Spectral Unmixing with Sony Spectral Analyzers

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Abstract: Sony analyzer platforms use spectral unmixing to expand the way cellular and microbiological samples are analyzed to ensure accurate visualization of fluorescent populations. This paper discusses the method and benefits of spectral analysis methods of spectral analyzers over conventional flow cytometry compensation calculations.

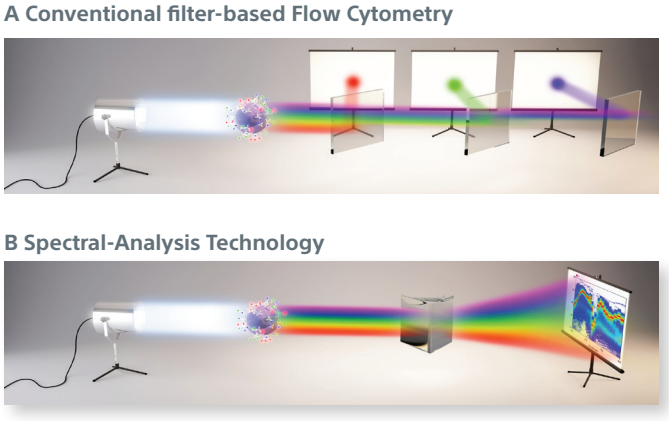

Figure 1. *Concept of Spectral-Analysis in Flow Cytometry*

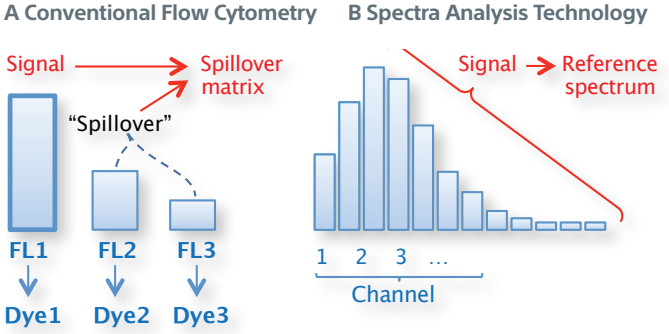

Figure 2. *The conventional flow cytometry data and the spectral analysis data.*

*Illustrations **A** and **B** show how conventional flow cytometry and spectral analysis technology handle detected signals from single fluorochrome, dye 1. In conventional flow cytometry, each detector is correlated with single fluorochrome. For dye 1 fluorescent emission, signals in FL1 is considered as a "signal" and signals in FL2 and FL3 are called as the spillover. The signal and the spillover compose the spillover matrix. Spectral analysis technology, uses signals from all detection channels to produce the emission signal of the dye1, to compose the reference spectrum.*

Data collection methods of conventional flow and spectral technology

Conventional flow cytometry analyzers use band pass filters and single channel PMT's (Figure 1) to collect cytometric data. In contrast to these systems, Sony Spectral Analyzers use a series of prisms that spectrally decompose emitted fluorescent light passing it through to a 32 channel array photomultiplier tube (PMT) to measure spectral information of each individual particle.

In spectral analysis, signals from all detected channels are used regardless of the number of fluorochromes analyzed. Thus, for 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 the detectors matches with the number of fluorochromes. When the fluorescent signal from a single fluorochrome (dye1) is acquired, the detected signal in FL1 is considered as the "signal" and signals in other channels are described as "photon spillover". Signals from these other channels make up the spillover matrix that is used in the compensation calculation (Figure 2).

In contrast, spectral analysis uses signals from all detection channels regardless of the number of fluorochromes analyzed. Since the number of detectors is usually greater than the number of fluorochromes analyzed, each dye is measuring from 420nm to 800nm. Thus for spectral analysis technology, mathematically the number of detectors is greater than or equal to the number of dyes. Using spectral analysis the single fluorochrome, dye1, uses all the detection channels to produce a spectral emission signal. The spectral emission signal is used to create the reference spectrum of the dye1, which, in turn, is applied in the spectral unmixing calculation (Figure 2).

Mathematically the spillover matrix and the reference spectrum are similar, such is the same for compensation and the spectral unmixing. A three color calculation is illustrated in Figure 3 comparing a conventional flow cytometry compensation with spectral unmixing. Conventional flow cytometry compensation calculation uses spillover data (from the spillover matrix) to resolve the detected signal intensity.

In spectral analysis, reference spectra are used for the three dyes to perform spectral unmixing calculations. Though these calculations are similar, spectral analysis allows researchers to see the full emission signal without using bandpass filters and enables auto-fluorescence to be handled as a separate color.

