



# Understanding Spreading Error in Spectral Flow Cytometry: A Case Study to Evaluate Software Features

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## Introduction

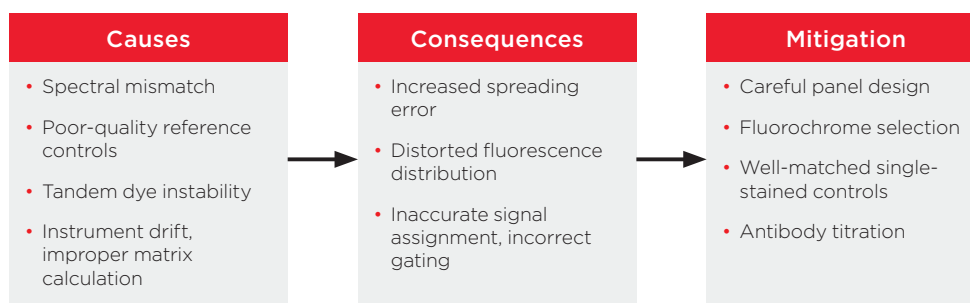
Spectral flow cytometry captures the full emission spectrum of fluorochromes, enabling precise detection of dim markers and separation of overlapping fluorescent signals. However, it can be affected by spreading errors and unmixing errors, which occur when signals from different fluorochromes are not accurately separated, leading to compromised data quality. These issues are especially problematic in complex multicolor panels. The CytoFLEX mosaic Spectral Detection Module addresses this with unmixing algorithms that use high-resolution spectral data and single-stained controls to accurately distinguish fluorochromes. This application note will help you better understand these challenges and how the CytoFLEX system could help reduce spreading errors.

## Unmixing Error

In spectral flow cytometry, particularly when using large panels, a distinct challenge known as unmixing error has been observed. This issue commonly arises when the spectral profile of a fluorochrome in the reference control does not accurately match its profile in the experimental sample. Because spectral flow cytometry relies on computational unmixing algorithms to resolve signals from fluorochromes with overlapping emission spectra, even small mismatches can lead to significant errors in data interpretation.

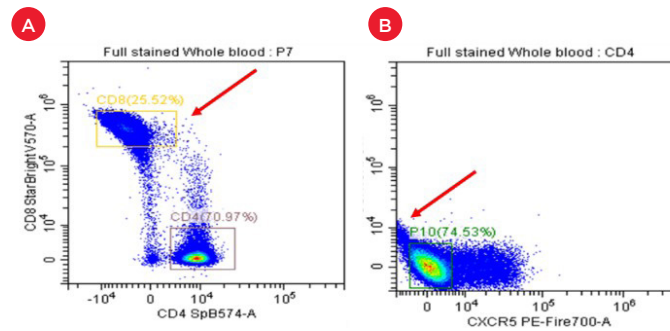
One of the most critical consequences of such unmixing errors is increased spreading error, where signal from a bright fluorochrome is incorrectly assigned to other detection channels. This misassignment results in elevated background noise, reduced ability to resolve dim or rare populations, and an overall decline in sensitivity and resolution.

Additionally, unmixing errors can distort fluorescence intensity distributions, leading to inaccurate signal assignment, incorrect gating, and increased variability between samples or experimental runs. These effects collectively reduce the reliability and reproducibility of cytometric data.



**Figure 1. Overview of Unmixing Error in Flow Cytometry.** This schematic diagram illustrates the concept of Unmixing Error through three interconnected sections: Causes, Consequences, and Mitigation.

It is important to note that while spectral mismatch is a major driver of unmixing error, other contributors include poor-quality reference controls, tandem dye instability, autofluorescence variation, instrument drift, and improper matrix calculation. Therefore, managing unmixing errors requires careful attention to panel design, fluorochrome selection, and the use of well-matched, high-quality single-stained controls under conditions that closely replicate the experimental setup.



**Figure 2. Unmixing error examples.** The unmixing error is indicated by the arrow and appears as a tilted pattern in the population.

