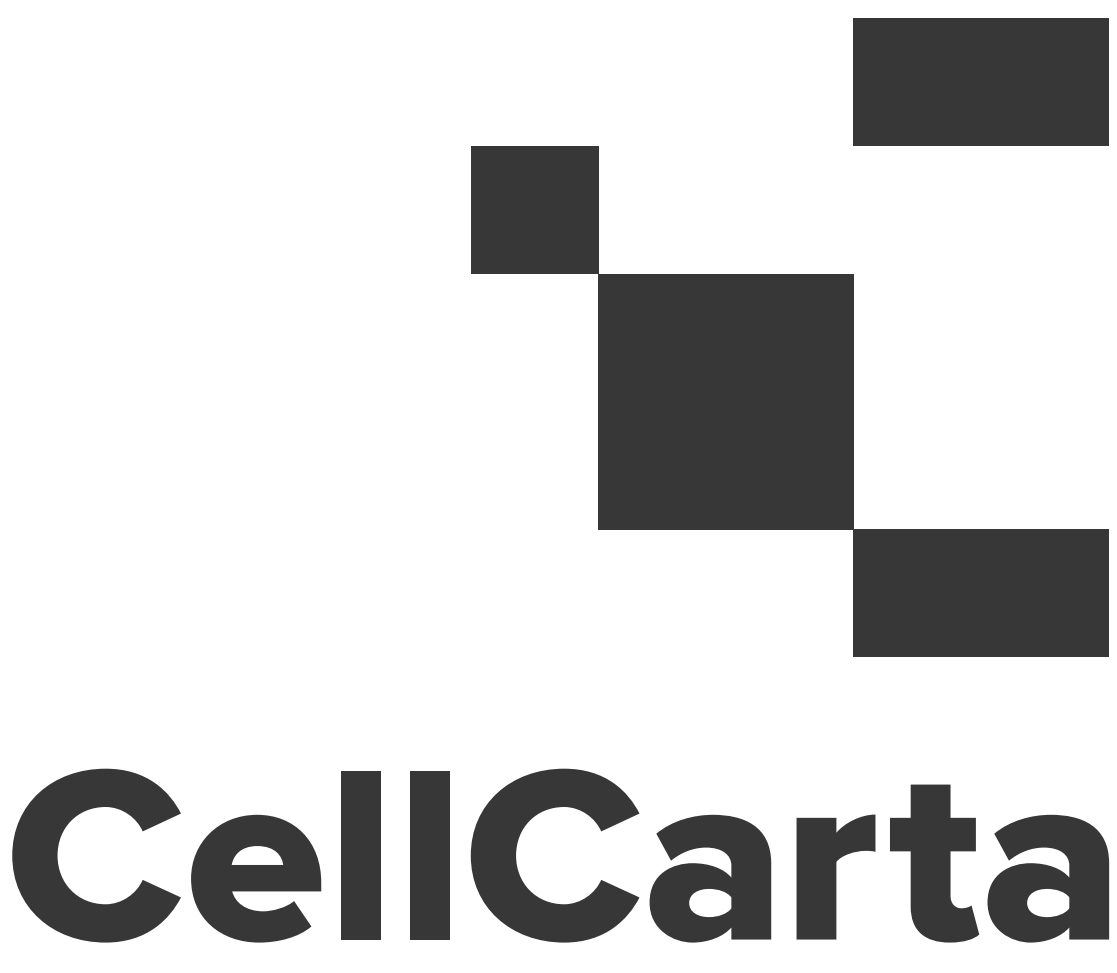


Lyophilized CompBeads for Flow Cytometer Alignment: When Does Instrument Drift Actually Matter?



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INTRODUCTION

In CellCarta laboratories, fluorescent reference materials, including lyophilised CompBeads (LyoBeads), are used for routine monitoring of flow cytometer performance. Deviations in LyoBeads mean fluorescence intensity (MFI) from predefined target values may prompt instrument re-alignment. However, the relationship between these deviations and cellular assay performance is not well defined.

Fluorescence intensity variations detected by LyoBeads may not necessarily changing interpretation of cellular populations. This distinction is particularly relevant to assays based on visual gating of discrete phenotypic populations, in which modest fluorescence shifts may not impact the frequency of events classified as positive.

This study assessed the relationship between controlled, channel-specific LyoBead MFI deviations and a CD4+ lymphocyte phenotyping readout. We evaluated whether positive and negative deviations had comparable effects, whether sensitivity differed between fluorochromes, and whether bead-level deviations predicted changes in cellular classification.

