Application Note

Cell Analysis

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# Capability of Spectral Flow Cytometry for Resolving Fluorochromes with Highly Overlapping Spectra


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## Abstract

Full spectrum flow cytometers use a series of photodetectors with corresponding narrow band-pass filters or diffraction gratings to measure the full spectral emission of fluorochromes across the entire spectrum with multiple excitation lasers. Unlike conventional flow cytometry, which employs compensation to correct the fluorescence spectrum spillover, spectral flow cytometry uses spectral unmixing to resolve the signal intensity of individual fluorochromes. This process involves a mathematical algorithm to decompose the spectral signature of a multicolor sample into a set of fluorochromes and their corresponding abundance, based on the unique spectral signature of each fluorochrome. This method enables the differentiation of fluorochromes with nearly identical peak emissions but varying off-peak emissions, which is usually challenging for the conventional flow cytometer.

In this application note, we present the capability of the Agilent NovoCyte Opteon spectral flow cytometer to resolve fluorochromes with similar spectra using several designed multicolor panels. Despite their highly overlapping spectra, these fluorochromes can be effectively resolved when used together within a panel, demonstrating the superior spectral unmixing capabilities of the NovoCyte Opteon spectral flow cytometer. This capability expands the possibility for more flexible fluorochrome selection to profile more biomarkers within the same panel.

## Introduction

In conventional flow cytometry, fluorochromes with close emission peaks are detected in the same channel and cannot be used in together. For example, APC and AF647, with the maximum emission wavelengths of 660 and 671 nm respectively (Figure 1A), are typically detected in the same channel excited by a red laser with a central wavelength of around 660 nm. Spectral flow cytometry, however, captures the emission profiles of fluorochromes from all available lasers across the entire spectrum. It then uses spectral unmixing to determine the contribution of each fluorochrome on each channel based on the unique spectral signature of each. This process allows for the distinction of each fluorochrome provided their spectral signatures differ. Using APC and AF647 as examples, their emission spectra are very similar in the red laser-excited channels, with only slight emission differences in the R661 and R681 channels, they exhibit significant differences in channels excited by other lasers, particularly ultraviolet(UV), violet, and blue channels. These differences alllow spectral unmixing to distinguish their signals easily. Other examples include PerCP-Cy5.5/PerCP-eFluor 710 (Figure 1B), and PerCP-Cy5.5/PE-Cy5.5 (Figure 1C), BV421/Pacific Blue (Figure 1D), Qdot 705/BV711 (Figure 1E), and BB515/FITC (Figure 1F).

Although spectral unmixing can distinguish fluorochrome signals based on their spectral signatures, spectral spillover can propagate errors when measuring multiple fluorochromes. Spectral unmixing cannot eliminate these errors. The closer the spectra are, the greater the spillover spreading error between fluorochromes. This phenomenon emphasizes the importance of selecting fluorochromes with minimal spectral overlap in panel design to reduce spillover spreading. Here, we first present several panels containing fluorochromes with similar spectra using the Agilent NovoCyte Opteon spectral flow cytometer, compared in parallel with panels containing the same markers but conjugated to fluorochromes with different spectra. The spectrum-similar fluorochrome combinations include BB515/FITC, Pacific Blue/BV421, APC/Alexa Fluor 647, PerCP-Cy5.5/PE-Cy5.5, and PE-Cy5.5/PE-AF700. Fluorochromes in these combinations can be resolved when used together, although the spillover spreading of spectrum-similar fluorochromes is much larger.

Secondly, we compared FITC with different spectrally similar fluorochromes using the NovoCyte Opteon spectral flow cytometer. As the similarity increased, the fluorescent signal spreading increased significantly, and the resolution of FITC decreased correspondingly. When FITC was used with Alexa Fluor 488 or KB520, the Similarity Index was 1.00, indicating almost identical spectra, which prevented signal resolution due to such high similarity.

Lastly, we used a mixture of compensation beads labeled with different fluorochromes excited by one laser (405 nm violet, 488 nm blue, or 640 nm red) to simulate the scenario where multiple fluorochromes are used together. In this context, the NovoCyte Opteon spectral flow cytometer effectively distinguished the signals of multiple fluorochromes.

