Development of a human T cell backbone flow cytometry panel enabling flexibility in reagent choice while minimizing panel design challenges

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Abstract

Expansion of existing flow cytometry panels with new markers of interest can result in suboptimal resolution and, in some cases, the need to design a new panel, increasing cost and increasing time to insight. To minimize these challenges and to provide increased flexibility, we have developed a human T cell backbone panel strategically designed to be complemented with 4–5 drop-in fluorochromes and markers of choice, depending on instrument configuration, with minimal panel design effort. The backbone panel contains five T cell markers (CD3, CD4, CD8, CD45RA, CCR7) conventionally used to identify different maturational states of CD4+ and CD8+ T cells (naïve, central memory, effector memory, effector memory RA). We will show how this backbone panel meets four fundamental requirements: i) clear resolution of major T cell subsets; ii) the fluorochromes used in the backbone panel have minimal resolution impact on the detectors allocated for drop-ins; iii) the fluorochromes assigned to drop-ins have minimal impact on the resolution of the backbone panel; iv) the fluorochromes assigned to drop-ins do not impact each other. We will provide examples of different drop-in combinations for the study of T cell and regulatory T cell biology. Performance of the backbone panel on different instruments will be shown to demonstrate assay consistency across platforms. The compatibility of the backbone panel with intracellular stain and transcription factor analysis will be further demonstrated.

Backbone

Marker	Fluorochrome
CD3	BV510
CD4	BV786
CD8	R718
CD45RA	PE-Cy7
CD197	BV711

3–4 laser flow cytometer

Drop-in fluorochromes
BV421/V450
FITC/BB515/AF488/GFP
PE/RY586
APC/AF647

5 laser flow cytometer

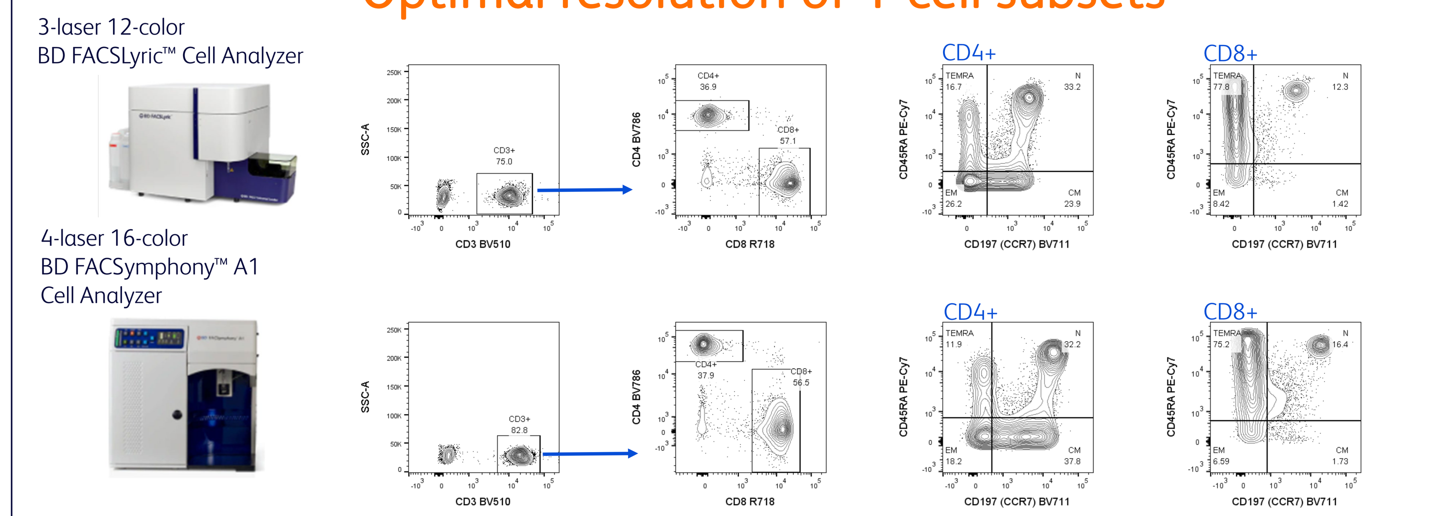
Drop-in fluorochromes
BV421/V450/
FITC/BB515/AF488/GFP
PE/RY586
APC/AF647
BUV395