## Spillover Spreading Error

Spectral overlap and spread are critical considerations in flow cytometry panel design, as they can significantly impact resolution and data interpretation. In spectral flow cytometry, the issue of overlapping emission spectra from multiple fluorophores is addressed using unmixing algorithms, which computationally separate the signals detected across the full spectrum. However, even after accurate unmixing, spread remains a challenge.

Spread, more precisely referred to as Spillover Spreading Error (SSE), is a fundamental limitation stemming from the physical and statistical nature of fluorescence detection. It occurs when the signal from one fluorophore introduces variability—or increased measurement noise—in detectors assigned to other fluorophores, due to the shared spectral regions. This results in broader distributions and can reduce the ability to resolve dim or rare populations, especially when markers are co-expressed.

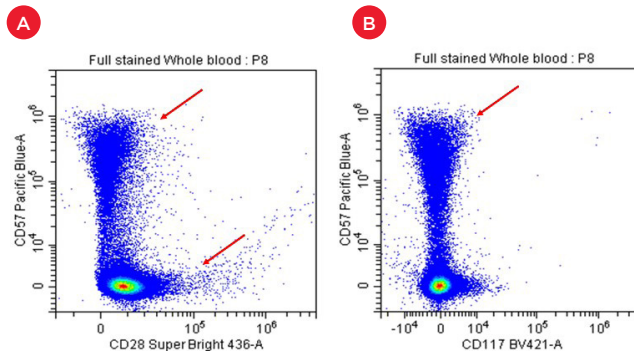
SSE arises from a combination of factors:

- The Poisson-based nature of photon detection, where brighter signals inherently produce more statistical variation.
- Electronic noise in the detection system.
- Spectral overlap, particularly with bright fluorophores or when highly expressed markers share detectors with others.

Importantly, spreading error cannot be fully eliminated—even with perfect unmixing. Its impact becomes more pronounced in high-dimensional panels and in the analysis of co-expressed markers.

To mitigate the effects of SSE:

- Careful panel design is crucial—fluorophore selection and should minimize spread between co-expressed markers.
- Use of proper controls, such as Fluorescence Minus One (FMO) sample, helps identify the impact of spread on gating accuracy.



**Figure 3. Spillover spreading error examples.** Spread errors here, seen as an "umbrella pattern" where the Pacific Blue signal spills into SuperBright 436 (A) and BV421 (B), causing signal distortion and making populations appear broader than they are. In Figure A, SuperBright 436 spreads into Pacific Blue, further contributing to signal overlap.

Category	Factor	Description
Fluorochrome Usage	Number of Fluorochromes in One Detector	More fluorochromes per detector increase spectral overlap and spread.
	Fluorochrome Brightness	Brighter dyes emit more photons, increasing the chance of spillover.
	Spectral Overlap	Similar emission spectra make separation difficult, leading to spreading.
	Fluorophore Combinations	Certain dye pairs are more prone to interference and should be avoided.
Biological Factors	Fluorochrome Stability and Photobleaching	While less directly linked to spreading error, fluorochrome instability or photobleaching during acquisition can affect signal quality and complicate unmixing.
	Antigen Density	High expression levels produce intense signals that can cause more spread.
	High Autofluorescence	If not modeled correctly, it spills across channels and increases background.
	Variable Autofluorescence Across Cell Types	Makes it hard to apply a single autofluorescence type, increasing spread.
Instrument Factors	Cell Viability and Sample Preparation	Dead cells or debris can increase autofluorescence and nonspecific signals, worsening spreading error.
	Detector Sensitivity	Variability in detector performance affects signal resolution and spread.
	Instrument Configuration	Misalignment or poor calibration can worsen spectral separation.
	Detector Noise	Electronic noise can exacerbate spread and reduce resolution.
Control & Setup Issues	Electronic Noise Factors	Photon counting statistics (shot noise) can increase spreading error, especially for dim signals.
	Poor-Quality Single-Stain Controls	Inaccurate spectral references lead to poor unmixing and signal spread.
	Dim or Over-Saturated Controls	Low signal-to-noise or saturation distorts spectral shape, causing misassignment.
	Beads vs. Cells for Controls	Beads may not replicate cellular spectral behavior, leading to mismatches.
Software & Algorithm	Numerical Instability in Unmixing	Matrix inversion errors can cause false signal assignment.
	Underfitting the Unmixing Model	Inadequate modeling forces signal into incorrect channels.
	Poor Autofluorescence Handling	Residual signals may be misattributed to other fluorochromes.

