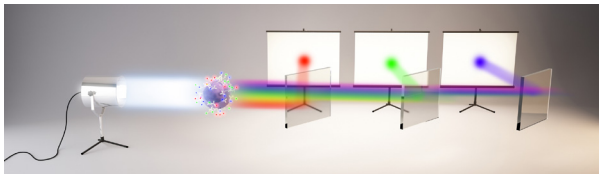
Spectral Unmixing in Sony Spectral Analyzers—A Review

Sony spectral cell analyzer platforms including the SP6800, SA3800, and ID7000™, use spectral unmixing to expand the way biological samples are analyzed to ensure accurate visualization of fluorescent populations. This technical note describes the method and benefits of spectral analysis, using the Weighted Least Squares Method, over conventional flow cytometry compensation calculations.

Conventional and spectral flow cytometry signal detection comparison

Conventional flow cytometry analyzers use bandpass filters and single channel photomultiplier tubes (PMTs) to measure fluorescence signals. In contrast, Sony spectral cell analyzers use prisms or diffraction gratings to spectrally decompose fluorescent light and pass it to multichannel PMT arrays to measure the full spectral signature of each fluorochrome.

A Conventional Flow Cytometry



B Spectral Flow Cytometry

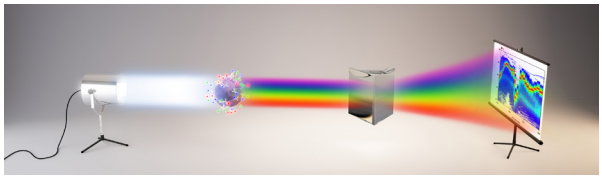


Figure 1. Conventional vs spectral flow cytometry signal detection

In conventional flow cytometry, the number of detectors is equal to the number of dyes. Conventional flow cytometers correlate each detection channel with each individual fluorochrome (for example, FL1 for FITC, FL2 for PE). Thus, the number of detectors matches the number of fluorochromes in the experiment. When the fluorescent signal from a single fluorochrome (dye1) is acquired, the detected signal in FL1 is considered the primary signal, and its fluorescence in other channels is described as "spillover." Signal from these other channels makes up the spillover matrix that is used in the compensation calculation **(Figure 2A)**.

In contrast, spectral analysis uses signals for all detection channels regardless of the number of fluorochromes analyzed. Because the number of detectors is typically greater than the number of fluorochromes, each dye is measured across all channels, with a maximum detection range of 360 nm to 920 nm with 184 fluorescence detectors in the ID7000 7-laser system. Thus, for spectral analysis, mathematically the number of detectors is greater than or equal to the number of dyes. This is the theoretical limit for number of colors that can be run in a single panel. Therefore, the maximum number of colors that can be run on the ID7000 spectral cell analyzer is 184.

Using spectral analysis, a single color control fluorochrome, dye1, is detected across all channels to produce a spectral emission signal. The spectral emission signal is used to create the reference spectrum of dye1, which, in turn, is applied in the spectral unmixing calculation to separate its spectral signature from all other signatures in the multicolor sample **(Figure 2B)**. This lets scientists better visualize and gain more insight into each fluorochrome marker, because the entire spectral signature is detected and not just a single emission peak in one detector. It also allows more colors to be added to a given experiment because the minor differences in each fluorochrome's emission can be visualized across the detector array. This provides researchers panel design flexibility because they can now use fluorochromes with very similar emission signatures and increase panel size to deliver a comprehensive picture of rare populations.

A Conventional Flow Cytometry B Spectral Flow Cytometry

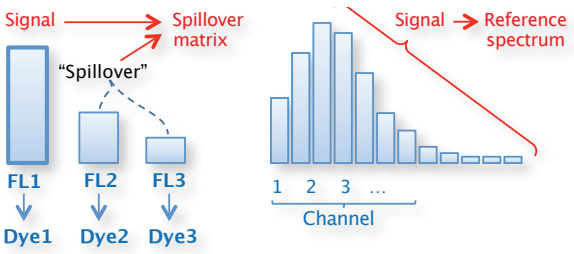


Figure 2. Comparison of conventional flow cytometry compensation spillover matrix and spectral flow cytometry spectral reference curve calculations

