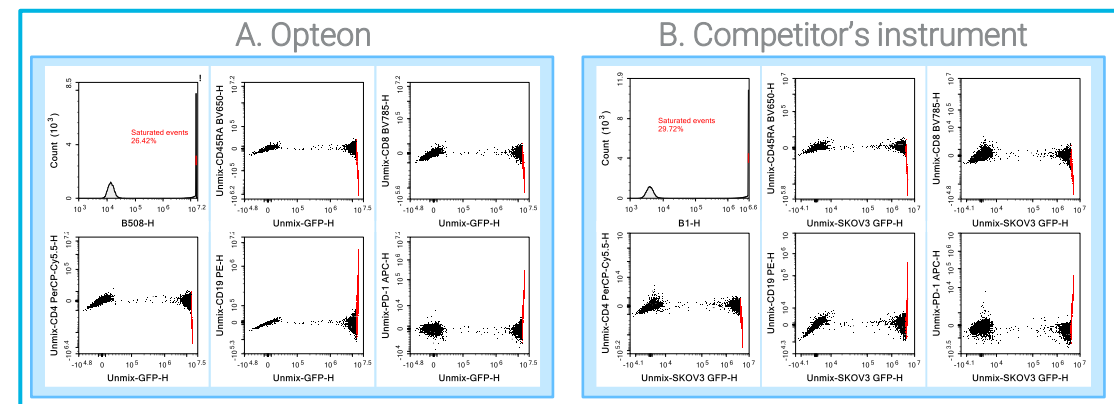


## Abstract

- When samples with highly fluorescent proteins, dyes, or probes are used, the signals they generate are of extremely high intensity, posing a risk of signal saturation. Such saturated signals lead to inaccurate compensation or unmixing, undermining the quality of data analysis. Even when in the absence of signal saturation, high fluorophore brightness can cause signal spreading errors, complicating the accurate identification of co-expressed signals.
- To tackle this issue, the Agilent NovoCyte Opteon spectral flow cytometer offers a solution with user-adjustable laser power. By appropriately reducing the laser power, saturation signals can be lowered to within the detection range and minimize the spreading error from high-brightness signals. This adjustment improves the accuracy of unmixing and enhances overall data reliability.
- Several applications were used to evaluate the performance of the NovoCyte Opteon at different laser power settings. A key characteristic of these applications is the bright signals associated with fluorescent proteins or various fluorescent probes, while some markers in multicolor immunophenotyping exhibit relatively weak expression.

