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Application Note

July. 2024

Advancing Precision in Flow Cytometry: Multiple Autofluorescence Subtraction with Spectral Analysis.

In cancer, autoimmune diseases, and other pathological conditions, abnormal cellular interactions often underlie disease mechanisms. Consequently, analyzing the dynamics of the entire cellular population within a disease microenvironment is crucial for accurately interpreting the state of the disease.

Flow cytometry (FCM) allows for the measurement of multiple proteins pre-stained with fluorescently labeled antibodies or other markers on a per-cell basis, capturing data at rates up to 10,000 cells per second. Thus, FCM is an exceptionally powerful tool for obtaining a comprehensive snapshot of cellular populations in various microenvironments.

However, tissue-derived samples often contain high levels of fluorescent proteins and metabolites such as collagen, NADH, and riboflavin. These substances can induce autofluorescence, an inherent fluorescence that significantly impedes accurate quantitative analysis of target epitopes in FCM, which relies on fluorescence-based detection.

In 2012, Sony pioneered the integration of spectral unmixing in FCM analysis. This technique enhances FCM by enabling (1) compensation-free analysis and (2) effective subtraction of autofluorescence. The latter has notably transformed the analysis of cells from tissues rich in autofluorescent compounds. The importance of spectral unmixing in FCM has increasingly been recognized, leading to the release of numerous FCM devices equipped with this technology by various manufacturers. While these devices offer the functions described in (1) and (2), it is important to note that the removal of autofluorescence described in (2) can sometimes be challenging, especially when samples contain multiple distinct autofluorescent populations.

This paper highlights the advanced 'multi' autofluorescence subtraction capabilities of Sony's spectral flow cytometry, demonstrating how it can deliver accurate quantitative assessments in samples with diverse autofluorescent populations.

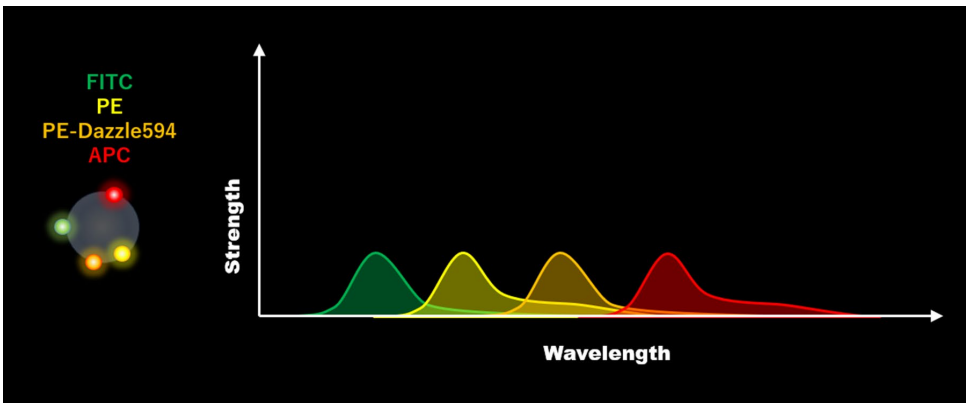

Figure 1. Fluorescence Spectra
Each fluorophore possesses a unique fluorescence signature, often referred to as its 'fingerprint'. This characteristic spectrum can be visualized by plotting the distribution of fluorescence across wavelengths on the horizontal axis against signal intensity on the vertical axis. When the concentration of the fluorescent dye varies, the spectrum's amplitude changes correspondingly, though its shape remains constant. In this scenario, a proportional relationship is maintained between the amount of fluorescent dye and the spectral height, ensuring consistent and predictable spectral behavior.

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■ Quantitative Principle of the Spectral Method

Each fluorophore exhibits a distinct fluorescence fingerprint along the wavelength spectrum, as depicted in Figure 1. This characteristic pattern is commonly referred to as the fluorescence spectrum. The spectrum's height has a parallel relationship to the quantity of the fluorescent dye detected, yet its shape remains unchanged. When cells are stained with fluorescent antibodies, multiple dyes adhere to the cell surface. As these stained cells pass through a flow cytometer, each cell's unique information is individually scanned by a focused laser spot (Figure 2A). The resulting fluorescence spectrum from a single cell represents the cumulative effect of all fluorophores present on that cell (observed value).

