitting, and tandem dyes shown above.

Overall better performance by UltraComp Spectral eBeads™ SCC beads across dyes compared to commercial SCCs

UV	Violet	Blue	Yellow	Red
=BUV395	@BV421	^Alexa.Fluor.488	^PE	^APC
=BUV563	=Super.Bright.436	^Alexa.Fluor.532	^NovaFluor.Yellow.590	^Alexa.Fluor.647
^BUV661	^eFluor.450	^NovaFluor.Blue.660.40S	^PE.eFluor.610	! NovaFluor.Red.700
^BUV737	^BV480	^FITC	@PE.Cy5	^Alexa.Fluor.700
=BUV805	^eFluor.506	^NovaFluor.Blue.B510	^PE.Cy5.5	^APC.eFluor.780
	=Pacific.Orange	^BB700	^PE.Cy7	
	=BV570	^PerCP.Cy5.5		
	=Super.Bright.600	^PerCP.eFluor.710		
	=BV711			
	^BV750			
	^BV786			

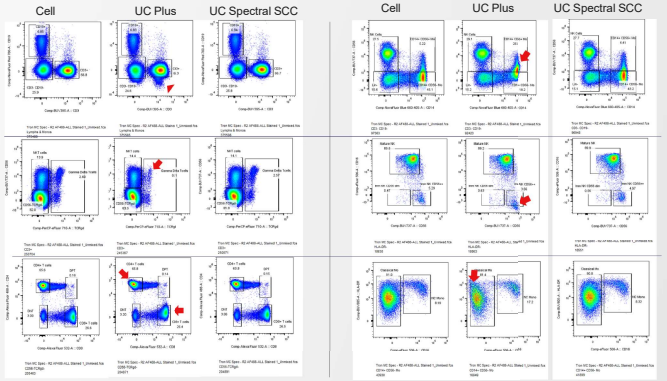
^	Improved performance
=	Equal performance
@	Lower performance in 1 panel
!	Lower performance in > 1 panels

Additional Dyes (13 total)

=Super Bright 702
=Super Bright 645
=BUV496
=BV650
^AF660
=PerCP

@BV605
=PE-AF700
^BV510
^APC-Fire750
@ BB515
= NFB585
^ NFB610-70s

UltraComp Spectral eBeads™ SCC had better performance in a 20-color panel



Representative comparison plots from a 20-color spectral flow cytometry panel

Summary of UltraComp Spectral eBeads™ unmixing beads vs. competitors' SCCs

Feature	UltraComp Spectal	UltraComp Plus	Competitor SCC I	Competitor SCC II	Competitor SCC III
Can bind mouse, rat, rabbit, hamster & human species and IgG isotypes with one product	✓	✓	☒	☒	✓
Compatible with UV laser	✓	✓	☒	✓	✓
Single Vial product format	✓	✓	☒	✓	✓
No. of washes	1	1	1	3	2
Scatter properties ≈ lymphs	✓	✓	☒	✓	✓
Frequency of unmixing errors vs cells	Lowest on market	Medium§	High§	Medium§	Medium§

§Note: Spectral unmixing performance vs. cells (PBMCs) is acceptable for some dye-Ab conjugates.

Conclusions: UltraComp Spectral eBeads™ unmixing beads react with mouse, rat, hamster, rabbit, recombinant human antibodies. They have similar FSC and SSC profiles to lymphocytes, facilitating easy instrument setup. These beads exhibit low autofluorescence, which is critical for minimizing unmixing errors, and feature matched autofluorescence between negative beads and capture beads, helping ensure more accurate results. Additionally, UltraComp Spectral eBeads™ unmixing beads require only one wash to simplify workflow, helping make the process more efficient. In a 20-color panel unmixing test, these single-color controls demonstrated greater unmixing similarity to cells than UltraComp eBeads™ Plus compensation beads and other commercial single-color controls. Overall, UltraComp Spectral eBeads™ unmixing beads offer exceptional unmixing performance. Their similarity to cell unmixability facilitates high-parameter multicolor panel design for spectral flow cytometry, making them a more suitable choice as spectral cytometry SCCs compared to other commercial products.