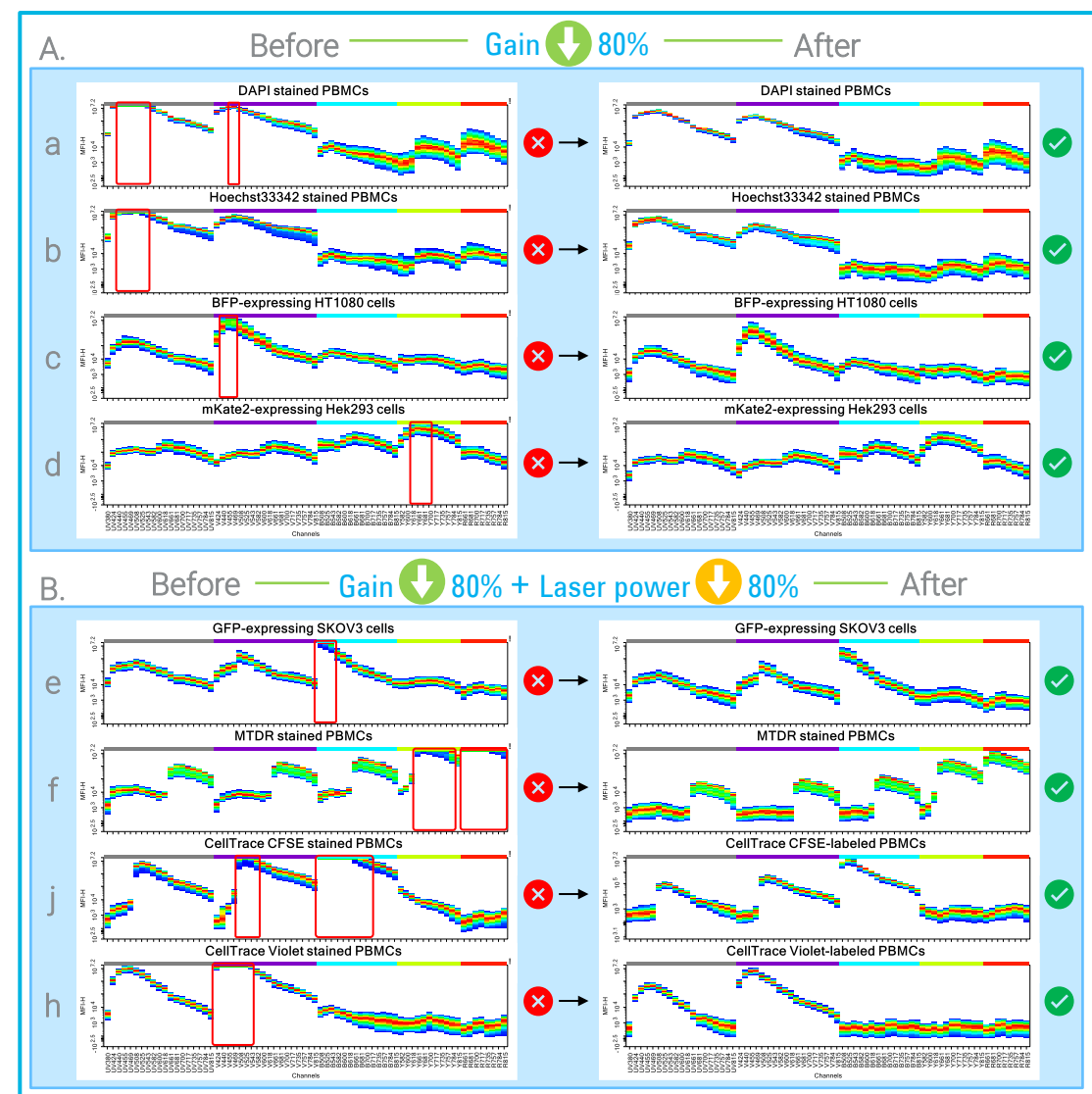
## Background

**Inaccurate unmixing of saturated signals.** Here is an example (Figure 1) to show the saturated GFP events(red color) were incorrectly unmixed.



**Figure 1. Saturated events can't be unmixed properly.** GFP stably expressing SKOV3 cells were acquired on (A) The Agilent NovoCyte Opteon is equipped with three lasers (VBR) and (B) competitor's instrument (VBR). Both datasets were recorded at QC settings and unmixed by least squares(LS) algorithm.

**Managing bright dyes: Begin by lowering the detector gain(Figure 2A), if insufficient, further reducing the laser power(Figure 2B).**



**Figure 2. The spectrum density plot of some bright dyes acquired under different instrument settings.** If the positive signal were off-scale at QC setting, the gain should be appropriately reduced to keep the signal on-scale, such as (A) DAPI, Hoechst 33342, BFP and mKate2. If reducing the detector gain alone doesn't bring the signal within the measurable range, further reducing the laser power is essential, such as (B) GFP, MitoTracker Deep Red(MTDR), CellTrace CFSE and CellTrace Violet

## Experimental

- Panel 1: T cell proliferation and immunophenotyping. Human PBMCs were labeled with CellTrace Violet or CellTrace CFSE and were stimulated with ImmunoCult Human CD3/CD28 T Cell Activator to activate T cells for six days. Activated or nonactivated control cells were stained with Live/Dead dye and immunophenotyping antibodies. Activation, differentiation, exhaustion, and proliferation of viable T cells were analyzed.
- Panel 2: CAR T cell killing and CAR T cell immunophenotyping. HEK293 stable expressing CD19 and mKate2, and SKOV3 stable expressing CD19 and GFP were used as target cells. The CD19 CAR T cells were cocultured with target cells at an E:T ratio of 1:1. 48 hours later, all cells were collected and stained with immunophenotyping antibodies, annexin V CF405M, and 0.2 µg/mL PI before being analyzed. The differentiation and exhaustion of live CAR T cells were assessed.
- Panel 3: mitochondrial membrane potential detection. Human cryopreserved PBMCs were stained with 20 µg/mL JC-1, followed by staining with an antibody mixture, and further stained with annexin V CF405M and 0.2 µg/mL PI before being analyzed. The ΔΨm of T cells was compared with CCCP-treated positive control.
- All samples of three panels were analyzed on a NovoCyte Opteon spectral flow cytometer equipped with five lasers. Laser power and detector gain settings used in each panel are shown in Table 1.

