Spectral Unmixing to Subtract Autofluorescence in Samples Collected with the ZE5 Cell Analyzer

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Introduction

In flow cytometry analysis, the background of autofluorescent samples can prevent the accurate determination of dimly expressed markers. Spectral unmixing algorithms have proved effective in reducing the background due to autofluorescence, thus providing improved detection of low expressing markers on challenging samples. The ZE5 Cell Analyzer is a flexible, high-parameter flow cytometer with up to 5 lasers and 30 detectors, which cover a large range of the emission spectrum. Previous work has established that data collected from the ZE5 Cell Analyzer can be successfully analyzed using spectral unmixing. Here, we examined the autofluorescence subtraction feature of the FCS Express spectral unmixing algorithm to improve resolution in a phagocytosis assay.

Methods

pHrodo Green Phagocytosis Assay. *E. coli* cells engineered to contain the pH-dependent fluorescent dye pHrodo Green (ex/em = 488/530 nm) were used to assay phagocytosis by J774 macrophages. Following the manufacturer's instructions, *E. coli* particles (Invitrogen #P35366) were resuspended at 1 mg/ml. 20 µl pHrodo Green *E. coli* stock solution was added to each tube of J774 macrophages in 100 µl DMEM with 10% FBS, and incubated at 37°C. To stop the reaction, tubes were placed on ice at 15-minute intervals.

Cell Staining. J744 cells were washed and resuspended in ice-cold stain/wash buffer, then stained in ice cold PBS/BSA, by incubation for 45 minutes with the following antibodies at manufacturer recommended concentrations: F4/80-SBUV510 (Bio-Rad #MCA497SBUV510) and CD11b-SBV515 (Bio-Rad #MCA711SBV515). For compensation and reference controls, cell samples were stained with single antibody or pHrodo Green BioParticles. Following staining, samples were washed three times by centrifugation and resuspended in ice-cold PBS/BSA. All data acquisition was performed using the ZE5 Cell Analyzer.

Setup of ZE5 Cell Analyzer as a Spectral Cytometer. For spectral analysis, all detectors were enabled, with the exception of the 640/20-561 channel. A blank (unstained) bead from Ultra Rainbow fluorescent particles set (Spherotech URCP-38) was acquired and PMT voltages were adjusted until the median fluorescent intensity was in channel 5-10. Next, the fully stained sample was acquired, and all plots reviewed to ensure populations were on scale. The spectral signature of each single-color reference control, including unstained J774 cells, was captured and saved.

FCS Express Spectral Analysis with Autofluorescence Subtraction. In FCS Express flow cytometry software (DeNovo), the Pipeline function was used to create a new merged spectra keywords file and a new unmixing function was launched. A single-cell gate was added to the scatter plot then applied to remaining samples. The unmixing wizard was completed and the new unmixing algorithm was applied. For traditional compensation, data parameters not in use were deselected, traditional compensation wizard steps were then followed.

Spectral Experiment Setup

Table 1: Experiment set-up for spectral unmixing..

Step	Traditional compensation on ZE5 Cell Analyzer	Spectral unmixing with ZE5 Cell Analyzer-generated data
Everest fluorophores selection	Everest enables detectors for selected fluorophores	Manually enable all detectors to capture the spectrum (except 640/20-561)
PMT voltage settings	• Use the unstained control to set PMT voltages. • Check single color controls to ensure positive stains are on scale	• Use the blank bead peak from 6 peak Ultra Rainbow beads set to MFI channel ~5-10. • Check fully stained sample to ensure all stains are on scale
Required controls	Unstained and single stain compensation controls.	Unstained and single stain reference controls
Analysis	Everest compensation wizard	FCS Express unmixing wizard

Summary of the key differences between experiment set-up on the ZE5 Cell Analyzer in Everest flow cytometry software for analysis using traditional compensation algorithms or for post-acquisition spectral unmixing.

Spectral Experiment Setup

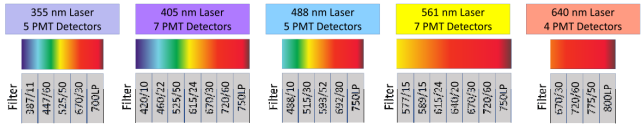

Figure 1: **ZE5 Cell Analyzer detector arrays by laser.** Detector coverage across the light spectrum using a standard five-laser configuration ZE5 Cell Analyzer.

