Method and Theory of the Autofluorescence Unmixing in SP6800 Spectral Cell Analyzer

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Abstract: Sony analyzer platforms use spectral technology to expand the way cellular and microbiological samples are analyzed to ensure accurate visualization of fluorescent populations. This paper discusses how spectral technology and conventional flow cytometry systems recognize and handle autofluorescence.

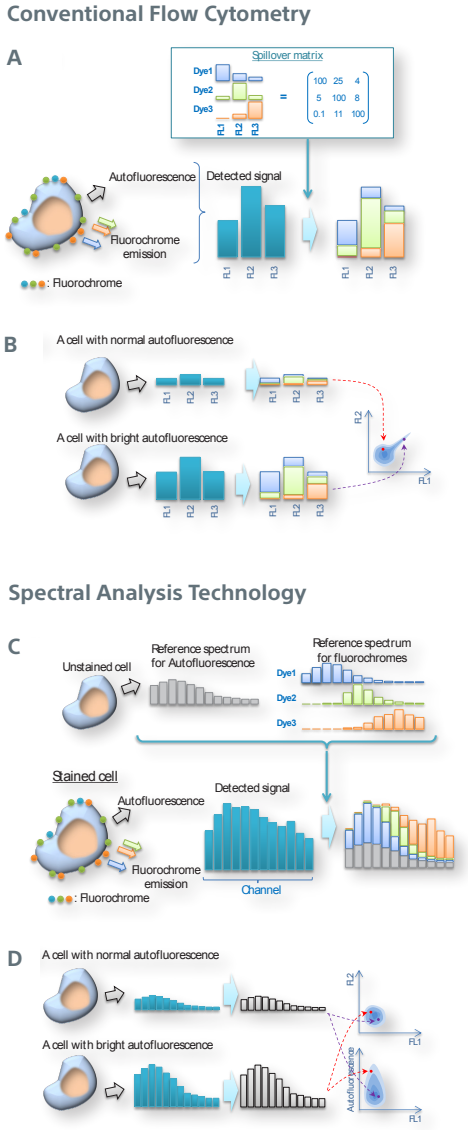

Figure 1. *Handling the cellular autofluorescence signal in conventional flow cytometry and using spectral analysis.*

*Figures **A** and **B**. Using conventional flow cytometry, cell autofluorescent signals are compensated with combination of dyes. This makes diagonal patterns in fluorescent scatter plots for bright autofluorescence populations. Figures **C** and **D** show how using spectral analysis, the reference spectrum for cell autofluorescence from the unstained control are used in combination with the reference spectra of fluorochromes. Cell autofluorescence is plotted on the autofluorescence axis, eliminating the diagonal patterns seen in conventional flow cytometry.*

Autofluorescence and conventional flow cytometry

Scientists engaged in biomedical discovery know that autofluorescence exists in all cells and that the intensity and pattern of autofluorescence can vary depending on factors such as type, size and cell condition. In addition to these natural factors, cell preparation reagents, such as fixation and permeabilization, can also alter the autofluorescence pattern.

In conventional flow cytometry, (Figure 1A and 1B), autofluorescence is "compensated" using a compensation matrix of fluorochromes. Since the autofluorescence spectrum is broad and overlaps with many fluorochromes, it is difficult to distinguish the autofluorescence as a signal to separate it from other signals using conventional flow cytometry. As a result, samples that contain a bright autofluorescent population show a diagonal pattern in fluorochrome scatter plots (Figure 1B). This diagonal pattern seen in both natural bright autofluorescent samples and those stained with cell preparation reagents, indicates a serious false positive result. Unfortunately, this pattern is frequently observed in conventional flow cytometry.

Autofluorescence using spectral analysis technology

In spectral analysis, (Figures 1C and 1D), reference spectra of fluorochromes are used for the unmixing calculation to determine the intensity of fluorochromes. It is also possible to use reference spectrum of cellular autofluorescence from the unstained cells. Importantly, since spectral analysis can both recognize autofluorescence as a separate signal and define it as a separate color, it can deliver more accurate results.