Conversely, if the spectra of individual fluorophores are pre-recorded in the system (Figure 2B), it becomes possible to compute a combined spectrum (simulated value) for any hypothetical arrangement of fluorophores (Figure 2C). With this setup, when a sample is measured, the system matches the observed values from each cell with the pre-calculated simulated values. Since the proportions of each fluorescent spectrum that contribute to these simulated values are known, these proportions are input as relative quantitative measures of each fluorescent dye (Figure 2D). The quantitative data obtained from this method can be seamlessly integrated into classical 2D plots, allowing users familiar with conventional flow cytometry to transition effortlessly without noticing any discrepancies. This computational process, known as Spectral Unmixing, operates without the need for traditional compensation adjustments.

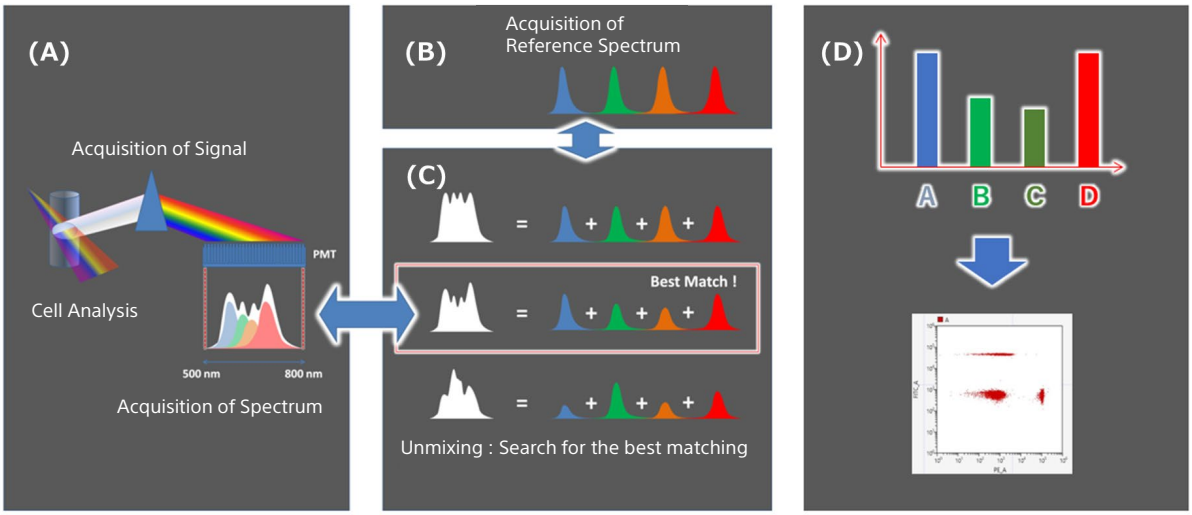

Figure 2.
(A) When the stained sample is run through the spectral FCM instrument, a combined spectrum consisting of multiple fluorochromes is scanned in each cell.
(B) Reference spectra of the fluorochromes are prepared in the database.
(C) By using the reference spectrum information, the simulation can produce the combined values with various combinations of the dyes. The computer then finds a simulated summed value that is highly homologous to the observed value. The amount of each spectral combination used to produce this summation is calculated as a relative quantitative value.
(D) From the quantitative value of each parameter, the data for one cell in question is determined. From this, a two-dimensional plot is developed using any two parameters, which is represented as a 2D-Plot used in general FCM analysis.

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■Principle of Autofluorescence Subtraction