METHODS

Two identically configured five-laser, 18-parameter BD LSRFortessa instruments were monitored using custom lyophilised CompBeads (LyoBeads) during monthly alignment assessments over five months. LyoBeads were generated from BD CompBeads individually stained with CD4 antibodies representing all 18 fluorescence channels and lyophilised by the BD Biosciences Custom Technology Team. To model the channel-specific variation observed during routine monitoring, baseline LyoBead mean fluorescence intensity (MFI) values were established on one instrument. Target LyoBead MFI values corresponding to -20%, -15%, -10%, -5%, +5%, +10%, +15%, and +20% deviations from baseline were calculated, and PMT voltages were independently adjusted in each channel to achieve these conditions. Stabilised whole blood from healthy donor, collected in Cyto-Chex tubes, was stained separately with the corresponding CD4 antibody for each fluorochrome and acquired at baseline and under each PMT-adjustment condition. CD4+ lymphocytes were gated at baseline, and the same gates were applied unchanged to all corresponding conditions. Cellular MFI and the proportion of CD4+ lymphocytes classified as positive were compared with baseline. Classification error was calculated as the absolute difference in percentage-positive events relative to baseline, expressed as percentage points (pp). Results were evaluated with classification error of at least 2 pp considered a practical threshold for potential assay-relevant impact.

RESULTS

Monthly LyoBead monitoring conducted over five months demonstrated closely overlapping fluorescence distributions across all 18 channels on two identically configured BD LSRFortessa instruments, supporting stable overall inter-instrument alignment (Figure 1). Small channel-specific MFI variation was nevertheless detectable between instruments and across monthly assessments, raising the question of whether these minor fluctuations affect cellular assay interpretation.

To model this variation, PMTs were independently adjusted to generate controlled LyoBead MFI deviations from -20% to +20% (Figure 2). These adjustments produced measurable, channel-dependent changes in CD4+ lymphocyte MFI across all fluorochromes. However, the magnitude of cellular MFI change was not consistently proportional to the corresponding LyoBead MFI deviation (Table 1). Negative and positive deviations also produced asymmetric responses in some channels, including BUV395 and BUV737.

Across 144 fluorochrome-deviation conditions, mean classification error was 1.11 percentage points (pp), with 14 conditions (10%) exceeding the predefined 2-pp threshold (Table 2). PE and PE-CF594 were the most sensitive channels. The maximum classification error was 4.14 pp for PE at a +15% LyoBead MFI deviation. Conversely, BUV395 demonstrated the largest cellular MFI change (42% at -20%), yet classification error remained below 2 pp at every tested condition (Figure 3).