## Experimental

### Instrument configuration

Agilent NovoCyte Opteon UVBYR spectral flow cytometer equipped with five lasers (349, 405, 488, 561, and 637 nm) and 70 fluorescence detectors.

### Examples of spectrum similar fluorochrome combinations

**Table 1.** Antibody information.

| Marker | Fluorochrome | Clone | Vendor | Part number |
|---|---|---|---|---|
| CD45 | PE | HI30 | BioLegend | 304007 |
| CD45 | BV510 | HI30 | BioLegend | 304036 |
| CD45 | FITC | HI30 | Agilent | 8921014 |
| CD3 | PerCP-Cy5.5 | SK7 | Agilent | 8931015 |
| CD3 | FITC | SK7 | Agilent | 8921016 |
| CD4 | BV421 | RPA-T4 | BioLegend | 300532 |
| CD4 | Qdot705 | S3.5 | Thermo Fisher | Q10060 |
| CD4 | BB515 | RPA-T4 | BD | 564419 |
| CD4 | Alexa Fluor 647 | RPA-T4 | BioLegend | 300520 |
| CD4 | PE-Cy5.5 | SK3 | Thermo Fisher | 35-0047-41 |
| CD8 | Pacific Blue | SK1 | BioLegend | 344718 |
| CD8 | FITC | SK1 | Agilent | 8920038 |
| CD8 | APC | SK1 | Agilent | 8920265 |
| CD19 | APC | HIB19 | Agilent | 8730007 |
| CD19 | PerCP-eFluor 710 | J3-129 | Thermo Fisher | 46-0197-42 |
| CCR6 (CD196) | PE | G034E3 | BioLegend | 353410 |
| CXCR3 (CD183) | BV711 | G025H7 | BioLegend | 353732 |
| CXCR3 (CD183) | PE-Cy7 | G025H7 | BioLegend | 353720 |

**Table 2.** Other reagents used.

| Materials | Part number | Manufacturer |
|---|---|---|
| AceaLyse solution | 894B604 | Agilent |
| Phosphate buffered saline (PBS) | GNM20012-2 | GENOM BIO |
| 4% paraformaldehyde in PBS (PFA) | P395744-100 mL | Aladdin |

### Sample preparation

Add the appropriate volume of antibody and EDTA-anticoagulated human whole blood to a tube, ensuring no blood adheres to the tube walls. Vortex immediately and incubate at room temperature for 15 minutes, protected from light. Add 2 mL of 1x AceaLyse solution, vortex and incubate for 10 minutes under the same conditions. Centrifuge at 500 × g for 5 minutes at room temperature, discard the supernatant and wash the sample twice with PBS. Resuspend the sample in PBS with 1% PFA.

## FITC combined with fluorochromes exhibiting varying degrees of similarity

**Table 3.** Antibody information.

| Marker | Fluorochrome | Clone | Vendor | Part number |
|---|---|---|---|---|
| CD4 | FITC | SK3 | Agilent | 8921018 |
| CD4 | BB515 | RPA-T4 | BD | 564419 |
| CD4 | KIRAVIA Blue 520 | SK3 | BioLegend | 344660 |
| CD4 | Alexa Fluor 532 | SK3 | Thermo Fisher | 58-0047-42 |
| CD4 | APC | SK3 | Agilent | 8931019 |
| CD4 | AF488 | RPA-T4 | BioLegend | 300519 |
| CD3 | PE-Cy7 | UCHT1 | Agilent | 8920263 |

**Table 4.** Other reagents and materials used.

| Materials | Part number | Manufacturer |
|---|---|---|
| Leukopak | FPB007-5 | Oribiotech |
| Human TruStain FcX | 422302 | BioLegend |
| Phosphate buffered saline (PBS) | GNM20012-2 | Genom |
| Fetal bovine serum (FBS) | A5669701 | Gibco |
| 4% paraformaldehyde in PBS (PFA) | P395744-100mL | Aladdin |
| FVS620 | 564996 | BD |

### Sample preparation

Thaw peripheral blood mononuclear cells (PBMCs), wash once with PBS, and resuspend in PBS. Add the appropriate volume of antibodies and FVS620 dye solution to the cell suspension, then vortex immediately. Incubate for 30 minutes at room temperature, protected from light. Wash cells twice with staining buffer (PBS containing 2% FBS) and resuspend in PBS with 1% PFA.