Results

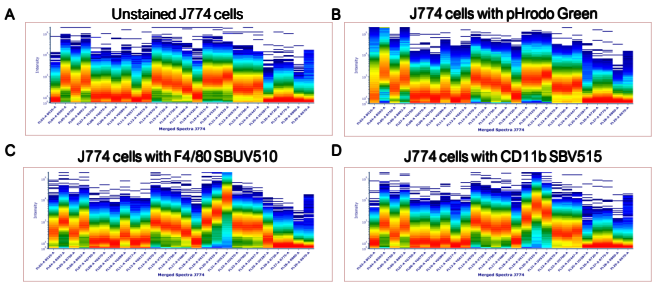

Figure 2: Spectral analysis plots for single color controls. Unstained cell spectra shows background fluorescence in several detectors (A). pHrodo reference control (B) shows intensity increase in 488nm laser channels, consistent with the fluorescent properties of the dye. Spectral signature of cells stained with F4/80 SBUV510 shows increased intensity over unstained, primarily in the 525/50-488 detector (C). Spectral signature of cells stained with CD11b-SBV515 shows increase in intensity primarily in the 525/50-405 detector (D). Intensity indicates detection of light by a particular PMT.

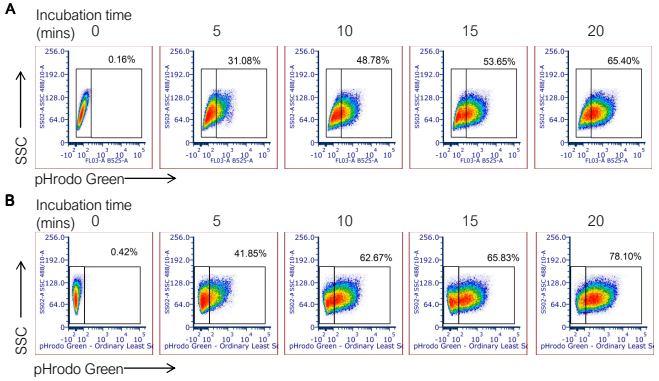

Figure 3: Autofluorescence subtraction improves *E. coli* phagocytosis detection. Cells were incubated at 37°C with pHrodo Green *E. coli* particles and removed at 5 minute intervals. Data acquisition was performed using spectral analysis without (A) or with (B) autofluorescence subtraction. The ability to remove autofluorescence improves the resolution between pHrodo Green positive and negative cells, revealing a higher percentage of phagocytosis at all timepoints.

Results

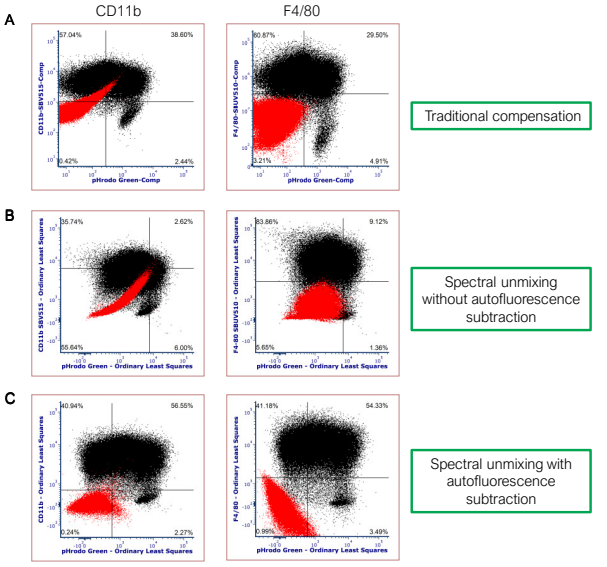

Figure 4: Autofluorescence subtraction improves detection of multiple markers. Cells were incubated with *E. coli* for 15 minutes then co-stained with CD11b SBV515 and F4/80 SBUV510. Data were analyzed using conventional compensation (A) spectral unmixing without (B), or with (C) autofluorescence subtraction. Plot overlays show unstained sample (red) and fully stained sample (black). Autofluorescence subtraction improves accurate signal detection.

Conclusions

- **ZE5 Cell Analyzer has a large number of detectors which, when enabled, provide sufficient coverage of the light spectrum to complete spectral analysis with software packages such as FCS Express**
- **Autofluorescence subtraction can improve the resolution of multiple markers that are masked when analysis is performed with traditional compensation**
- **Autofluorescence subtraction improves resolution of pHrodo Green phagocytosis in J774 murine macrophages, allowing for more accurate determination of bacteria uptake**

For more information on the ZE5 Cell Analyzer visit Bio-rad.com/ZE5