Review of Autofluorescence Unmixing in Sony Spectral Cell Analyzers

Sony cell analysis platforms use spectral technology to expand the way cellular and microbiological samples are analyzed to ensure accurate visualization of fluorescent populations. This technical note describes how autofluorescence is detected and managed in Sony spectral cell analyzers and highlights how this is distinct from the approach used in conventional flow cytometry.

Autofluorescence in conventional flow cytometry

It is well known that cells have some intrinsic level of autofluorescence and that the intensity and pattern of the autofluorescence can vary depending on factors such as cell type, size, and condition. In addition to these natural factors, sample preparation reagents used for fixation and permeabilization may also contribute to or alter the cell's native autofluorescence pattern. In conventional flow cytometry (Figures 1A and 1B), autofluorescence is "compensated" using a compensation matrix of fluorochromes. The autofluorescence spectrum may be broad and can overlap with multiple fluorochromes across multiple detectors, making it difficult to distinguish and separate from other signals using conventional flow cytometry. As a result, samples that contain a bright autofluorescent population exhibit a diagonal pattern in the fluorochrome scatter plots (Figure 1B). This diagonal pattern, seen in both naturally bright autofluorescent samples as well as those stained with certain cell preparation reagents, can lead to false positives in experimental data. The pattern has been frequently observed in conventional flow cytometry results.

Autofluorescence in spectral flow cytometry

In spectral analysis (Figures 1C and 1D), individual fluorochrome reference spectra are used in an unmixing calculation to determine the intensity of each fluorochrome in a multicolor sample. It is also possible to use the reference spectrum of cellular autofluorescence from the unstained cells. Since autofluorescence can be treated in the same way as a fluorescence signal, it can be defined and managed as a separate parameter, so that spectral flow cytometry can deliver more accurate results.

Comparison of autofluorescence using conventional and spectral flow cytometry

A mouse bone marrow sample was acquired and analyzed with a Sony spectral analyzer. Figure 2A shows data from the unstained bone marrow cells. The diagonal plot pattern populations were produced from cellular autofluorescence in three slightly different angles as separate populations. These populations were gated in individual spectral plots (Figure 2B). The reference spectrum of each population was used to generate autofluorescences AF1, AF2, and AF3, which were used as reference spectra for unmixing (Figure 2C). Using these

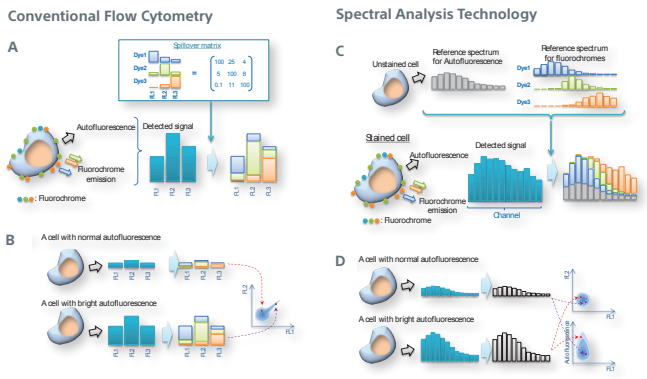

Figure 1. Comparison of cellular autofluorescence signal management between conventional and spectral flow cytometry

A and B. Using conventional flow cytometry, signals from cellular autofluorescence are compensated with a combination of dyes. This results in diagonal patterns in fluorescence scatter plots for bright autofluorescent populations. **C and D** show how, using spectral analysis, a reference spectrum for cellular autofluorescence can be generated using the unstained control sample. This can be used with the reference spectra of fluorochromes to unmix individual components of a multicolor sample, eliminating the diagonal patterns seen in conventional flow cytometry.

autofluorescence reference spectra, the unstained data was analyzed for unmixing, and the AF1, AF2, and AF3 populations were plotted on these axes (Figure 2D). As a result, the diagonal plot patterns originally observed on the fluorochrome axes were eliminated (data not shown).