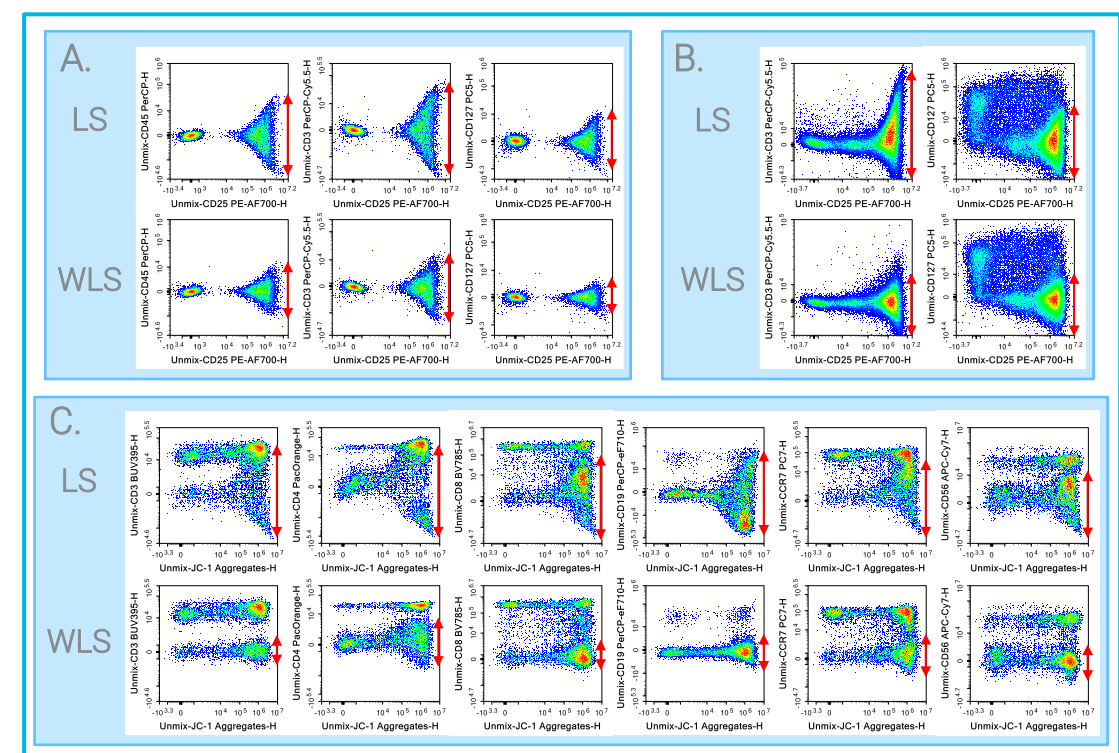
Instrumental Settings		Setting 1 Default	Setting 2 -50%	Setting 3 -80%
Laser Power (mW)	349 nm	20	10 ⬇️ 50%	4 ⬇️ 80%
	405 nm	130	65 ⬇️ 50%	26 ⬇️ 80%
	488 nm	100	50 ⬇️ 50%	20 ⬇️ 80%
	561 nm	100	50 ⬇️ 50%	20 ⬇️ 80%
	637 nm	120	60 ⬇️ 50%	24 ⬇️ 80%
Detector gain	UV380 - UV815	Panel 1 : QC gain		
	V424 - V815	Panel 2 : ⬇️ 80%		
	B508 - B815	Panel 3 : ⬇️ 50%		

**Table 1. Instrumental settings used for recording the datasets.** ⬇️ denotes the decrease of laser power, ⬇️ reindicates the decrease of detector gain.