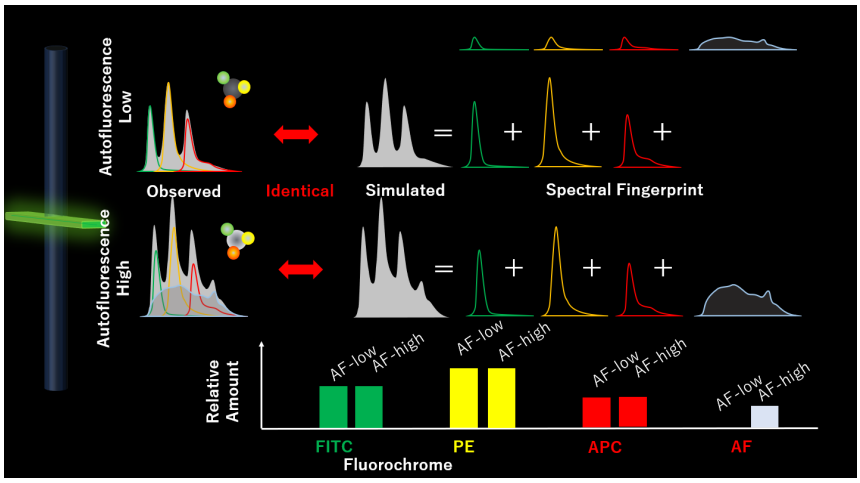
In this case of three-color staining, the principle of autofluorescence subtraction can be elucidated. When cells exhibit no autofluorescence, the spectrum observed from each cell is simply the sum of the spectra of the fluorescent dyes attached to the cell, as previously discussed. In traditional FCM using bandpass filters, filters are employed to isolate only the regions near each spectrum's peak, allowing for quantitative analysis based on the corresponding detector signals. This classical method is effective for quantitative analysis in the absence of autofluorescence.

However, challenges arise when cells exhibit autofluorescence. In such cases, the autofluorescence spectrum is superimposed on the observed values. As autofluorescence levels can vary within the same cell population, correct quantitative analysis of specific fluorochromes cannot be achieved by merely subtracting uniform values across the data. Consequently, the classical filter-based FCM, which isolates specific wavelength regions, fails to accurately quantify highly autofluorescent cells (Figure 3A).

Conversely, the spectral methodology treats the autofluorescence spectra, extracted from unstained samples, as additional "fluorescent dyes" in the spectral unmixing process. In this approach, components derived from autofluorescence are integrated into newly generated parameters representing autofluorescence. Subsequently, the autofluorescence component is removed from each specific parameter (Figure 3B).

This innovative methodology significantly enhances the quantative capacity beyond what traditional FCM analysis can offer, as it efficiently extracts and utilizes autofluorescence spectra from unstained samples.

(A)



(B)

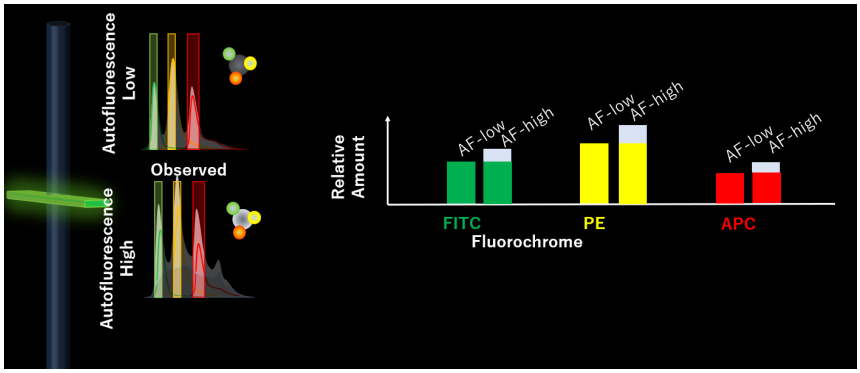


Figure 3.
(A) When autofluorescent cells are stained with three different fluorescent dyes and measured with classical FCM equipment, the autofluorescence contamination signal is mixed into each parameter and is difficult to be removed.

(B) By adding the autofluorescence spectrum as an additional fluorescent dye to the unmixing calculation, the autofluorescence-derived signal is integrated into the autofluorescence parameter, and the autofluorescence-derived contamination signal is removed from the various specific signals.

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■Multiple Autofluorescence Subtraction Methodology

The previous section highlighted the principle of autofluorescence subtraction to achieve high quantitation. However, samples derived from tissues often contain multiple autofluorescence subpopulations. The spectral method's utility has gained recognition recently, with many companies now offering FCM systems that employ this technique. Yet, most of these systems can only identify a single autofluorescence population from unstained samples. In contrast, Sony's spectral FCM system is capable of defining multiple autofluorescence populations and executing multi-autofluorescence subtraction, as detailed below.