Examples of fluorochromes that can be added as drop-ins using equivalent instrument and filter configurations

BUV: BD Horizon Brilliant™ Ultraviolet; BB: BD Horizon Brilliant™ Blue; R: BD Horizon™ Red; RY: BD Horizon RealYellow™; V: BD Horizon™ Violet; AF= Alexa Fluor™

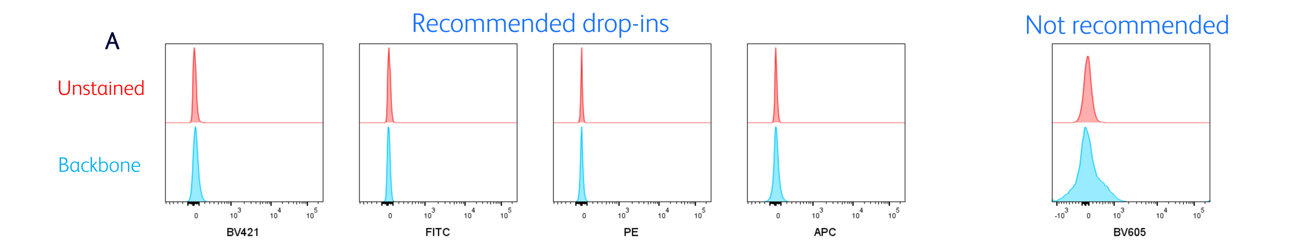
Strategically designed to enable marker addition with minimal panel design and risk of resolution loss

Optimal resolution of T cell subsets



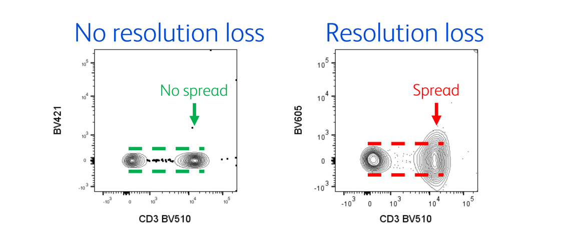
Lysed whole blood from a healthy donor was stained with the backbone panel and acquired on a BD FACSLyric™ and a BD FACSymphony™ A1 Cell Analyzer. Clear resolution of all the T cell subsets of interest was observed irrespective of the instrument used.

