Proceedings of ICBioMed 2026 Symposium: Biomedical Engineering and Smart Medicine
DOI: 10.54254/2753-8818/2026.HZ37227

# *Dim-Marker Resolution in Spectral Flow Cytometry: Reference-Control Mismatch and Spectral Collinearity*

**Yida Yan**

*School of Biological Sciences, University of California, Irvine, Irvine, USA*
*yiday6@uci.edu*

***Abstract.*** High-parameter spectral flow cytometry can measure lots of immune-cell markers in one sample, but the detection of dim markers remains sensitive to errors that created during spectral unmixing. This review examines two related but statistically distinct mechanism of poor dim-marker resolution. First, a reference-control mismatch happened when the spectrum measured from a single-stained control that does not accurately represent the spectrum in the fully stained biological sample. This mismatch may produce biased signals and residual patterns. Second, spectral collinearity occurs when several fluorochromes have highly similar emission profiles. In this situation, unmixing can increase the measurement variance and produce unmixing-dependent spreading, even when an average signal value seemed correct. Both mechanisms will decrease the separation between negative and weakly positive populations. The effects are important for immune phenotyping because low-expression markers often define rare or biologically meaningful cell subsets. Evidence from high-color immune panels, antibody titration studies, autofluorescence analysis, and measurable residual disease testing shows that poor control selection or panel design can change biological interpretation. Matched reference controls, careful fluorochrome assignment, reagent titration, and unmixing quality checks can reduce these risks.

***Keywords:*** spectral flow cytometry, spectral unmixing, dim markers, reference controls, immune phenotyping

## 1. Introduction

Spectral flow cytometry has greatly increased the number of proteins that can be measured on a single cell. A recent optimized panel measured 50 fluorochromes to describe major immune-cell lineages, T-cell states, and dendritic-cell subsets in human samples [1]. Standardized whole-blood panels with more than 30 fluorescent markers have also been developed for clinical and translational immune monitoring [2]. These studies show the biological value of collecting many markers from a limited sample. However, adding more fluorochromes also makes the unmixing problem more difficult and increases the need for strong quality control.

The problem is very important for dim markers. Bright lineage markers such as CD3 or CD4 can often remain visible after a moderate loss of signal quality. In short words, a weak activation marker, chemokine receptor, or a stem-cell marker may sit close to the negative distribution. A small shift or

© 2026 The Authors. This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (https://creativecommons.org/licenses/by/4.0/).

DOI: 10.54254/2753-8818/2026.HZ37227

an increase of spread can therefore change the number of events classifies as positive. The resulting error is not only technical. More importantly, this technical error can change the estimated size of an immune-cell population and therefore affects the biological interpretation of the data.

This review focused on two major sources of this problem: reference-control mismatch and spectral collinearity. The first point mainly introduces systematic bias, while second point mainly increases variance during unmixing. This difference provided a useful statistical framework for explaining why dim populations may shift, widen, or disappear in a high-parameter panel.

## 2. Spectral unmixing and dim-marker resolution

### 2.1. Basic model of spectral unmixing

In spectral flow cytometry, fluorescence from each cell is collected across many detectors. Each fluorochrome is represented by a spectral signature, which describes the relative signal measured across those detectors. Single-stained control group are used to build a spectral matrix, and an unmixing algorithm estimated how much of each fluorochrome is present in a multicolor event [3]. This approach differs from conventional compensation because the number of detectors can be larger than the number of fluorochromes, allowing different mathematical solutions to estimate fluorochrome abundance [4].

A simple linear model helps explain this process. The detector signal is treated as the spectral matrix multiplied by the fluorochrome abundance, and plus measurement noise. Unmixing attempts to recover the abundance values. An accurate recovery depends on two conditions: the reference spectra must match the experimental sample, and the spectra in the matrix must contain enough independent information. Failure of either condition reduces the reliability of the unmixed values.

### 2.2. Why dim markers are vulnerable

Marker resolution depends on both signal intensity and width of the negative population, therefore fluorochrome brightness is not the only factor. Spectral overlap from a bright and co-expressed marker can add variations to another parameter and reduces its effective sensitivity [5]. The stain index describes this relationship by comparing the distance between positive and negative populations with the spread of the negative population.

Nguyen and colleagues developed the spillover spreading matrix to measure how one fluorochrome will increase the spread observed in another detector [6]. Their multicolor example showed that different fluorochrome assignments for CD45RA changed the ability to resolve dim CD25 expression. Although that study used conventional flow cytometry, the central principle remains the same: a correct average correction will not remove measurement variance, and the remaining spread can hide weak biological signals.

## 3. Reference-control mismatch

An unmixing control should reproduce the spectrum of the same fluorochrome in the experimental sample. Cells are often considered an ideal control because the fluorochrome is measured in a biological environment similar to the test sample. However, some antigens are too rare or too dim to produce a clear single-stained population. Antibody-capture beads are useful in this case because strong positive and negative populations can be prepared without depending on antigen expression.