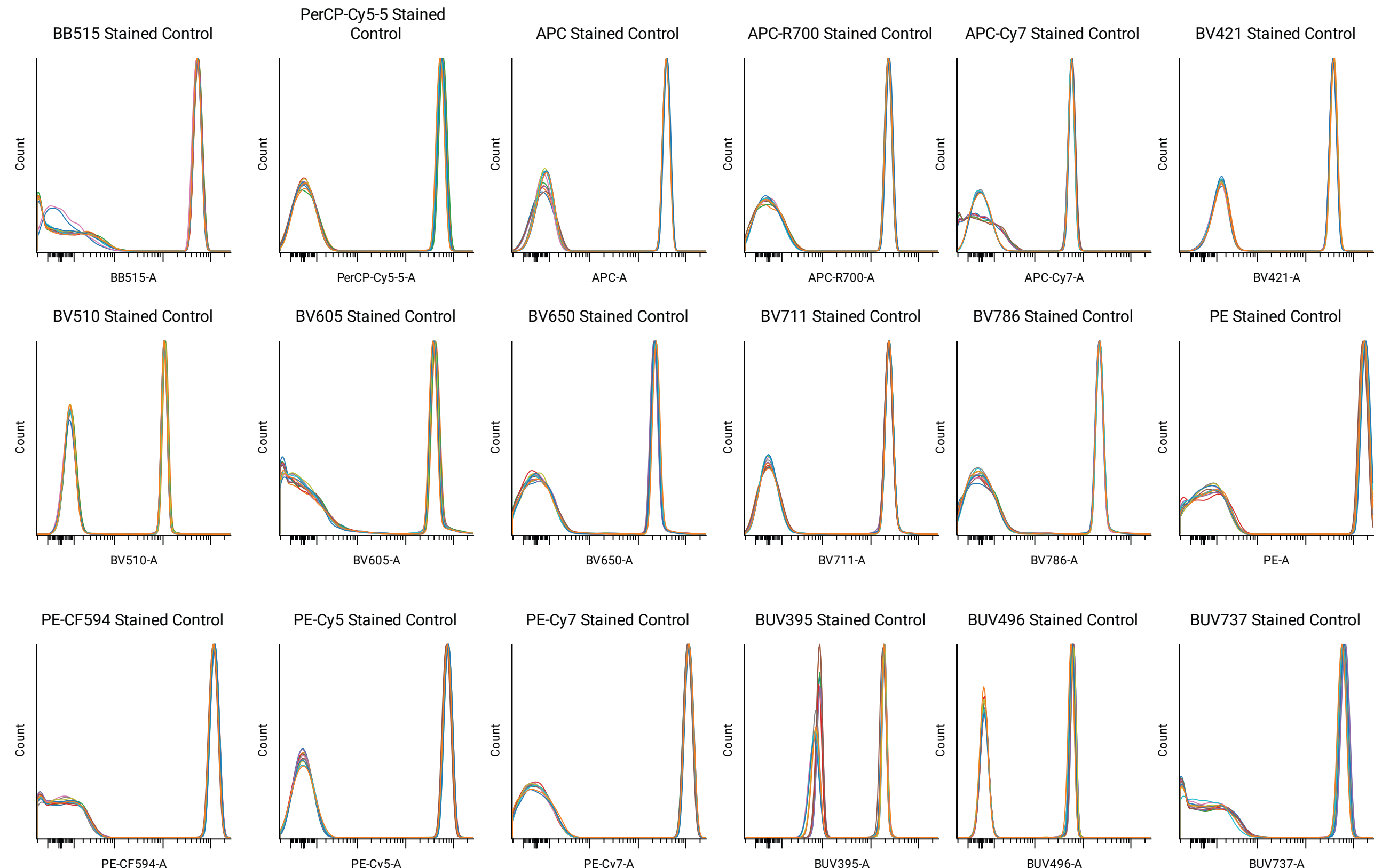


Figure 1. Routine LyoBead monitoring demonstrates stable inter-instrument alignment with small channel-specific variation. Overlaid LyoBead histograms from monthly alignment assessments performed over five months on two identically configured 5-laser, 18-parameter BD LSRFortessa instruments. Each panel represents one fluorescence channel. The closely overlapping distributions indicate stable overall alignment between instruments. Minor channel-specific fluorescence variation remains detectable across routine monitoring timepoints. This observed variation provided the rationale for evaluating the extent to which LyoBead MFI deviations affect cellular assay readouts.