The backbone does not impact resolution of the drop-ins



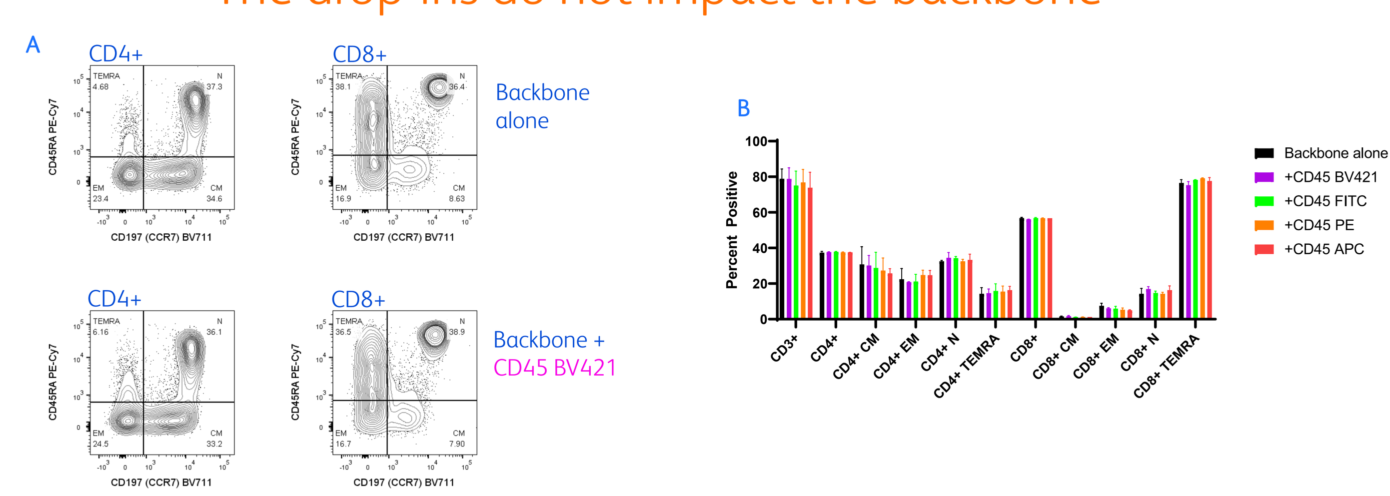
B

Total Spread Matrix		Into recommended drop-ins				
		BV421	FITC	PE	APC	BV605
From Backbone	BV510	13	8	0	0	290
	BV711	12	0	0	33	15
	BV786	32	0	7	14	0
	PE-Cy7	18	8	25	18	10
	R718	0	0	0	69	14



A) Visualization of spread in open detectors on unstained sample and sample stained with the backbone panel. B) Representative Total Spread Matrix. The backbone panel introduces negligible spread into the recommended drop-in BV421, FITC, PE and APC channels as compared to a drop-in like BV605, which is not recommended. Data generated on a 4-laser BD FACSymphony™ A1 Cell Analyzer.

The drop-ins do not impact the backbone



A) Resolution of representative T cell population before and after addition of CD45 conjugated to BV421. B) Quantification of T cell subsets before and after individual addition of CD45 conjugated to the recommended drop-ins. No impact to resolution or quantitation was observed upon addition of there commended drop-ins, despite CD45 being highly expressed and co-expressed by all T cells. Data generated on a BD FACSymphony™ A5 SE Cell Analyzer.

The recommended drop-ins do not impact each other

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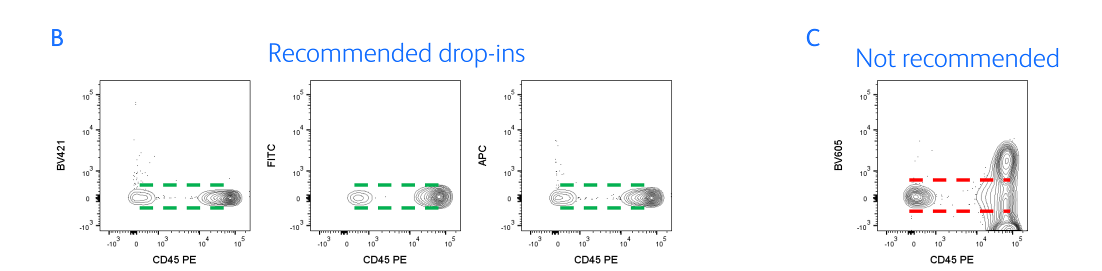
Total Spread Matrix		Into recommended drop-ins				
		BV421	FITC	PE	APC	BV605
From Drop-in	BV421	0	0	0	0	52
	FITC	0	0	0	0	54
	PE	0	0	0	0	1405
	APC	0	0	0	0	15



A) Total Spread Matrix. B) Representative spread visualization. The recommended drop-ins BV421, FITC, PE and APC are spatially separated and do not impact each other's resolution. C) BV605 is shown here as a drop-in that would be significantly impacted by PE. Data generated on a 4-laser BD FACSymphony™ A1 Cell Analyzer.