Incubate FVS620 stained cells with TruStain FcX for 10 minutes, then add the antibody mixture and incubate for 30 minutes in the dark at 4 °C. Wash the stained cells twice with staining buffer and resuspend in PBS with 1% PFA.

Mix CD3 PE-Cy7/CD4 FITC-stained PBMC samples with those labeled with CD3 PE-Cy7 and different fluorochrome-conjugated CD4 antibodies.

## Multiple fluorochrome combinations excited by the 405, 561, or 637 nm lasers

**Table 5.** Antibody information.

| Group | Marker | Fluorochrome | Clone | Vendor | Part number |
|---|---|---|---|---|---|
| Violet-excited fluoro-chromes | CD4 | BV510 | OKT4 | BioLegend | 317444 |
| | CD4 | 317444 | RPA-T4 | BioLegend | 300532 |
| | CD4 | BV421 | RPA-T4 | BD | 746541 |
| | CD4 | BV480 | RPA-T4 | Thermo Fisher | 69-0049-42 |
| | CD4 | eFluor 506 | RPA-T4 | Thermo Fisher | 79-0049-41 |
| | CD11c | Pacific Orange | 3.9 | Thermo Fisher | 48-0116-42 |
| Violet-excited fluoro-chromes | CD4 | PE | SK3 | Agilent | 8920028 |
| | HLA-DR | CF568 | TAL 1B5 | Biotium | BNC682187-500 |
| | CD4 | PE-Dazzle 594 | RPA-T4 | BioLegend | 300548 |
| | CD4 | PE-Alexa Fluor 610 | S3.5 | Thermo Fisher | MHCD0422 |
| | CD4 | PE-Fire 640 | SK3 | BioLegend | 344663 |
| | CD4 | PE-Cy5 | RPA-T4 | BioLegend | 300510 |
| | CD117 | PE-Cy5.5 | YB5.B8 | Novus | NBP1-43358PECY55 |
| | CD25 | PE-Alexa Fluor 700 | CD25-3G10 | Thermo Fisher | MHCD2524 |
| Blue-excited fluoro-chromes | CD4 | Alexa Fluor 700 | RPA-T4 | BioLegend | 300526 |
| | CD4 | APC | SK3 | Agilent | 8931019 |
| | CD4 | Alexa Fluor 647 | RPA-T4 | BioLegend | 300520 |
| | CD4 | Spark NIR 685 | SK3 | BioLegend | 344658 |
| | CD4 | APC-R700 | RPA-T4 | BD | 564975 |

**Table 6.** Other reagents and materials used.

| Materials | Part number | Manufacturer |
|---|---|---|
| Compensation beads | CMIgP-30-2K | Spherotech |
| Zombie NIR | 423105 | BioLegend |
| Phosphate buffered saline (PBS) | GNM20012-2 | Genom |
| Bovine serum albumin (BSA) | 36101ES60 | YESEN |

### Sample preparation

Add appropriate antibodies and 50 µL of vortexed positive beads into the tubes, then vortex to mix. Incubate for 30 minutes at room temperature, protected from light. Wash twice with 1 mL PBS containing 0.2% BSA and resuspend. Mix the stained positive beads with an equal amount of negative beads to prepare single-stained and multiple fluorochrome combination samples.

## Results and discussion

### Examples of spectrum similar fluorochrome combinations

Several panels containing fluorochromes with similar spectra were designed and compared in parallel with panels containing the same markers but conjugated to fluorochromes with different spectra. The Similarity Index, ranging from 0 to 1, measures how closely one spectral signature matches another. Values near 0 indicate highly different signatures, while values near 1 indicate highly similar signatures. The spectrum-similar fluorochrome combinations include BV421/Pacific Blue, Qdot 705/BV711, BB515/FITC, APC/Alexa Fluor 647, PerCP-Cy5.5/PerCP-eFluor 710, and PerCP-Cy5.5/PE-Cy5.5. The Similarity Indices of these combinations are 0.66, 0.75, 0.99, 0.89, 0.89, and 0.76, respectively.