## Results and Discussion

### Weighted Least Squares (WLS) algorithm reduces spreading error of samples with extremely high signals

More spreading error occurs with brighter fluorescence intensity<sup>[1,2]</sup>, and the degree of spreading was extremely exaggerated on parameters with high signals (Figure 3). WLS algorithm is an extension of traditional LS, but it assigns weights to observations based on the variance of the errors. This helps to reduce the effect of noise heteroscedasticity and improve the accuracy of the spectral unmixing process. Both single and multicolor samples show in Figure 3, WLS yielded smaller spreading error, thus improving resolution. Therefore, all the data of panel 1, 2, and 3 were analyzed using the WLS algorithm, which is in development and will be released later.

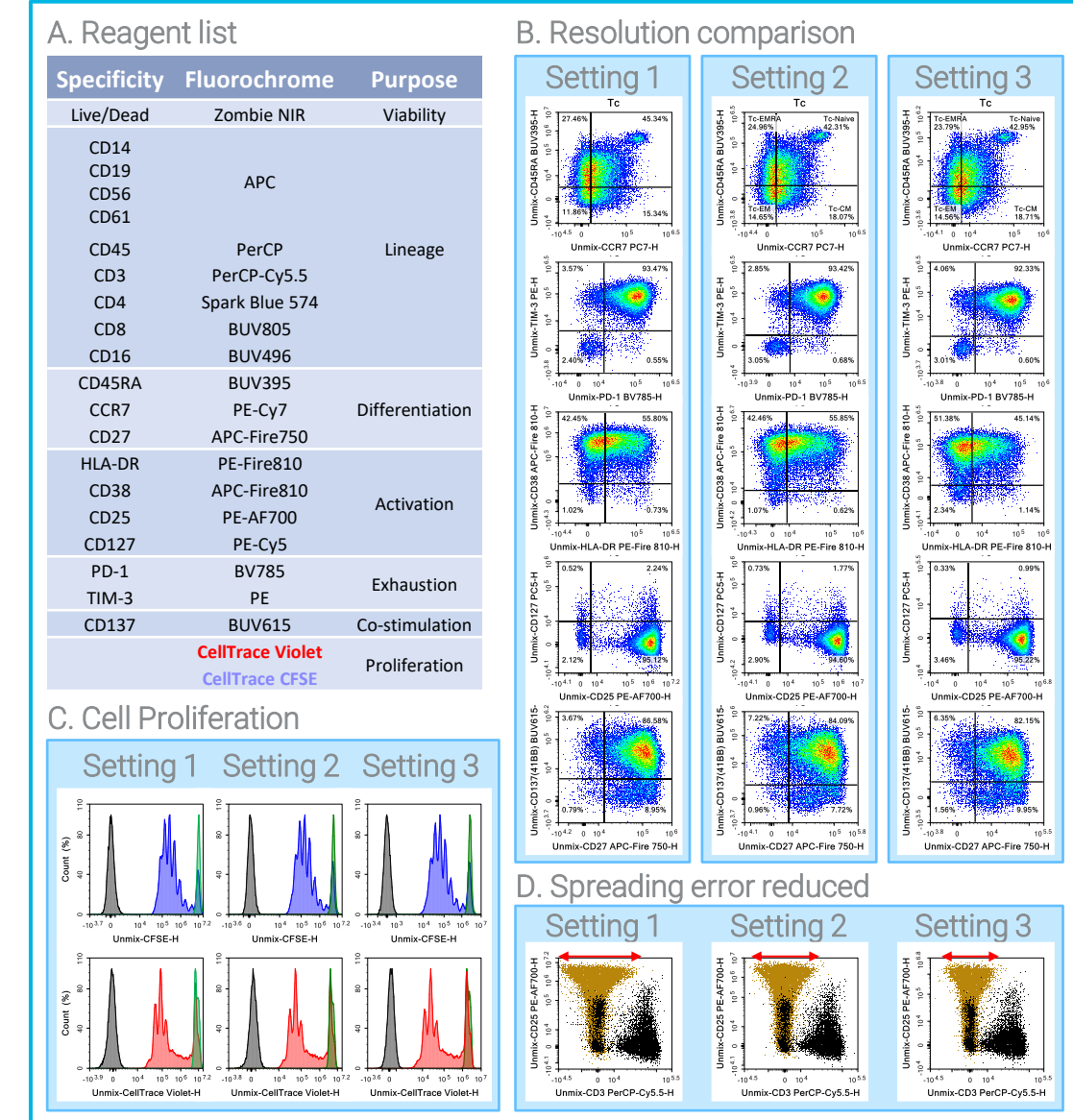


**Figure 3. Comparison of WLS and LS algorithms in unmixing samples with extremely high signals.** (A) CD25 PE-AF700 single-stained activated T cells. (B) Multicolor sample of panel 1. (C) Multicolor sample of panel 3.