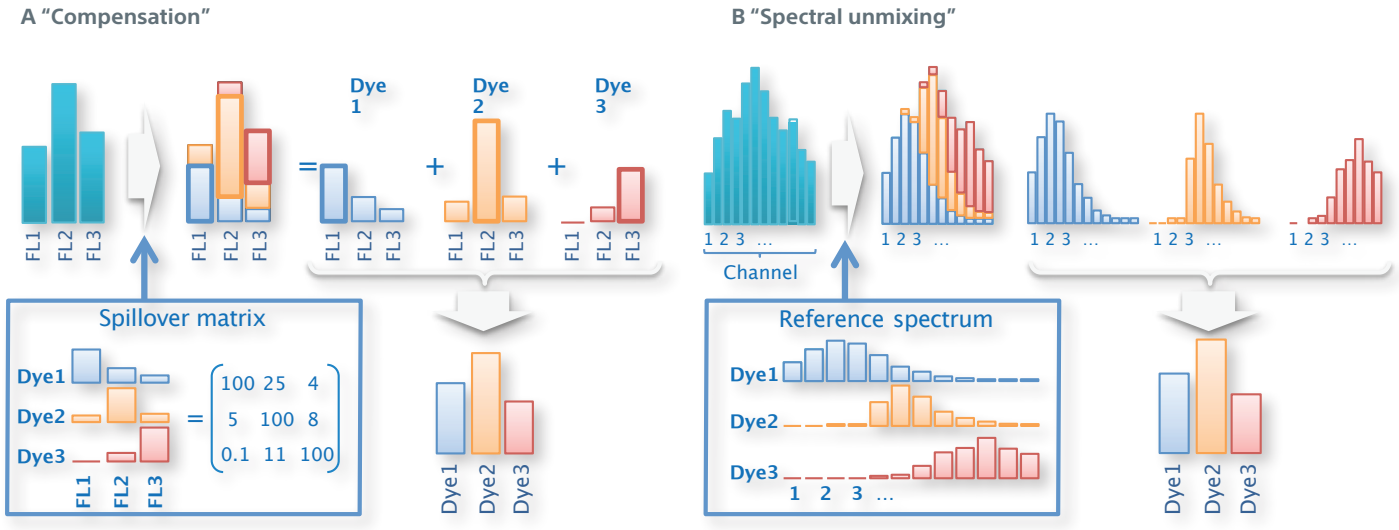

Figure 3. *Comparison of the "compensation" and the "spectral unmixing."*

*These figures illustrate the concepts of conventional compensation using the spillover matrix **A** and spectral unmixing using the reference spectra **B**. In both calculations, the detected signals are resolved to the combination of the emission spectra from dyes (dye1, dye2 and dye3), and the intensity of each dye is calculated.*

Noise management with conventional compensation and spectral unmixing.

The high number of detection parameters in spectral analysis allows greater flexibility in dealing with fluorescent 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 the system of individual matrix equations, which does not ideally manage residual noise. This is important since noise in detection signal directly affects the dye intensity. (Figure 4A). On the other hand, spectral analysis allows a higher number of inputs than outputs, and spectral analysis equations can be solved with minimal noise (Fig 4B). Spectral analysis equations can handle a variety of scenarios, thus the role of the calculation is to estimate the most reasonable result. The goal of this approach is to minimize the effect of the noise on dye intensity results.