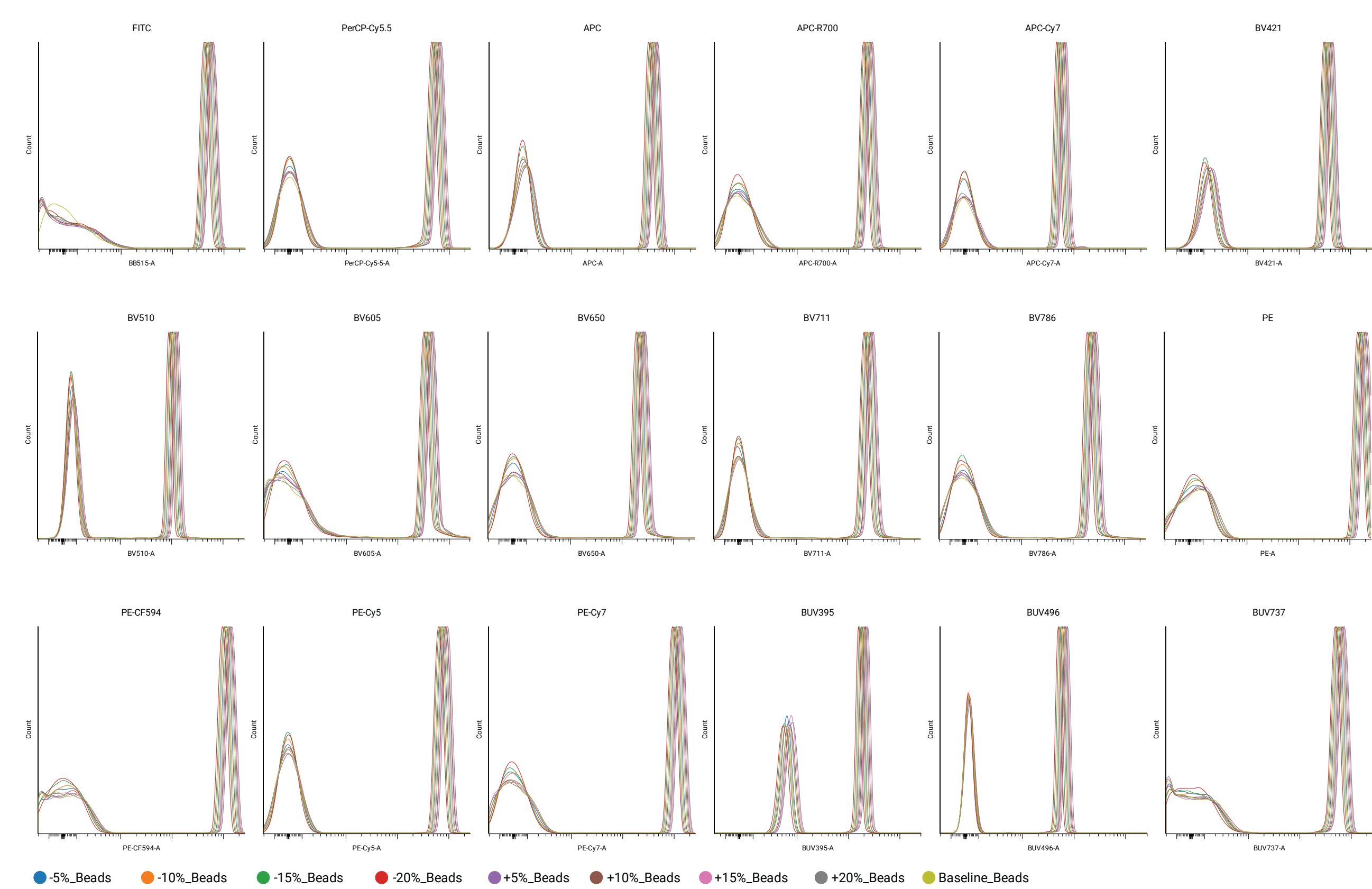


Figure 2. Channel-specific LyoBead fluorescence distributions following controlled PMT adjustment. Representative overlaid histograms of LyoBeads acquired on a five-laser, 18-parameter BD LSRFortessa after independent PMT adjustment in each fluorescence channel. PMT voltages were adjusted to generate LyoBead MFI deviations from baseline of -20%, -15%, -10%, -5%, +5%, +10%, +15%, and +20%. The overlaid distributions demonstrate the intended, channel-specific shifts in LyoBead MFI used to model controlled instrument-performance variation.

Table 1. Cellular MFI change across fluorochrome channels following controlled LyoBead MFI deviation. Percentage change in cellular MFI of CD4+ lymphocytes compared with baseline. PMT voltages were independently adjusted to achieve channel-specific LyoBead MFI deviations from -20% to +20%. Although cellular MFI changed across channels, the magnitude and direction of change were not consistently proportional to the corresponding bead-level deviation.

Fluoro-chromes	-20%	-15%	-10%	-5%	+5%	+10%	+15%	+20%
FITC	21%	15%	9%	4%	4%	10%	14%	20%
PerCP-Cy5.5	26%	17%	15%	8%	2%	7%	10%	16%
APC	25%	16%	13%	8%	3%	8%	12%	18%
APC-R700	14%	13%	11%	1%	3%	8%	11%	15%
APC-Cy7	27%	17%	17%	12%	3%	9%	12%	15%
BV421	34%	27%	15%	9%	4%	9%	15%	14%
BV510	21%	14%	8%	2%	6%	11%	16%	15%
BV605	29%	24%	13%	10%	2%	9%	11%	10%
BV650	27%	21%	12%	7%	4%	10%	14%	10%
BV711	32%	26%	11%	8%	5%	8%	12%	14%
BV786	31%	17%	4%	0%	10%	10%	11%	21%
PE	32%	13%	8%	5%	7%	9%	13%	18%
PE-CF594	23%	14%	8%	4%	6%	11%	14%	20%
PE-Cy5	26%	15%	7%	4%	3%	10%	12%	19%
PE-Cy7	21%	15%	9%	4%	4%	10%	14%	20%
BUV395	42%	29%	21%	15%	1%	4%	8%	6%
BUV496	27%	16%	7%	3%	4%	9%	14%	16%
BUV737	39%	26%	11%	11%	0%	7%	11%	16%

Table 2. Classification error across fluorochrome channels following controlled LyoBead MFI deviation. Absolute change in the percentage of CD4+ population classified as positive relative to baseline, expressed in percentage points (pp). PMT voltages were independently adjusted to produce channel-specific LyoBead MFI deviations from -20% to +20%. Red cells indicate classification error of at least 2 pp, the predefined threshold for potential assay-relevant impact.