DOI: 10.54254/2753-8818/2026.HZ37227

Beads are not always spectrally equal to cells. Shevchenko and colleagues compared two types of compensation beads with human and mouse primary leukocytes for 30 commonly used fluorochromes [7]. Most fluorochromes showed similar profiles, but several produced control-dependent differences, and some were better controlled with cells. This result is important because full-spectrum unmixing uses information from the complete emission profile. Even a small change across several detectors may influence the estimated signal in a complex panel.

Mismatch can also result from tandem-dye variation, different fixation or buffer conditions, autofluorescence, reagent aging, or a difference between the staining intensity of the control and sample. In statistical terms, the spectral matrix used by the algorithm no longer represents the true data-generating process. The estimated abundance may then contain systematic residuals. These residuals can appear as an unexpected shift, a tilted population, or signal in a parameter that should be negative. For a dim marker, the shift may be large compared with the biological signal.

## 4. Spectral collinearity and unmixing-dependent spreading

A different problem occurs when several fluorochromes have highly similar spectral signatures. Similar spectra provide partly repeated information to the unmixing model. Mage and colleagues described the resulting effect as unmixing-dependent spreading (UDS) [8]. In panels containing strongly overlapping fluorochrome combinations, UDS progressively reduces the unmixed signal-to-noise ratio. Importantly, the same raw events can show more spread when unmixed with a larger and more collinear spectral matrix.

The statistical reason is variance inflation. When two or more columns of the spectral matrix are close to collinear, several combinations of fluorochrome abundances can explain nearly the same detector signal. Small amounts of photon noise or detector noise can then produce larger changes in the estimated abundances. The means of negative populations may remain near zero, but their distributions become wider and may develop covariance with other parameters. High cosine similarity between one pair of dyes is not sufficient to predict the complete effect because the full set of spectra determines the condition of the matrix [8].

Algorithm choice can further influence the observed result. Bhowmick and Bushnell compared unmixing approaches across some large published panels and found that algorithm differences could cause loss of resolution, population misidentification, and incorrect biological interpretation [9]. Kmet and Novo also show that including dyes in the mixing matrix can increase the variance of an event even when the dyes are not presented on that event [10]. Their biologically informed approach reduced variance in both small and 40-color panels. These findings showed that panel size is not the main problem. The spectral relationships among these dyes and the structure of the unmixing method are more important.

Reference mismatch and collinearity should not be treated as the same error. Reference mismatch means that the matrix is not accurate enough and therefore tends to create bias. Collinearity means that the matrix has difficulty separating similar signals and therefore tends to increase variance. A sample can contain both errors at the same time. For example, a mismatch tandem-dye reference may shift the center of a negative population, while a collinear group of spectra widens the same population. The combined change can be much more harmful for a dim marker than either error alone [7-10].

DOI: 10.54254/2753-8818/2026.HZ37227

## 5. Biological consequences for immune-cell analysis

Many immune-cell subsets are defined by weak or continuous expression rather than a clearly separated positive population. Regulatory T cells, activation states, exhaustion states, and some myeloid populations may depend on markers located near the gating boundary. Increased spread can move negative events into the positive region and positive events into the negative region. The first change may overestimate population frequency, while the second would reduce sensitivity. In both cases, a technical change can be mistaken for biological differences.

Antibody concentration also involved in this problem. Insufficient antibody reduced positive signal, while excessive antibody can increase the background, nonspecific staining, and spread. Bonilla and colleagues compared stain index, separation index, signal-to-noise ratio, and positive-cell frequency during titration of immune-cell markers [11]. The results support selection of a concentration that provides strong separation rather than simply the highest positive median. This step is important when a bright marker contribute spread into a dim, co-expressed marker.

Autofluorescence provided another source of spectral variation. Different cell types, and even different states within one cell type, can have different autofluorescence profiles. Roet and colleagues used clustering of unstained cells to identify several autofluorescence signatures and include them during unmixing [12]. This approach reduced confounding spread into external phenotyping markers. The study demonstrates that a single autofluorescence reference may be insufficient for a heterogeneous sample.

The effect also matters in clinical applications. A 22-color assay for measurable residual disease in acute myeloid leukemia used peripheral-blood leukocytes as widely available unmixing controls [13]. The assay succeeds reproducible detection and strong agreement with a five-tube reference method. This example does not prove that one control type is always best, but it shows that reference-control design is part of biological assay validation rather than a minor software setting.

## 6. Practical strategies to protect dim-marker resolution

The first strategy is to select reference controls according to each fluorochrome and sample type. A bead control should be compared with cells when possible, especially for tandem dyes or dyes previously associated with control-dependent spectra [7]. Controls should use the same fluorochrome conjugate, similar preparation conditions, and sufficient signal. Unmixed single-stained controls should be inspected for shifts, tilted negatives, and unexpected residual patterns before the full experiment is interpreted.

The second strategy is to design the panel by assigning fluorochromes according to the co-expression relationships among markers. Dim targets should receive bright fluorochromes that receive limited spread. Very bright and highly expressed markers should not be assigned to dyes that produce strong spread into important dim parameters [3, 5, 6]. Collinear groups of fluorochromes should be evaluated in the context of the full matrix, not only by examining pairs [8]. Reagent titration should then balance positive signal against negative spread [11].