EDTA-anticoagulated human peripheral blood was stained. Red blood cells were then lysed and afterward, the samples were washed and analyzed on a five-laser NovoCyte Opteon flow cytometer. Spectrum density plots generated by the NovoCyte Opteon flow cytometer and theoretical emission spectra of the spectrum similar fluorochromes are shown in Figure 1, visually presenting the overlap and similarity of similar spectra.

The plots in Figure 1 show that fluorochromes in these combinations can be resolved when used together and the subpopulation identifications are not affected.

Although spectral unmixing can distinguish fluorochrome signals based on their spectral signatures, spectral spillover can propagate errors when measuring multiple fluorochromes simultaneously and these errors cannot be eliminated by spectral unmixing. The closer the spectra are, the greater the spillover spreading error between

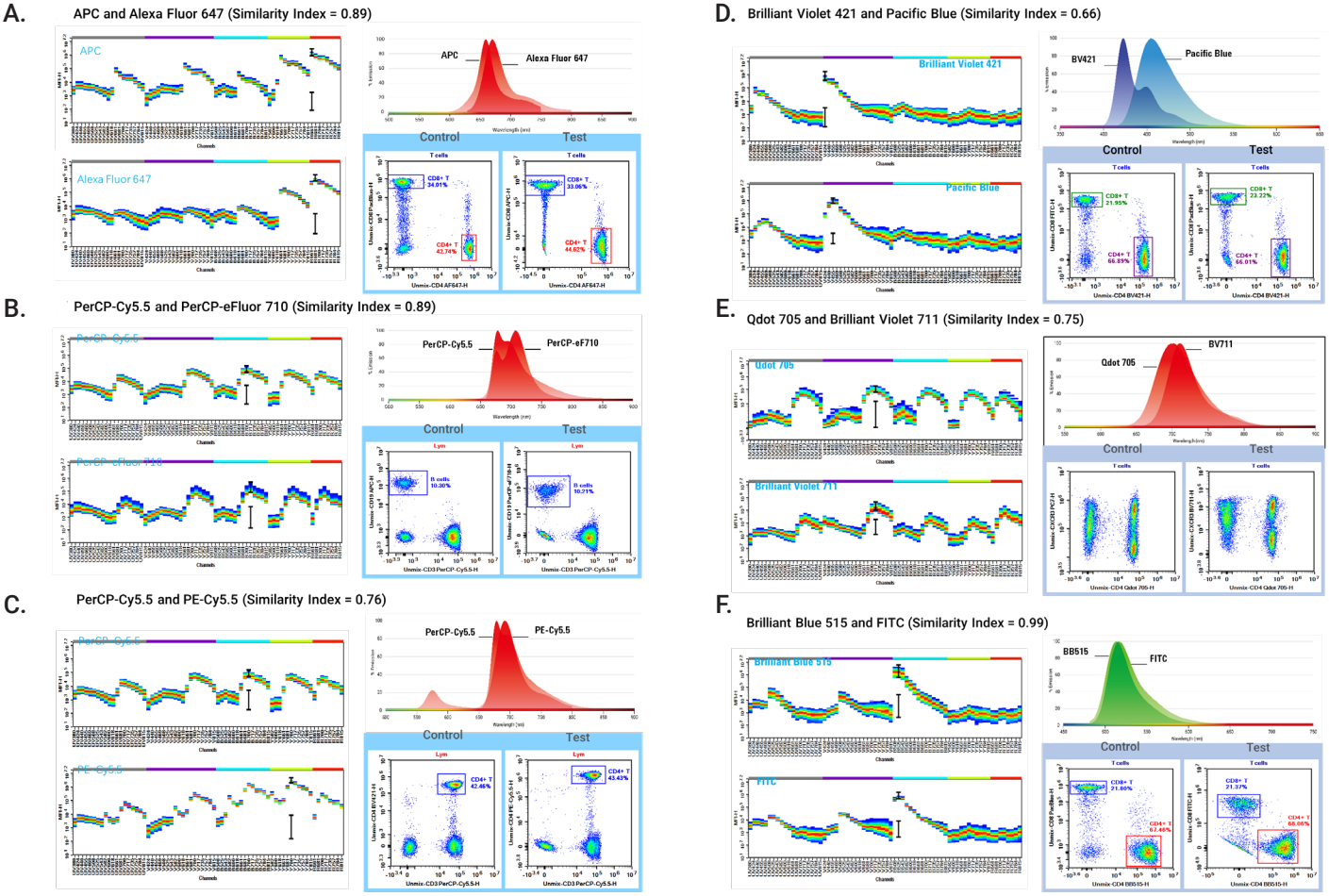


**Figure 1.** The detection of spectrum-similar fluorochrome combinations. The combinations include APC/Alexa Fluor 647(A), PerCP-Cy5.5/PerCP-eFluor 710(B), and PerCP-Cy5.5/PE-Cy5.5(C), BV421/Pacific Blue(D), Qdot 705/BV711(E), BB515/FITC(F). Control: no spectrum-similar fluorochromes included. Test: spectrum-similar fluorochromes included.

fluorochromes. This phenomenon reminds us to select fluorochromes with minimal spectral overlap in panel design to reduce spillover spreading.