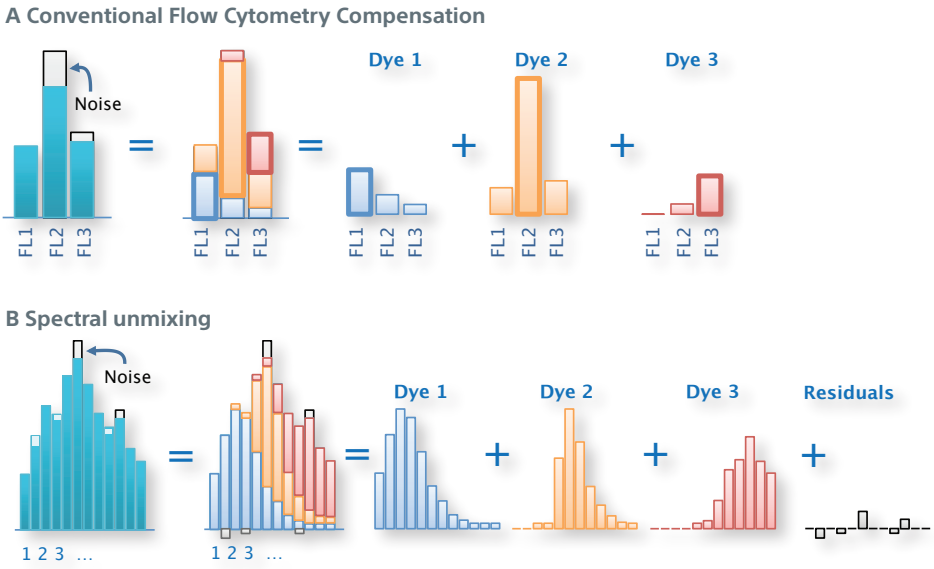

Figure 4. *Different approaches to noise handling.*

*This figure describes how noise in the conventional flow cytometry compensation **A** and in spectral unmixing **B**. In conventional flow, the compensation calculation always produces one unique result, so the noise in the detected signal directly affects the result for dye intensities. In spectral unmixing is accounted for as residuals in the equation. Ideally, most of the noise is calculated as the residuals.*

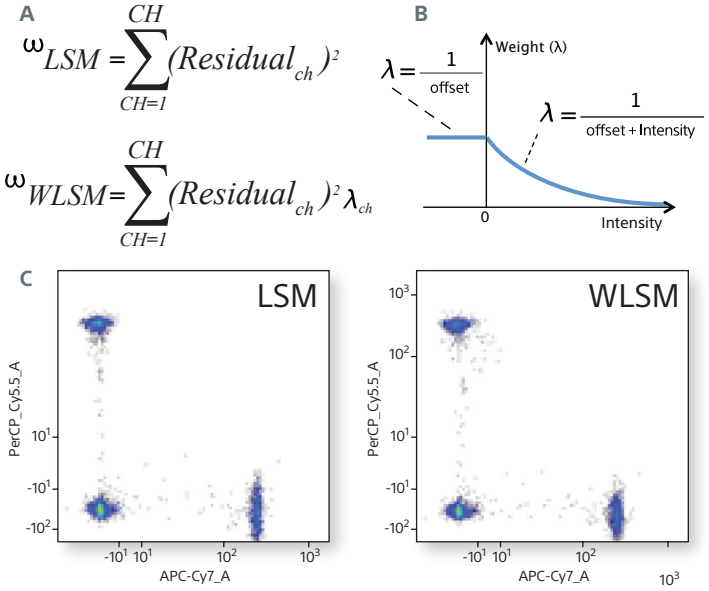

Figure 5. *Least Square Method (LSM) and Weighted Least Square Method (WLSM) algorithms.*

A *Equations used in LSM and WLSM. In LSM, the square sum of the residual for all channels (ω↓LSM) is minimized. In WLSM, weight value λ↓(ch) is imposed to the square value of the residual of each channel to calculate ω↓WLSM.* ***B*** *Weight value λ used is calculated from the signal intensity of the channel as illustrated here.* ***C*** *Same data with mixed beads sample of APC-Cy7 single positive, PerCP-Cy5.5 single positive and unstained sample is analyzed with LSM and WLSM.*

Algorithms for the spectral unmixing.

One of the simplest algorithms is the Least Square Method (LSM). It minimizes minimizes the square sum of the residuals in the results. The LSM assumes that dispersion appears evenly through all channels like a white noise. However, this is incorrect as the level of the dispersion differs for each detection channel, depending on the signal intensity. Since the LSM does not take this into account, results are over-fitted to the bright channels. To improve accuracy, Sony has introduced the Weighted Least Square Method (WLSM) algorithm. In WLSM, the square value of residuals are 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 takes into account 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 where PerCP-Cy5.5 and APC-Cy7 separation in single stained beads, is calculated with LSM and WLSM for unmixing. The comparison result shows that WLSM provides a more concentrated, less deviating population, especially for APC-Cy7 positive beads.

Conclusion

In conclusion, spectral unmixing separates the individual spectral fingerprints to let scientists better visualize and gain more insight into each fluorochrome marker. It also delivers a more comprehensive picture of rare populations while decreasing the complexities associated with working with fluorescent proteins and multi laser excited fluorochromes. Spectral analysis collects photons from 420nm – 800nm and eliminates bright auto-fluoroscence to optimize sensitivity and enhance dim signal detection. These capabilities allow scientists to accurately visualize fluorescent populations. Overall spectral technology simplifies multicolor panel design, by eliminating the use of bandpass filters and conventional compensation matrices. It also allows for greater flexibility in workflow and analysis.

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