Finally, quality control should include more than the appearance of the final gates. Single-stain mismatch measures, robust spread statistics, autofluorescence checks, and fluorescence-minus-one controls can identify problems before a biological conclusion is made [9, 12]. New unmixing methods may reduce variance by using biological information about which dyes can be present on an event [10]. However, algorithmic improvement cannot replace poor reference controls or weak experimental design. A reliable workflow combined matched controls, careful panel design, titration, and direct inspection of dim populations.

DOI: 10.54254/2753-8818/2026.HZ37227

## 7. Conclusion

High-parameter spectral flow cytometry gives detailed immune-cell information, but larger panels do not automatically produce reliable results. Reference-control mismatch mainly creates bias, whereas spectral collinearity increases the variance and UDS. Dim markers are affected first because their positive and negative distributions are very close. The result may be a shifted gate, a missed rare population, or an incorrect change in cell frequency. Separating bias from the variance makes troubleshooting clearer. Matched references, careful fluorochrome assignment, titration, autofluorescence modeling, and unmixing checks can protect weak signals.

## References

[1] Konecny, A. J., Mage, P. L., Tyznik, A. J., Prlic, M., & Mair, F. (2024). OMIP-102: 50-color phenotyping of the human immune system with in-depth assessment of T cells and dendritic cells. *Cytometry Part A, 105*(6), 430-436. https://doi.org/10.1002/cyto.a.24841

[2] Dott, T., Culina, S., Chemali, R., Mansour, C. A., Dubois, F., Jagla, B., et al. (2024). Standardized high-dimensional spectral cytometry protocol and panels for whole blood immune phenotyping in clinical and translational studies. *Cytometry Part A, 105*(2), 124-138. https://doi.org/10.1002/cyto.a.24801

[3] Ferrer-Font, L., Small, S. J., Lewer, B., Pilkington, K. R., Johnston, L. K., Park, L. M., et al. (2021). Panel optimization for high-dimensional immunophenotyping assays using full-spectrum flow cytometry. *Current Protocols, 1*(9), e222. https://doi.org/10.1002/cpz1.222

[4] Novo, D. (2022). A comparison of spectral unmixing to conventional compensation for the calculation of fluorochrome abundances from flow cytometric data. *Cytometry Part A, 101*(11), 885-891. https://doi.org/10.1002/cyto.a.24669

[5] Maecker, H. T., Frey, T., Nomura, L. E., & Trotter, J. (2004). Selecting fluorochrome conjugates for maximum sensitivity. *Cytometry Part A, 62*(2), 169-173. https://doi.org/10.1002/cyto.a.20092

[6] Nguyen, R., Perfetto, S., Mahnke, Y. D., Chattopadhyay, P., & Roederer, M. (2013). Quantifying spillover spreading for comparing instrument performance and aiding in multicolor panel design. *Cytometry Part A, 83*(3), 306-315. https://doi.org/10.1002/cyto.a.22251

[7] Shevchenko, Y., Lurje, I., Tacke, F., & Hammerich, L. (2024). Fluorochrome-dependent specific changes in spectral profiles using different compensation beads or primary cells in full spectrum cytometry. *Cytometry Part A, 105*(6), 458-463. https://doi.org/10.1002/cyto.a.24836

[8] Mage, P. L., Konecny, A. J., & Mair, F. (2026). Measurement and prediction of unmixing-dependent spreading due to collinearity in spectral flow cytometry. *Cytometry Part A, 109*(6), 436-455. https://doi.org/10.1002/cyto.a.70044

[9] Bhowmick, D., & Bushnell, T. P. (2025). Impact of panel size, fluorochrome selection, and unmixing algorithms on ultra-high parameter flow cytometry analysis. *Cytometry Part A, 107*(10), 683-694. https://doi.org/10.1002/cyto.a.24960

[10] Kmet, R., & Novo, D. (2025). Reducing spreading: Removing the impact of irrelevant dyes improves unmixed flow cytometry data. *Cytometry Part A, 107*(9), 573-586. https://doi.org/10.1002/cyto.a.24957

[11] Bonilla, D. L., Paul, A., Gil-Pulido, J., Park, L. M., & Jaimes, M. C. (2024). The power of reagent titration in flow cytometry. *Cells, 13*(20), 1677. https://doi.org/10.3390/cells13201677

[12] Roet, J. E. G., Mikula, A. M., de Kok, M., Chadick, C. H., Garcia Vallejo, J. J., Roest, H. P., et al. (2024). Unbiased method for spectral analysis of cells with great diversity of autofluorescence spectra. *Cytometry Part A, 105*(8), 595-606. https://doi.org/10.1002/cyto.a.24856

[13] Waclawski, J. P., Arnhardt, I., Fokken, H., Kattre, N., Maetzig, T., Herbst, J., et al. (2026). A clinically applicable unmixing approach for spectral measurable residual disease detection in acute myeloid leukemia. *Cancers, 18*(14), 2323. https://doi.org/10.3390/cancers18142323