Fluoro-chromes	-20%	-15%	-10%	-5%	+5%	+10%	+15%	+20%
FITC	1.37	1.31	2.28	1.27	0.55	0.14	0.08	0.14
PerCP-Cy5.5	0.51	0.15	0.5	0.55	0.2	0.19	0.71	0.9
APC	0.83	0.66	0.85	1.49	1.32	0.55	0.98	1.47
APC-R700	1.95	1.04	0.56	1.83	0.39	0.14	0.11	0.98
APC-Cy7	1.93	0.05	1.02	0.9	0.67	0.67	0.23	0.44
BV421	0.16	0.76	1.08	1.12	0.65	0.18	0.81	0.09
BV510	1.43	1.98	1.15	0.61	0.57	1.35	2.8	0.47
BV605	2.35	1.33	2.01	0.17	0.48	1.45	0.28	1.95
BV650	0.07	2.96	0.39	0.68	0.53	0.69	0.28	1.01
BV711	0.22	1.16	2.1	1.75	1.64	1.18	2.76	2.26
BV786	2.13	1.34	0.33	1.26	1.01	0.06	0.02	0.5
PE	0.17	0.95	3.39	3.05	2.91	3.01	4.14	3.04
PE-CF594	2.71	0.95	3.41	1.35	1.01	1.52	1.48	2.16
PE-Cy5	0.69	0.56	0.94	1.52	1.46	1.63	2	2.09
PE-Cy7	3.19	0.11	2.34	1.29	0.06	1.3	1.43	0.47
BUV395	1.99	1.09	0.24	0.12	0.01	0.36	0.6	0.2
BUV496	0.86	0.55	1.57	0.42	0.68	0.72	0.97	0.62
BUV737	1.47	2.61	0.49	1.61	1.2	0.32	0.74	0.22

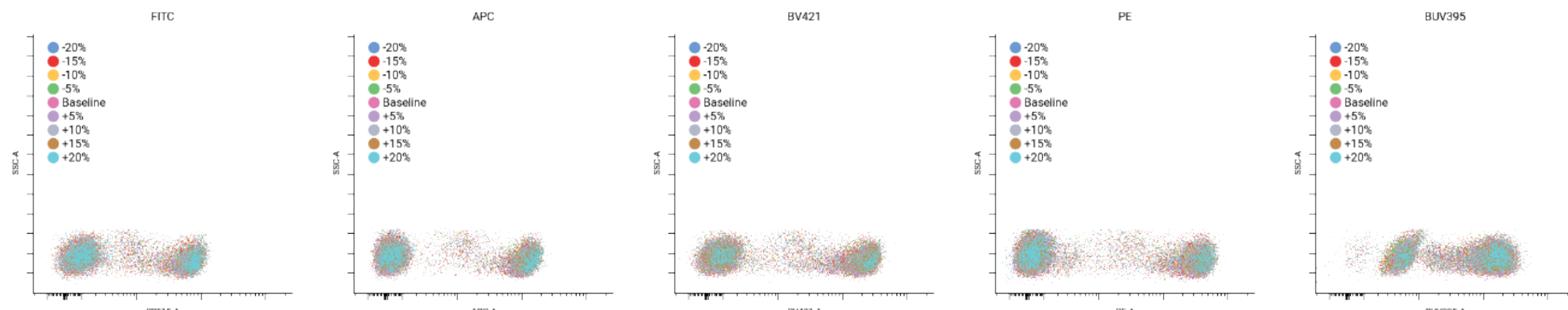


Figure 3. CD4+ lymphocyte fluorescence distributions remain stable following controlled, channel-specific PMT adjustment. Overlaid CD4+ lymphocyte events from stabilised healthy-donor whole blood acquired at baseline and after PMT adjustments designed to produce LyoBead MFI deviations of -20%, -15%, -10%, -5%, +5%, +10%, +15%, and +20%. Representative primary fluorochrome channels (FITC, APC, BV421, PE, and BUV395) are displayed. Despite controlled LyoBead shifts across the tested range, cellular fluorescence distributions showed minimal visual displacement, supporting limited impact on standard visually gated phenotyping readouts.

CONCLUSION

The LyoBead-based alignment strategy is a robust approach for inter-instrument standardisation and ongoing performance monitoring. Small, channel-specific LyoBead MFI fluctuations were nevertheless detectable during routine assessments. In the controlled single-donor experiment, these modest deviations did not consistently translate into impactful changes in the visually gated CD4+ lymphocyte readout. The relationship between bead-level MFI deviation, cellular MFI change, and classification error was channel-dependent and non-linear. These data therefore support using LyoBeads not only to detect variation, but also to guide proportionate, channel- and assay-specific decisions about when intervention or re-alignment is warranted. MESF or QSC beads remain appropriate where absolute fluorescence quantification is required.

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