## Results and Discussion

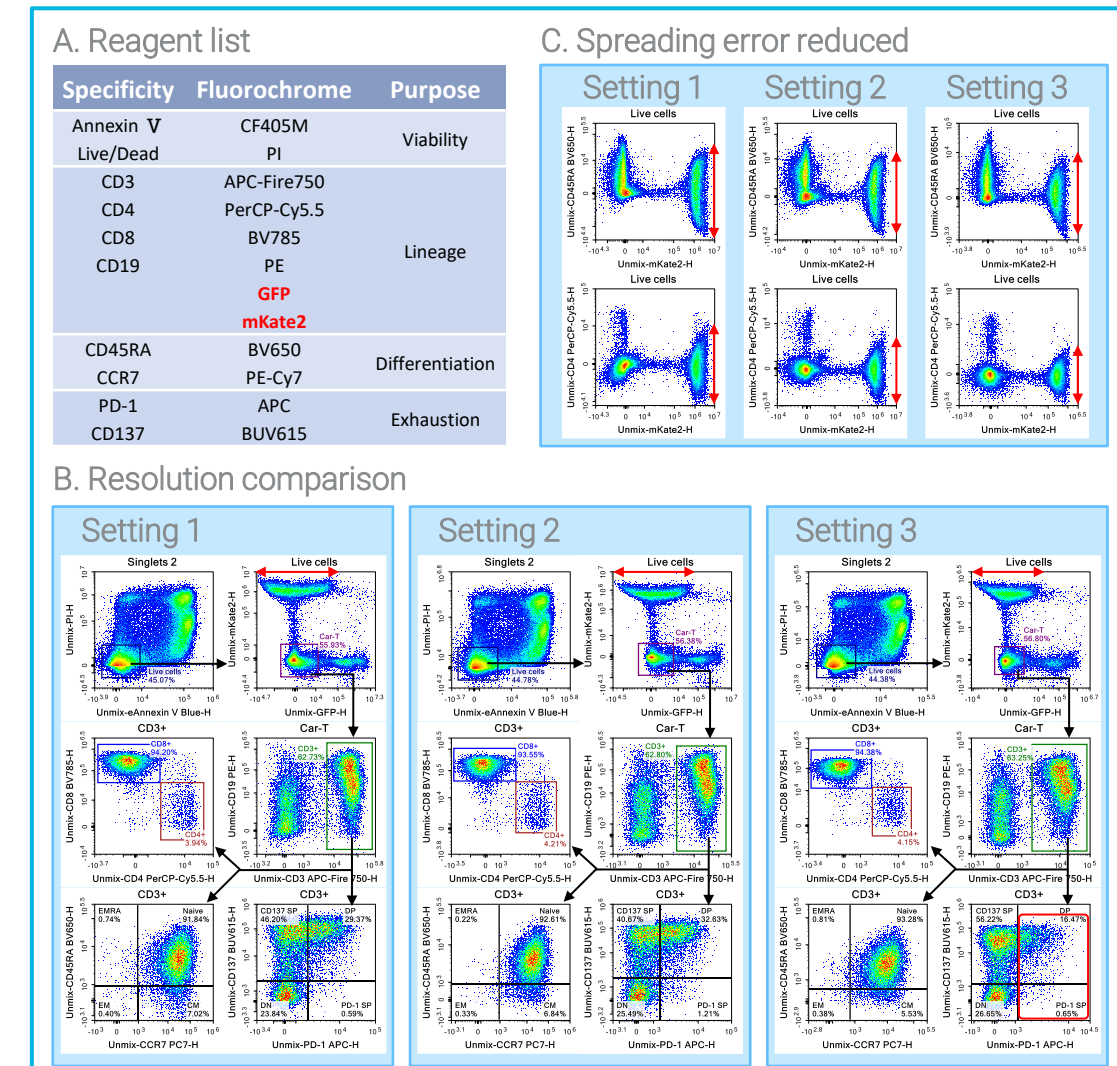
### Markers with low expression could still be resolved and the spreading error from high-brightness signals were reduced(Figure 4,5,6).