**Table 1.** Comprehensive Factors Influencing Spread in Spectral Flow Cytometry.

## Case Study: 10-Color Panel Design Challenges

The current panel includes the following fluorophore-marker combinations:

Marker	Fluorophore	Catalogue #	Company
CD45	SP395	304096	BioLegend
CD3	PerCP	300428	BioLegend
CD4	BV650	300536	BioLegend
CD8	BUV496	612943	BD
CD45RA	PED594	304146	BioLegend
CD56	AF647	981204	BioLegend
HLA-DR	BUV661	612981	BD
CD14	PC5	301864	BioLegend
CD19	PEF700	363050	BioLegend
CD197	PC7	353226	BioLegend

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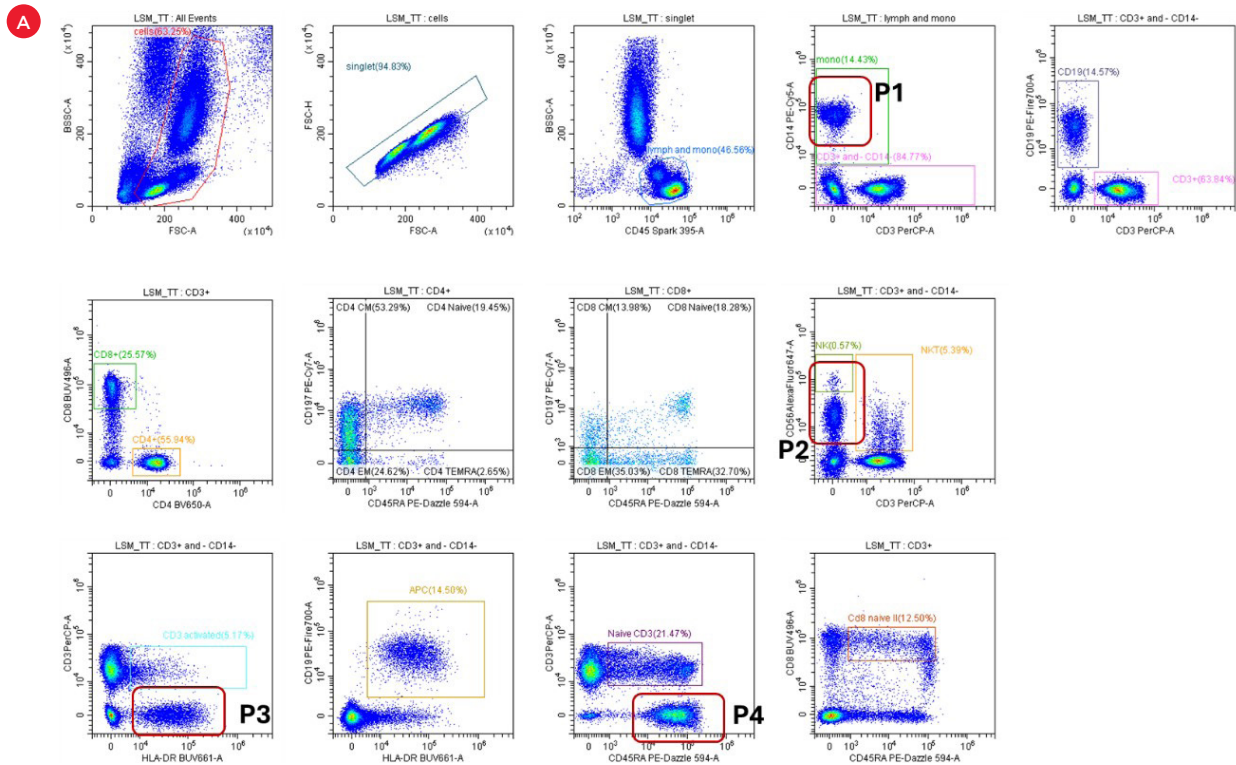
For this 10-color panel, we used fresh blood samples and ran them on the CytoFLEX LX flow cytometer equipped with the CytoFLEX mosaic 88 spectral detection module. Figure 4 presents the results obtained using two unmixing algorithms: the Least Squares Method (LSM) and a proprietary Poisson Hybrid algorithm. Further optimization of this 10-color flow cytometry panel could have improved data quality. For instance, highly expressed markers such as CD45RA and CD14 were assigned to very bright fluorophores—PED594 and PECy5, respectively—potentially leading to signal saturation and reduced resolution for other markers. This pairing increases the risk of signal spillovers into adjacent channels and can contribute to challenges in spectral unmixing and increased spreading error, ultimately affecting the reliability and resolution of the data.

