Fred Hutch Cancer Center

Flow Cytometry

Research Administration

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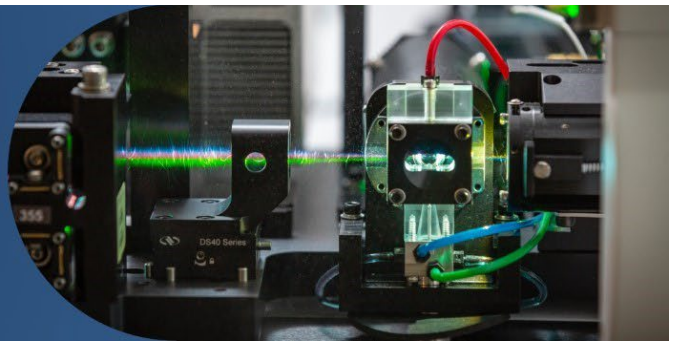


Fred Hutch's Shared Resources are catalysts for lifesaving discoveries. This uniquely centralized program of 15 specialized core facilities and scientific services drives advances by integrating dedicated experts and cutting-edge technologies across the entire research pipeline, from basic science to clinical trial.

Understanding Traditional Compensation and Spectral Unmixing Processes

Both Compensation and Spectral Unmixing are mathematical models used to separate and describe the mix of different fluorescent signals conjugated to antibodies or biological markers. Separation of fluorescent signals or dyes is essential for correctly identifying and quantifying a marker the dye is associated with. The mixing primarily occurs because emission signals are broad. Even though they display the highest intensity in their primary detectors, the signal spills into other detectors (Figure 1A). In the Compensation method, each fluorochrome is assigned a single primary channel, and the spillover into the other detectors is subtracted. Uncompensated data appears to be positive in both detectors, when compensation is applied, the signal is displayed in the primary detector (Figure 1B).

On the other hand, in the Spectral unmixing, the emission of a fluorochrome is observed across all detectors as a spectral trace or signature, and the spillover subtraction is performed across the entire detector array (Figure 2).

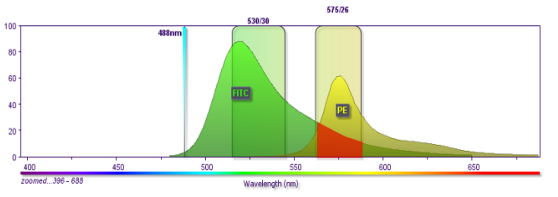


Figure 1A. 488nm laser excitation of FITC and PE with the with bandpass filters near the emission max (Em_{max}) for each fluorochrome. The red area in PE is the spillover of FITC into that detector that must be compensated out.

Compensation spillover visualization

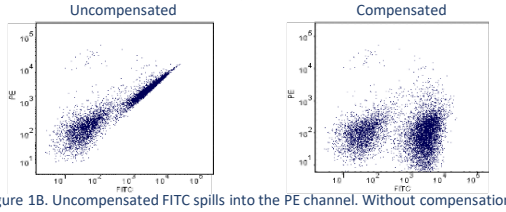


Figure 1B. Uncompensated FITC spills into the PE channel. Without compensation it appears to be double positive for FITC and PE. When compensation is applied, the signal is visualized in the FITC channel and true double positive can be resolved and quantified..

Spectral spillover visualization

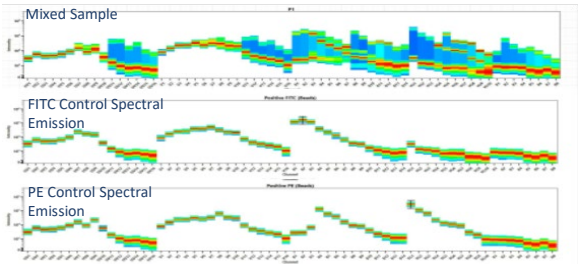
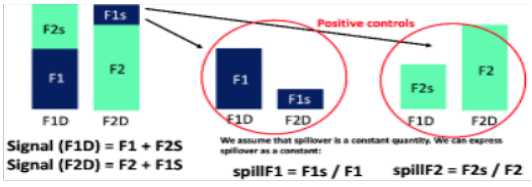


Figure 2. FITC and PE spectral signature excited by a 5 lasers Cytek Aurora. The unmixed sample shows the combination of spectral signatures, BUV661, PerCP, and APC in addition to FITC and PE.