#### Panel 1 T cell proliferation and immunophenotyping



**Figure 4. Comparison of different laser powers based on panel 1.** (A) Reagents used in this panel. (B) The different laser power settings produce similar resolution for various markers in panel 1. (C) In vitro activated T cell proliferation was followed using the CellTrace CFSE (blue) or CellTrace Violet (red) histogram overlaid with that of unstimulated unstained control (black) and unstimulated parent generation (green). (D) Spreading errors were reduced with lowered laser power.

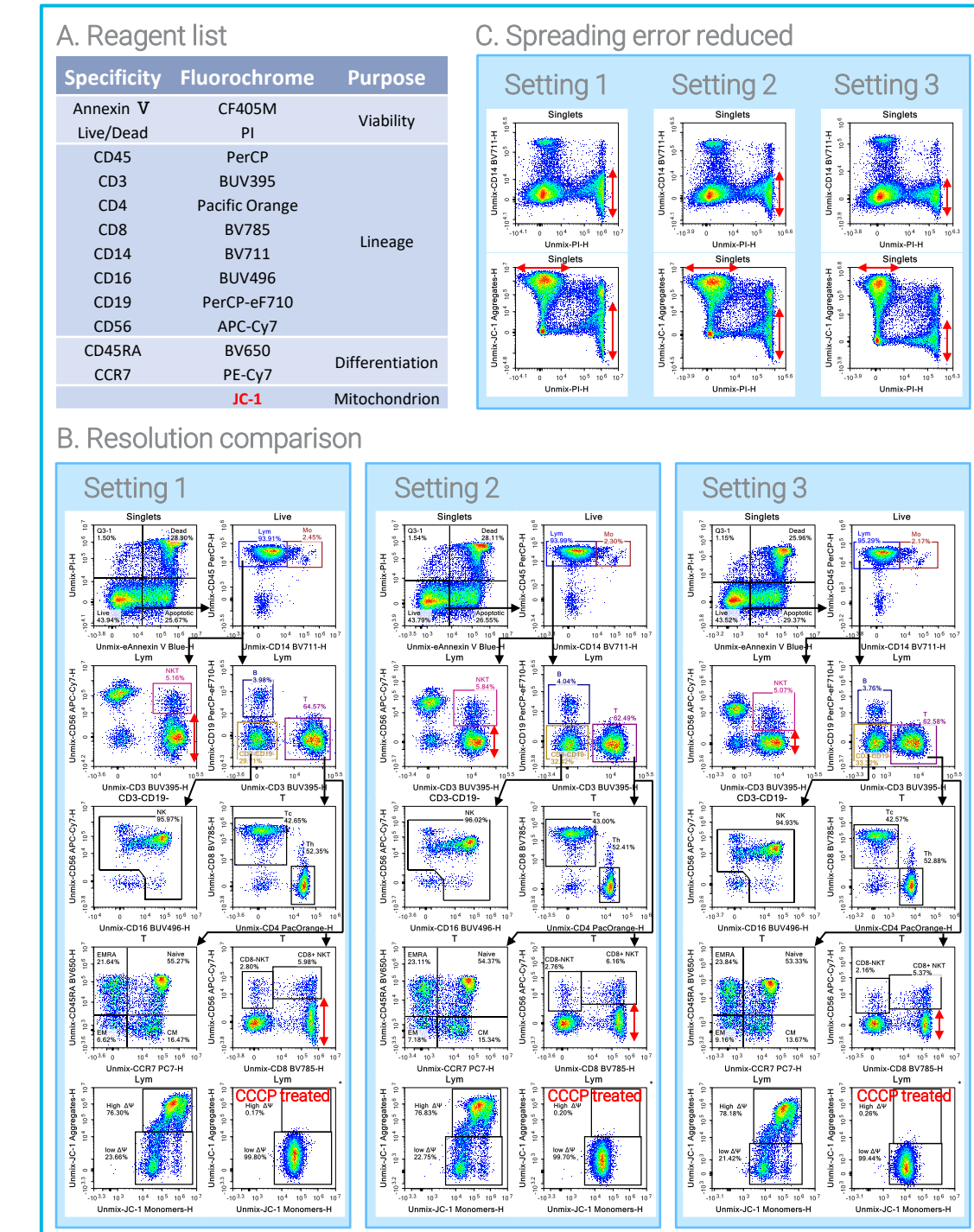
#### Panel 2 CAR T cell killing and CAR T cell immunophenotyping



**Figure 5. Comparison of different laser powers based on panel 2.** (A) Reagents used in this panel. (B) When the laser power was reduced by 80%, the resolution of PD-1+ was reduced since APC is highly sensitive to laser power. (B, C) With laser power decreased, the spreading error from mKate2 to GFP, BV650 and PerCP-Cy5.5 were reduced.