### Unmixing Algorithm Solutions: Poisson Hybrid vs. LSM

The CytoFLEX mosaic spectral detection module provides users with two unmixing algorithms: the widely recognized LSM and a proprietary Poisson hybrid algorithm. The LSM unmixing algorithm is the simplest and most widely used approach. It assumes that the mean of the noise is constant and that all detectors have the same variance, regardless of the signal level. However, in practical scenarios, this assumption often doesn't hold, leading to a mismatch that can cause population distortion, such as spreading of the data.

The Poisson Hybrid unmixing algorithm, proprietary to Beckman Coulter Life Sciences, combines Poisson statistics with LSM. It uses the LSM result as the initial parameter estimate and then incorporates the Poisson nature of both signal and noise.

This hybrid approach enhances both efficiency and accuracy, particularly in datasets with variable signal characteristics, as illustrated in Figure 4. Populations P1, P2, P3 and P4 demonstrate distinct differences in signal spreading between the two unmixing algorithms (Figures 4A and 4B). The Poisson hybrid method is especially recommended, as it models noise based on the inherent randomness of photon emission (Poisson statistics), offering a more realistic representation of signal variability. While it generally performs well in minimizing spreading error, it is computationally slower compared to the LSM algorithm. These differences are further supported by the relative Standard Deviation (rSD) plot in Figure 4C, which quantitatively illustrates the variation in signal spread across channels for each algorithm. rSD (relative Standard Deviation) is a normalized measure of variability, calculated as the standard deviation divided by the mean, often expressed as a percentage. In the context of spectral unmixing, rSD is important because it reflects the consistency and precision of signal separation. Lower rSD values indicate tighter, more reliable population clustering, which is critical for accurate identification and quantification of cell populations in high-dimensional flow cytometry. The Spectral Spillover Matrix (SSM) shown in Figure 4D is used to measure how much signal from one fluorochrome overlaps into other detection channels. It plays a key role in the unmixing process, which separates overlapping signals so each fluorochrome can be accurately identified. A lower SSM value means less overlap between fluorochromes, resulting in cleaner data, better population separation, and more reliable analysis (Figure 4D).





D

LSM											
Sum=101.9758											
Channel	AF647-A	BUV496-A	BUV661-A	BV650-A	PC5-A	PC7-A	PE-Dazzle 594-A	PE-Fire 700-A	PerCP-A	Spark395-A	Sum
AF647-A	0	0	0	0	1.47811	0.222104	0.50897	0.719403	1.62256	0	4.551147
BUV496-A	0.529275	0	1.2889	0	0.430355	0	0	0	0.532645	0	2.781175
BUV661-A	5.94967	0	0	2.20033	3.55275	0	0	1.47046	2.66262	0	15.83583
BV650-A	2.47053	0	5.40429	0	1.67764	0.408964	0	1.09409	1.90774	0	12.96325
PC5-A	3.0951	1.66514	3.42029	1.43575	0	1.15896	0	4.29922	5.91706	0.166313	21.15783
PC7-A	0	0.999854	0	0	0.598611	0	0	0	0.885498	0	2.483963
PE-Dazzle 594-A	1.16267	1.77225	1.75643	1.69817	5.22883	0.887045	0	2.56899	2.76348	0.0822821	17.92015
PE-Fire 700-A	0.833853	0.821006	0.818693	0	1.74127	1.46659	0	0	2.49632	0	8.177732
PerCP-A	2.28181	1.11519	2.6965	1.47302	3.20082	0	0.16522	2.03597	0	0	12.96853
Spark395-A	0	2.12748	0	0.298441	0.143616	0.0699834	0	0	0.478638	0	3.118158
Sum	16.322908	8.50092	15.385103	7.105711	18.052	4.2136464	0.67419	12.188133	19.26656	0.2485951	101.9578