### FITC combined with fluorochromes exhibiting varying degrees of similarity

FITC used in conjunction with fluorochromes exhibiting varying degrees of similarity was compared using the NovoCyte Opteon spectral flow cytometer. The fluorochromes include Alexa Fluor 488, KIRAVIA Blue 520, Brilliant Blue 515, Alexa Fluor 532, and APC. The spectral overlap of these combinations gradually decreased, and the Similarity Indices were 1.00, 1.00, 1.00, 0.99, 0.56, and 0.08, respectively. As the Similarity Index decreases, the fluorescent spreading error becomes smaller, and the resolution increases. When FITC was used in conjunction with BB515, the Similarity Index was 0.99, indicating that the spectra of these two dyes were almost identical. Despite such high similarity, their signals still can be resolved (Figure 2).

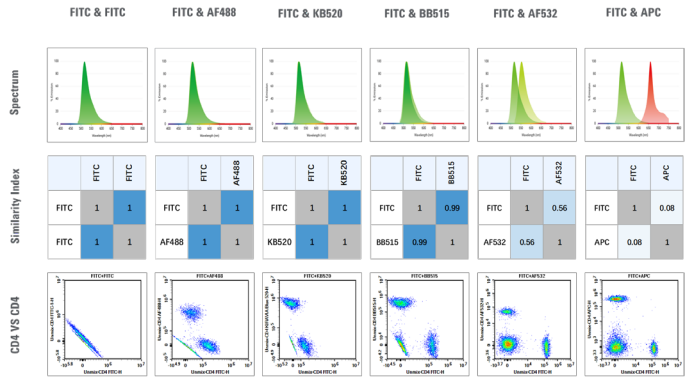


**Figure 2.** FITC combined with fluorochromes exhibiting varying degrees of similarity. PBMCs were stained with CD3 PE-Cy7 and FITC, Alexa Fluor 488, KIRAVIA Blue 520, Brilliant Blue 515, Alexa Fluor 532, or APC-conjugated CD4 antibody. CD3 PE-Cy7/CD4 FITC-stained PBMC samples were mixed with samples labeled with CD3 PE-Cy7 and different fluorochrome-conjugated CD4 antibodies correspondingly and analyzed on a five-laser Agilent NovoCyte Opteon spectral flow cytometer.

### Multiple fluorochrome combinations excited by the 405, 561, or 637 nm lasers

Compensation beads were stained with different fluorescent antibodies. Those beads labeled with fluorochromes primarily excited by one laser (violet, yellow, or red) were mixed together to simulate multicolor panels and acquired on the NovoCyte Opteon spectral flow cytometer. Unmixed single-stained compensation bead samples were used to generate spectrum signatures for each fluorochrome. The unmixed results are presented in Figure 3. All populations can be distinguished well.