Figures 2A and **2B** show how conventional flow cytometry and spectral flow cytometry handle detected signals from a single fluorochrome, defined here as dye1. In conventional flow cytometry, each detector is correlated with a single fluorochrome. For dye1, the fluorescence emission peak in FL1 is used as the final output signal for experimental plots, and the additional signals from dye1 in FL2 and FL3 are defined as spillover values and are removed from the main emission signal coming from dye2 and dye3 during compensation. Spectral flow cytometry uses the detected signal from dye1 across all channels in the detector array to produce a spectral reference curve of the entire emission signal that can be used to unmix dye1 from a multicolor sample.

Spillover values in a compensation matrix and spectral reference curves in an unmixing matrix are mathematically similar, and so too are the respective calculations.

A three-color calculation is illustrated in **Figure 3**, comparing conventional flow cytometry compensation with spectral unmixing. The conventional flow cytometry compensation calculation uses spillover to resolve the detected signal intensity of each fluorochrome in a multicolor sample (**3A**). In spectral flow cytometry, single color fluorochrome reference spectra are generated for each of the three dyes and used (**3B**) to perform spectral unmixing calculations to determine the final intensity of each fluorochrome.

Though compensation and spectral unmixing are similar, spectral analysis allows researchers to see the full emission signal, without using bandpass filters, and enables one or more autofluorescence populations to be treated in the same way as fluorescence signals. Autofluorescence is handled as a separate color, using the spectral reference signature generated from the intrinsic autofluorescence properties of the unstained sample.

Figure 3 illustrates the concepts of conventional compensation using a spillover matrix (**3A**) and spectral unmixing using reference spectra (**3B**). In both calculations, the detected signals for all dyes in the multicolor sample (dye1, dye2, and dye3) are resolved from each other, and the individual intensity signal for each dye is calculated.

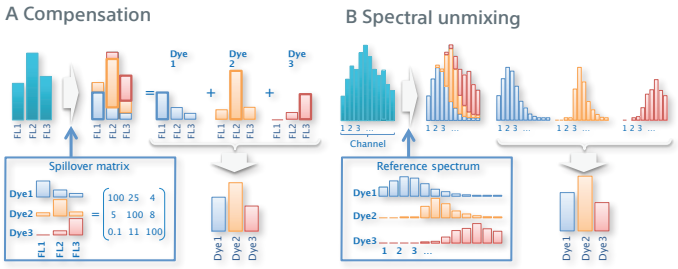


Figure 3. Comparison of compensation and spectral unmixing.

Noise management with conventional compensation and spectral unmixing

The high number of detection parameters in spectral analysis allows greater flexibility in dealing with fluorescence noise. Conventional flow cytometry compensation requires the number of the input parameters (detectors) to equal the number of the output parameters (dyes). Mathematically, it usually produces one unique answer by solving a system of individual matrix equations, which does not ideally manage residual noise. This is important because noise in a detection signal directly affects the dye intensity (**Figure 4A**). Spectral analysis allows a higher number of inputs than outputs, and spectral analysis equations can be solved with minimal noise (**Figure 4B**). Spectral analysis equations can handle a variety of scenarios. Thus, the role of the calculation is to estimate the most reasonable result, which minimizes the effect of the noise on intensity calculations.

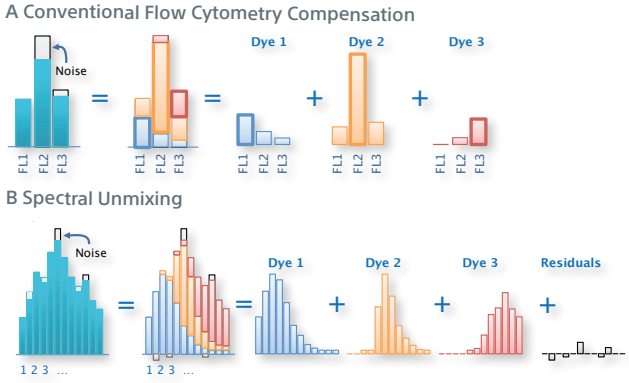


Figure 4. Noise handling approaches in conventional and spectral flow cytometry

Figure 4 describes how noise is handled in conventional flow cytometry compensation (**4A**) and in spectral unmixing (**4B**). In conventional flow, the compensation calculation always produces one unique result, so the noise in the detected signal

directly affects the result for each dye's intensity. In spectral unmixing, noise is accounted for as residuals in the equation.