Poisson Hybrid											
Sum=98.47297											
Channel	AF647-A	BUV496-A	BUV661-A	BV650-A	PC5-A	PC7-A	PE-Dazzle 594-A	PE-Fire 700-A	PerCP-A	Spark395-A	Sum
AF647-A	0	0	0	1.1078	1.85812	1.02199	0	1.48604	1.96301	0	7.43696
BUV496-A	0.552617	0	1.33481	0.774682	0.4974	0	0	0.153892	0.214752	0.179315	3.707468
BUV661-A	5.44791	0	0	2.56591	3.17107	0.801888	0.258739	1.51182	1.97819	0	15.73553
BV650-A	2.56537	0	5.26796	0	2.02452	0.798031	0.593005	1.28443	2.11974	0	14.65306
PC5-A	3.41984	0	1.35957	1.52527	0	1.26875	0	3.27519	2.98483	0	13.83345
PC7-A	0	0	0	0.59968	0.353715	0	0.578132	0.882043	0	0	2.41357
PE-Dazzle 594-A	1.10777	0.644453	1.45712	1.87271	5.24434	1.08556	0	2.20283	1.62541	0	15.24019
PE-Fire 700-A	1.16	0	0.115543	0.571815	1.4783	1.91182	0.462068	0	1.63515	0	7.334696
PerCP-A	2.42771	0.550779	2.67693	2.54455	2.95874	1.42414	0.374095	2.29749	0	0	15.25443
Spark395-A	0.145208	2.19685	0	0.406799	0.114754	0	0	0	0	0	2.863611
Sum	16.826425	3.392082	12.211933	11.96922	17.70096	8.312179	2.266039	13.093735	12.52108	0.179315	98.47297

**Figure 4.** Comparison of Poisson hybrid and LSM Algorithms in Spread Error Reduction. A 10-marker panel was used to detect major cell subpopulations in human peripheral blood, unmixed using Poisson hybrid and LSM algorithms. **(A)** Gating data unmixed strategy with the LSM (Least Squares Method) algorithm. **(B)** Gating data unmixed strategy with the Poisson hybrid algorithm. **(C)** Relative Standard Deviation (rSD) calculated for selected gates demonstrates reduced spread with the Poisson hybrid algorithm, highlighting improved resolution not captured by SSM scores alone. **(D)** Spillover Spreading Matrix (SSM) table comparing both algorithms shows similar spreading scores across detectors.

It is important to note that the results we have shown here may vary when using a different panel with more fluorochromes, highly overlapping dyes, or different types of samples, regardless of whether the antibodies used are titrated. Users are advised to switch between unmixing algorithms to determine which best fits their specific requirements and experimental conditions. That said, our studies show that the modified Poisson algorithm helps reduce spreading error, particularly with highly overlapping dye combinations and in panels where not all antibodies are fully titrated

## Summary

Spillover spreading error and unmixing error are major challenges in spectral flow cytometry, especially in high-dimensional panels. These errors stem from both biological and technical sources, including fluorochrome brightness, spectral overlap, instrument configuration, and control quality. While unmixing algorithms like the Poisson hybrid offer accuracy, careful panel design and control selection remain essential for minimizing spread and ensuring reliable data interpretation.

## Acknowledgement

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