## Results and Discussion

### Panel 3 mitochondrial membrane potential detection



**Figure 6. Comparison of different laser powers based on panel 3.** (A) Reagents used in this panel. (B) Although with laser power reduction, some of the separation was lowered, mitochondrial membrane potential detection and immunophenotyping of PBMCs was not influenced. (C) With laser power decreased, spreading errors were reduced.

## Conclusions

- When extremely strong signals are present, reducing laser power can help minimize fluorescence spillover error. Although we observed a slight reduction in the resolution of weak fluorescent signals while lowering laser power, this study shows minimal impact on immunophenotyping analysis. Overall, the results demonstrate that appropriately adjusting the laser power to the right level can expand the detector's dynamic range, enabling effective detection of both weak and strong signals in these panels.
- By offering user-adjustable laser power, the Agilent NovoCyte Opteon spectral flow cytometer optimizes the balance between detecting weak and very bright fluorescent signals. This enhancement not only improves the instrument's versatility but also meets the diverse needs of customers in operating more complex applications, providing more accurate and reliable results when detecting high-dynamic-range biological signals.

## References

- (1) Nguyen R, Perfetto S, Mahnke YD, et al. Quantifying spillover spreading for comparing instrument performance and aiding in multicolor panel design. *Cytometry A*. 2013 Mar;83(3):306-15.
- (2) Roederer M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry*. 2001 Nov 1;45(3):194-205.

## Introduction

**This template is designed for a 60" x 42" landscape poster.**

The page size is 24.76" x 17.33".

This is a full text box which is 5.57" wide and uses Roboto Light 16 pt. The text box has no indent and no internal margin so that text lines up with header bar.

Text is left justified. Line spacing is .9. Paragraph spacing is 6 pt.

## Experimental

**Subhead: Roboto Medium 16 pt Agilent Blue. Text is left justified. Line spacing is .9. Paragraph spacing is 6 pt.**

Text: Roboto Light 16 pt Black type. Text is left justified. Line spacing is .9. Paragraph spacing is 6 pt.

## Results and Discussion

**Subhead: Roboto Medium 16 pt Agilent Blue. Text is left justified. Line spacing is .9. Paragraph spacing is 6 pt.**

This is a split column text box which is 5.61" wide and uses Roboto Light 16 pt Black type. Text is left justified. Line spacing is .9. Paragraph spacing is 6 pt.

## Results and Discussion

**Subhead: Roboto Medium 16 pt Agilent Blue. Text is left justified. Line spacing is .9. Paragraph spacing is 6 pt.**

This is a split column text box which is 5.61" wide and uses Roboto Light 16 pt Black type. Text is left justified. Line spacing is .9. Paragraph spacing is 6 pt.

## Experimental

**Subhead: Roboto Medium 16 pt Agilent Blue. Text is left justified. Line spacing is .9. Paragraph spacing is 6 pt.**

Text: Roboto Light 16 pt Black type. Text is left justified. Line spacing is .9. Paragraph spacing is 6 pt.

Use this shape around figures if you wish. It can be resized but please do not change the color or the shape of the corners.

Figure Label. Roboto Light 16 pt Grey type.

## Conclusions

**Subhead: Roboto Medium 16 pt Agilent Blue. Text is left justified with 6 pt paragraph spacing.**

Text: Roboto Light 16 pt Black type. Text is left justified. Line spacing is .9. Paragraph spacing is 6 pt.

- Bulleted Text: Roboto Light 16 pt Black type.
- Paragraph spacing is 6 pt above and below.
- Use rounded bullets

## References

<sup>1</sup>References: Roboto Light 14 pt Black type. Text is left justified. Line spacing is .9. Paragraph spacing is 6 pt.