Consider a sample containing two distinct autofluorescence populations: (1) one characterized by a spectrum weighted toward shorter wavelengths, and (2) another biased toward longer wavelengths, as depicted in Figure 4. The spectral method, capable of scanning the entire wavelength spectrum, allows for the creation of "virtual" bandpass filters. These filters are specifically set to capture signals from designated wavelength bands. For instance, one virtual filter might be positioned on the short-wavelength side and another on the long-wavelength side. A key characteristic of autofluorescence within a specific population is that the spectrum's shape remains constant while its intensity varies. Consequently, the ratio of light captured by the virtual filters from the short to the long wavelength side remains consistent, even as autofluorescence intensity changes, creating a unique value for each autofluorescent population.

When analyzing a sample with mixed autofluorescence populations, the data gathered from the virtual filters set for short and long wavelengths can be represented on a two-dimensional plot. This visualization will show cells from each autofluorescence population positioned along distinct slopes, as illustrated in Figure 4.

To accurately distinguish between different autofluorescent populations, it is crucial to set up two "appropriate" virtual filters that provide different slopes for each population. This setup typically requires manual adjustment. Sony's spectral FCM system includes a support tool that facilitates easy configuration of these virtual filters. For detailed guidance on utilizing this tool, please contact Sony directly.

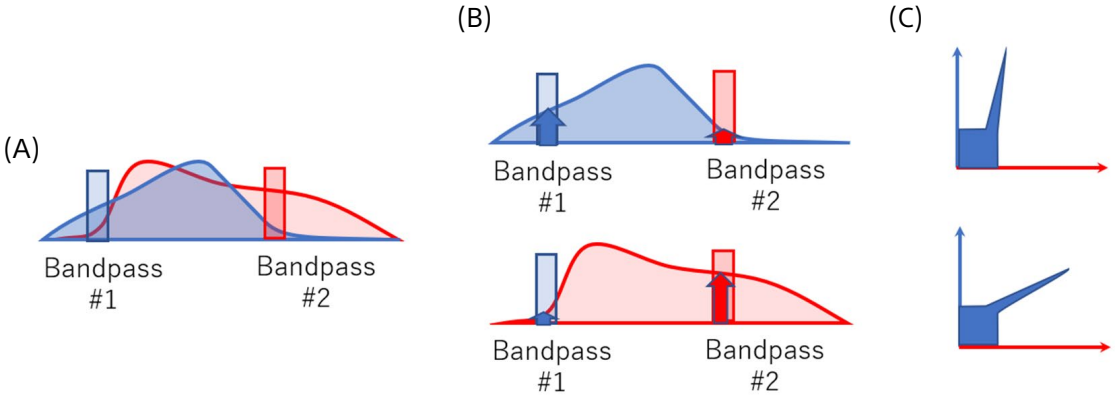

Figure 4.
(A) Assume that the two different patterns of autofluorescence populations are the blue and red shaped spectra.
(B) When two virtual bandpass filters, designated as #1 and #2, are independently configured for distinct wavelengths, the ratio of fluorescence intensities captured by each will differ across various autofluorescence spectra.
(C) Consequently, when plotting the signals derived from the two virtual bandpass filters, "horn-like" projections appear, each at distinct angles, representing the different autofluorescence patterns.

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■The Importance of Multi-Autofluorescence Subtraction

In this final chapter, we demonstrate the impact of multi-autofluorescence subtraction through actual sample results.

In Figure 5A, autofluorescence spectra from the same sample were analyzed under three distinct conditions: (1) without any autofluorescence subtraction, (2) with single autofluorescence subtraction, and (3) with multiple autofluorescence subtraction. It is important to note that the exact number of autofluorescence populations present in the samples was not predetermined at the time of measurement.

During the actual analysis, the 2D plots revealed multiple "hornlike" projections specific to certain parameters, prompting the implementation of autofluorescence subtraction. With the single autofluorescence subtraction approach in condition (2), residual false positives were observed, indicating incomplete subtraction. In contrast, the multi-autofluorescence subtraction approach in condition (3) effectively eliminated these false positives, establishing a solid foundation for accurate quantitative analysis, as shown in Figure 5B.