In Figure 3, bone marrow samples were stained using a 9-color panel and analyzed with nine fluorochromes and three autofluorescence reference spectra (AF1, AF2, and AF3). Unmixing calculations were then performed as shown in Figures 3B–3E. The density plots on PerCP-Cy™5.5 (CD4) and BV510 (CD8) are shown with and without the autofluorescence reference spectra being used as part of the unmixing matrix. In the plots without the autofluorescence reference spectra, some diagonal populations were observed. Since the same patterns were also observed in the unstained sample, these diagonal patterns are false-positive artifacts caused by cellular autofluorescence. These artifacts were successfully removed when the autofluorescence reference spectra were included in the unmixing matrix.

Summary

In the bone marrow example, using spectral analysis technology, false positives were eliminated and several different autofluorescence spectra were observed. Greater accuracy and more precise results delivered when using spectral technology enable researchers to use autofluorescence in useful new ways to support biomedical discovery. In these endeavors, Sony spectral cell analyzers can simplify workflow and deliver more accurate fluorescence data.

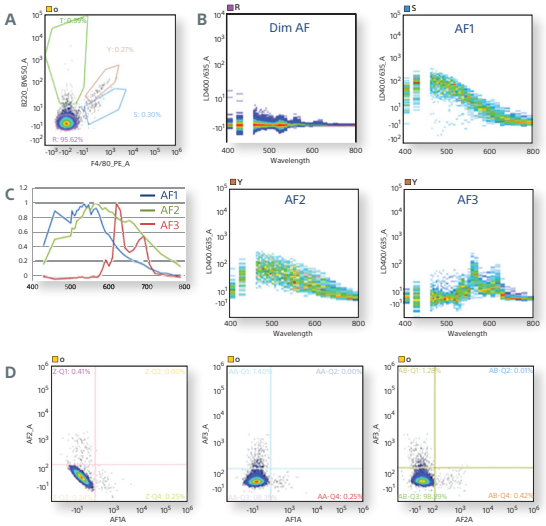

Figure 2. Defining and unmixing cellular autofluorescence in unstained mouse bone marrow
An unstained bone marrow sample was acquired with the SP6800. The events were gated with forward scatter and side scatter, as well as doublet discrimination.
A. The scatter plot with PE and BV650 axes shows three diagonal populations of autofluorescence. **B.** Spectrum plots for the regions shown in plot A indicate a different population of bright autofluorescent cells, labeled AF1 and AF2, and a dim population labeled Dim AF. **C.** From these spectra, the reference spectra for AF1, AF2, and AF3 were created. **D.** The unstained data was calculated using reference spectra and unmixing.

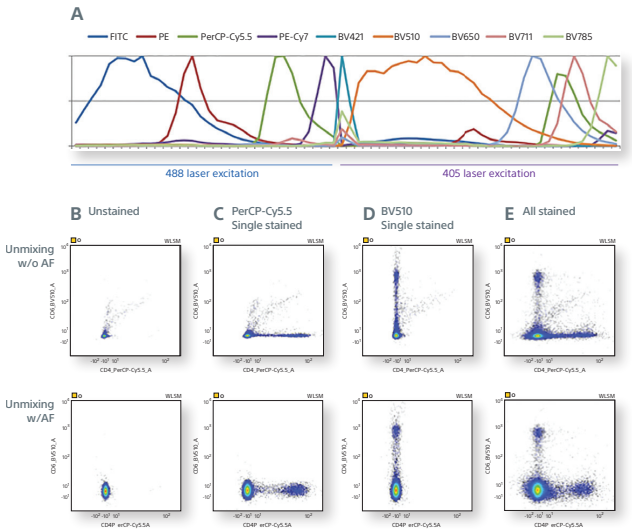

Figure 3. Unmixing autofluorescence in a 9-color bone marrow panel
A. The nine fluorochrome reference spectra used to unmix the multicolor bone marrow sample. **B–E.** PerCP-Cy5.5 and BV510 plots that have the unmixing with and without the three highly autofluorescent population reference spectra applied, bottom and top, respectively. **B.** The unstained bone marrow sample. **C.** The single stain for PerCP-Cy5.5. **D.** The single stain for BV510. **E.** The full stained sample with all nine colors.