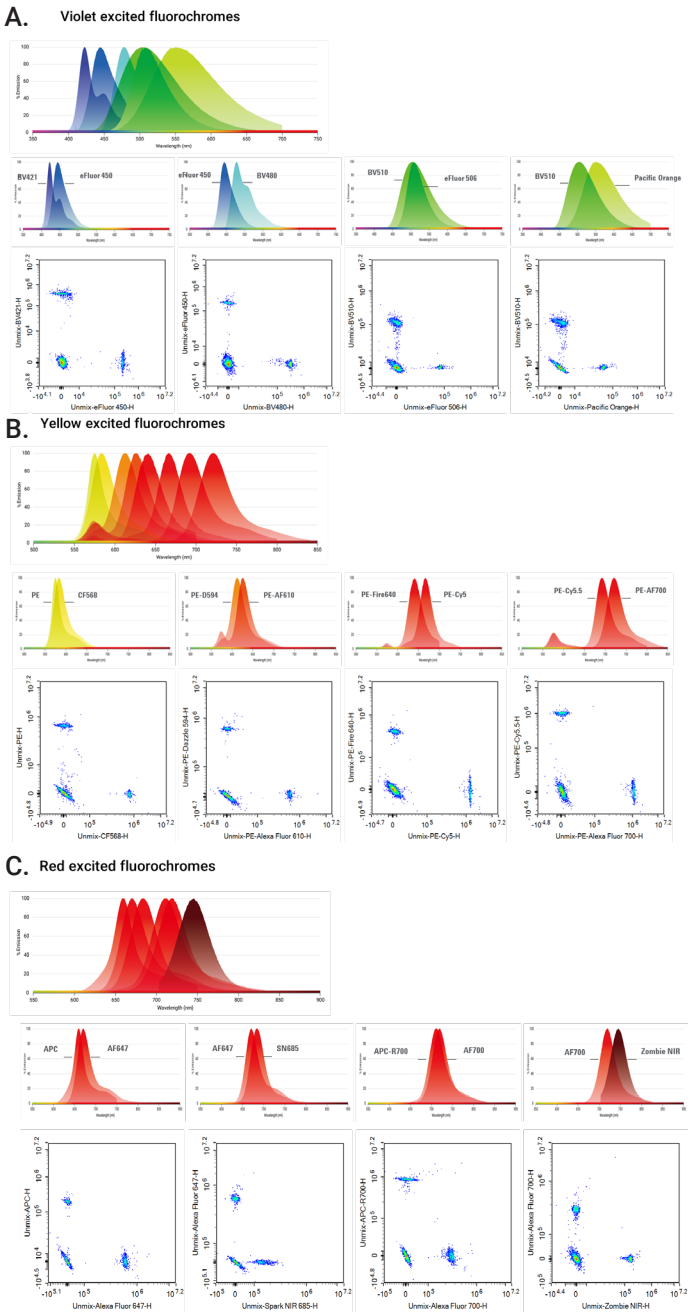


**Figure 3.** Unmixing of multiple fluorochromes with overlapping emission spectra excited by the 405, 561 or 637 nm lasers. (A) Combination of violet laser excited fluorochromes, BV421, eFluor 450, BV480, BV510, eFluor 506, and Pacific Orange. (B) Combination of yellow laser excited fluorochromes, PE, CF568, PE-Dazzle 594, PE-Alexa Fluor 610, PE-Fire 640, PE-Cy5, PE-Cy5.5, and PE-Alexa Fluor 700. (C) Combination of red laser excited fluorochromes APC, Alexa Fluor 647, Spark NIR 685, APC-R700, Alexa Fluor 700, and Zombie NIR.

## Conclusion

By testing various spectrum-similar fluorochrome combinations with multiple panel design, we demonstrated that the Agilent NovoCyte Opteon spectral flow cytometer can effectively distinguish fluorochromes with high spectral overlap. When comparing the use of FITC in combination with fluorochromes exhibiting varying degrees of similarity, we found that as the similarity of fluorochromes increased, the spillover spreading largely increased, leading to a corresponding decrease in the resolution of FITC. Notably, NovoCyte Opteon was able to distinguish fluorochromes with a Similarity Index as high as 0.99 (FITC and BB515). Additionally, the multicolor unmixing capabilities of NovoCyte Opteon were validated using multiple fluorochrome combinations excited by the additional 405, 561, or 637 nm lasers.

In summary, spectral flow cytometry allows for simultaneous use of fluorochromes with highly overlapping spectra, enhancing flexibility in fluorochrome selection. However, the inevitable spillover spreading between these fluorochromes must be considered in panel design to ensure accurate results.

## Products used in this application

### Agilent products

Agilent NovoCyte Opteon spectral flow cytometer

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