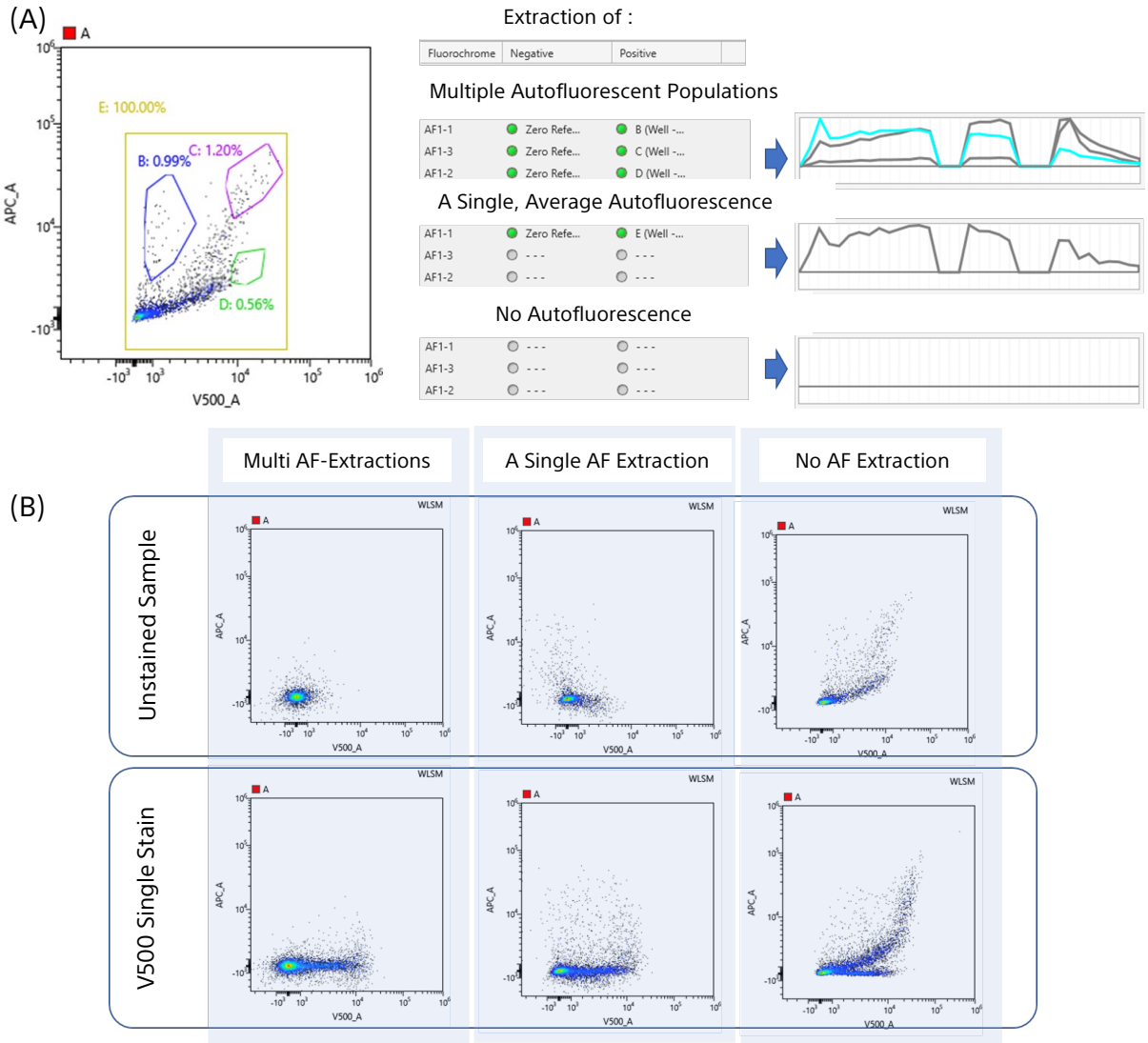

Figure 5.
(A) Defining 3 autofluorescent populations using distinct spectral references
(B) Plot of autofluorescence removal under 3 conditions for unstained and V500 single-stained samples

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■Summary

Spectral FCM analysis represents the pinnacle of flow cytometry, not only due to its streamlined process that eliminates the need for conventional compensation but also because of the enhanced accuracy it achieves through autofluorescence subtraction. Furthermore, Sony's spectral FCM system stands out by allowing for multiple autofluorescence subtractions, facilitating a level of quantitative accuracy unattainable with other systems. As research into the interactions among multiple cells grows in importance for understanding biological phenomena, spectral FCM is poised to become the new standard in the field.

Spectral Cell Analyzer ID7000™

Spectral Cell Sorter FP7000

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