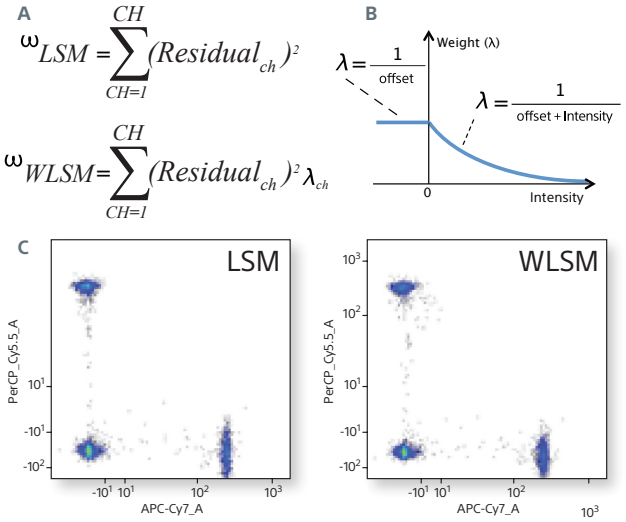


Figure 5. Least Squares Method (LSM) and Weighted Least Squares Method (WLSM) algorithms

Figure 5A. Equations used in spectral analysis are LSM and WLSM. In LSM, the square sum of the residual for all channels (ω↓LSM) is minimized. In WLSM, a weight value λ↓(ch) is imposed to the square value of the residual of each channel to calculate ω↓WLSM. **Figure 5B.** The weight value λ used is calculated from the signal intensity of the channel as illustrated here. **Figure 5C.** The same data with a mixed beads sample of APC-Cy™7 single-positive, PerCP-Cy™5.5 single-positive, and unstained sample was analyzed with LSM and WLSM.

Spectral unmixing algorithms

One of the simplest unmixing algorithms is the Least Squares Method (LSM). It minimizes the square sum of the residuals in the results. The LSM assumes that dispersion appears evenly through all channels like white noise. However, this is incorrect because the level of the dispersion differs for each detection channel, depending on the signal intensity measured on each fluorochrome. Since the LSM does not take this into account, results are over-fitted to bright channels. To improve accuracy, Sony has introduced the Weighted Least Squares Method (WLSM) algorithm. In WLSM, the square value of residuals is individually weighted (**Figure 5A**). The weight is imposed so that the residuals in bright channels are relatively under-weighted compared to dim channels (**Figure 5B**). The WLSM accounts for the actual noise pattern in the detected signal to ensure that the estimation of each dye's intensity is more accurate. This is illustrated in **Figure 5C**, in which the individual signals from PerCP-Cy5.5 and APC-Cy7 in a stained bead sample are separated using the LSM and WLSM algorithms. The comparison result shows that WLSM provides a more compact, less deviated population, especially for APC-Cy7 positive beads.

Summary

Spectral cytometry detects the entire spectral signature of each fluorochrome with a maximum detection range from 360 nm to 920 nm in the ID7000 7-laser system. Spectral unmixing uses single color control spectral references to separate the individual spectral signatures in a multicolor sample with the Weighted Least Squares Method (WLSM). The method utilized in Sony Spectral cell analyzers is weighted and thus better at reducing residual noise than other commonly used unmixing algorithms. Spectral cytometry decreases the complexities associated with working with fluorochromes with close emission peaks by eliminating the use of bandpass filters and conventional compensation matrixes to simplify panel design. Spectral cytometry also defines bright autofluorescence spectral signatures as additional "colors" that can be unmixed from the experimental sample to optimize sensitivity and enhance dim signal detection. Together, these capabilities allow scientists to accurately visualize more information from each fluorescent population to deliver a comprehensive picture of each sample.