Every detector generates signals that can be formulated as a linear equation. This equation is a composite of both primary fluorescent signals and spillover signals. In our software and literature, these linear equations are articulated using matrix notation. By solving these linear equations, we can derive the unmixed signals (Figure 3a and 3b).

Compensation Diagram



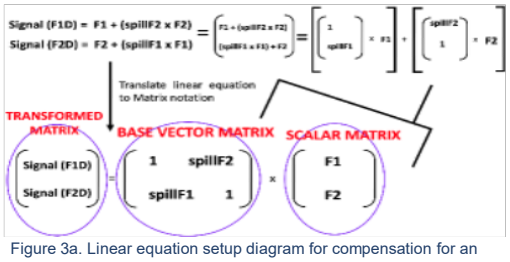


Figure 3a. Linear equation setup diagram for compensation for an experiment with 2 colors. Linear equations are setup to define spillover and main signals. After rearrangement, the equations are translated to matrix notation.

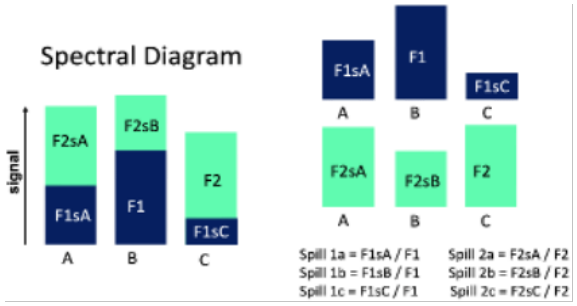


Figure 3b. Linear equation setup diagram for spectral unmixing for an experiment with 3 colors. This diagram shows that there are more spills to define for spectral experiments compared to compensation since there are more detectors than the number of colors. It should be noted that F1sB = F1 and F2sC = F2.

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The primary distinction between the two unmixing models lies in their equation systems. The compensation model comprises a full-rank system of equations, resulting in a unique value for the calculated unmixed fluorescence values. In contrast, the spectral unmixing model involves an overdetermined system. Due to the nature of overdetermined systems, it's impossible to find a unique solution for all sets of equations. Therefore, the values we determine for unmixed fluorescence are dependent on our approach to what we consider as the optimal value.

This also clarifies why we cannot calculate the inverse of mixing matrices observed in spectral platforms, as they are non-square (Figure 4). We address this issue by determining an approach to solve overdetermined equations, which varies across the different platforms of our spectral machines.

Most popular approaches to solve the equations of Spectral Unmixing are Weighted Least Squares and Ordinary Least Squares. WLS will prioritize the equations of high intensity detectors over lower ones. This idea comes from the variation of signal being greater in high intensities compared to low ones. OLS will not prioritize and choose a best fit for all detectors. In theory, Poisson statistics describe the emission process more accurately in comparison to Gaussian since emission is a stochastic process and variation is greater in higher intensity measurements (Figure 5).

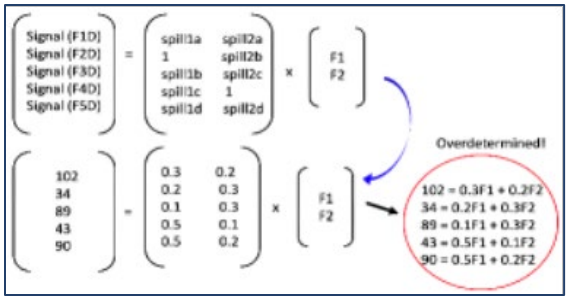
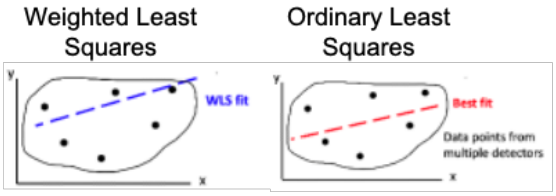


Figure 4. Translation of the matrix operations to their corresponding set of linear equations. The equations demonstrate that no unique solution for F1 and F2 abundances can solve all equations. This situation arises from the fact that our system being overdetermined. This also explains the reason why the inverse of non-square matrices are incalculable unless an approach for solution Is determined.



- Uses a Poisson statistical model
- Signal variation across the detectors is assumed to be greater in the high intensity channels
- The bright detectors are assigned higher priority

- Uses a Gaussian statistical model
- Signal variation across the detectors is assumed to be the same in all channels
- All detectors are assigned equal priority

Figure 5. Demonstrating the fundamental differences between WLS and OLS approaches to solve our overdetermined system of equations in spectral unmixing.