Figure 2

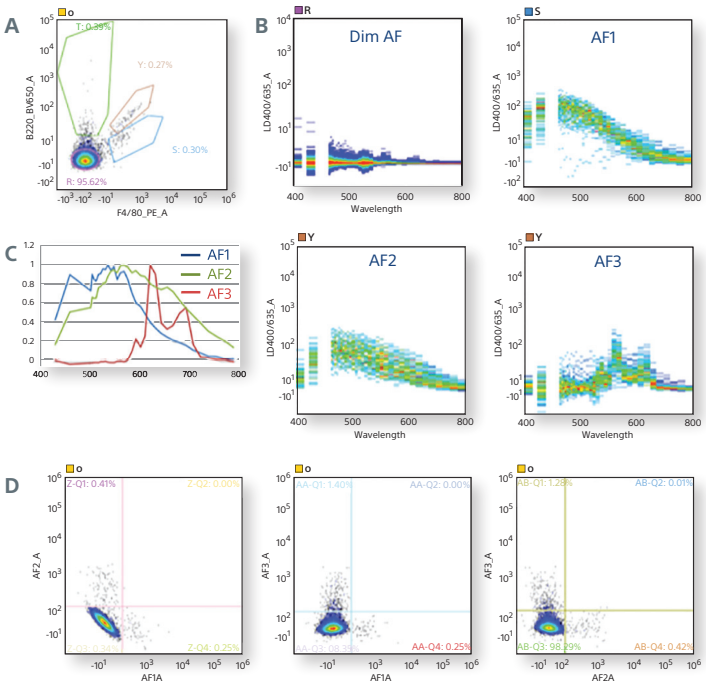

Figure 2. *Spectral analysis of unstained mouse bone marrow.*

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 in autofluorescence.* ***B****. Spectrum plots for the regions shown in plot A indicate a different population of bright autofluorescent cells, labeled AF1, 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* ***D****.*

Comparison of autofluorescence using conventional flow cytometry and spectral analysis

Mouse bone marrow sample was acquired and analyzed with the spectral analyzer SP6800. 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 spectra of each population was used to generate autofluorescences AF1, AF2 and AF3 that were used as reference spectrum for unmixing (Figure 2C). Using these autofluorescence reference spectra, the unstained data were 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 in fluorochrome axes were eliminated (data not shown).

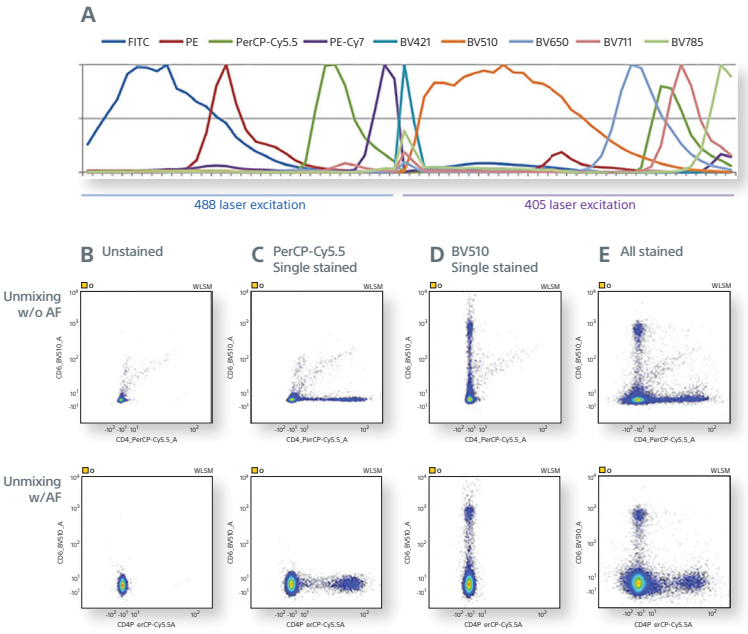

Figure 3. *Spectral analysis of bone marrow sample with 9-color panel.*

A *Fluorochromes were used here to stain the sample, and their emission spectra.* ***B*** *Scatter plots with PerCP-Cy5.5 and BV510 axes for unstained sample, single stained sample for PerCP-Cy5.5 and BV510, and the sample stained with all 9-colors were shown. Here, the unmixing calculation was done with the 9-color fluorochrome reference spectra only (upper, Unmixing w/o AF), or with the 9-color fluorochrome and 3 autofluorescence, as a total 12 reference spectra (lower, Unmixing w/AF).*

In Figure 3, bone marrow samples were stained with a 9-color panel and analyzed with 9-color fluoroschromes and 3 autofluorescence spectra (AF1, AF2 and AF3). Unmixing calculations were then performed shown in figures 3B-3E.

The density plots on PerCP-Cy5.5 (CD4) and BV510 (CD8) are shown without and with the autofluorescent reference spectra used. In the plots without the autofluorescence reference spectra, some diagonal populations are observed. The same patterns were observed in the unstained sample indicating they were the false positive artifacts derived from the cell autofluorescence. These artifacts were successfully removed when the autofluorescence reference spectra was included in unmixing.

Conclusion

In the bone marrow example, using spectral analysis technology, false positives were eliminated and several different autofluorescent spectrums were observed. Greater accuracy and more precise results delivered by spectral technology enable researchers to use autofluorescence in useful new ways to support biomedical discovery. In these endeavors, the SP6800 spectral cell analyzer can be counted on to simplify workflow and deliver more accurate fluorescent data.

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