Dissection of CD4+ Th cell subsets

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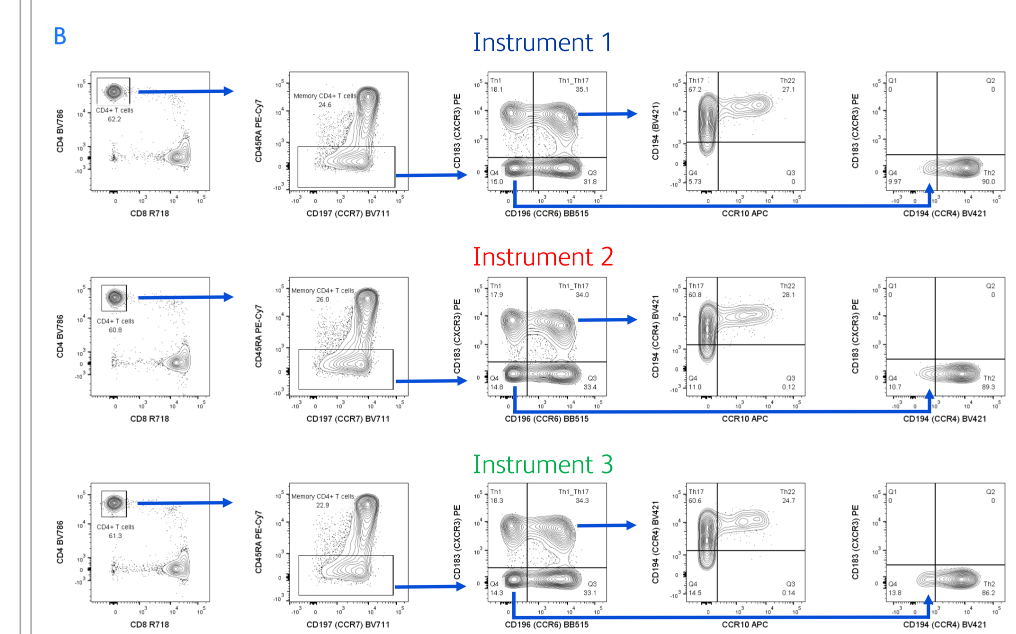
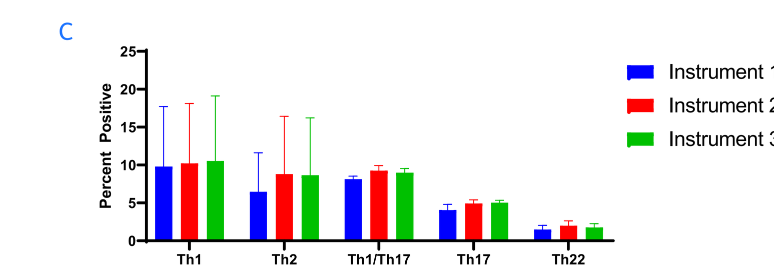
Backbone	Fluorochrome
CD3	BV510
CD4	BV786
CD8	R718
CD45RA	PE-Cy7
CD197	BV711

Drop-ins	Fluorochrome
CD194 (CCR4)	BV421
CD196 (CCR6)	BB515
CD183 (CXCR3)	PE
CCR10	APC
Live/dead	7-AAD





A) The backbone panel was complemented with a viability dye and four markers conventionally used for the identification of CD4+ Th1/Th2/Th17/Th22 subsets. B) PBMCs from N=2 healthy donors were acquired on three 3-laser 12-color BD FACSLyric™ Cell Analyzer. Clear resolution of all the subsets of interest was observed on each instrument. C) Subset relative quantitation revealed repeatable panel performance across three instruments

Methods

Sample handling and staining

- Normal donor samples included lysed whole blood, freshly prepared PBMCs, and cultured and activated PBMCs.
- BD Horizon™ Brilliant Stain Buffer Plus is used at 10 µL/sample and included in all compensation controls, single-color controls and panels.
- Backbone panel reagents were used at 5 µL/test.
- Cells are pre-stained with CD197 (CCR7) BV711 reagent at 37 °C for 10 minutes. The same protocol was followed for the stain of any chemokine receptor in the CD4+ Th subset panel.
- Cells are stained with the full cocktail for 30 minutes.
- The BD Pharmingen™ Transcription Factor Buffer Set was used for the intracellular staining of FoxP3, according to manufacturer's instructions. Briefly, cells were stained with surface markers prior to fixation and permeabilization then intracellular markers are added for an additional 30-minute incubation at 4 °C.
- BD Pharmingen™ 7-AAD was added 10 minutes prior to acquisition, whereas BD Horizon™ Fixable Viability Stain (FVS) 620 was added during panel incubation in protein-free PBS.
- T cells were activated with Dynabeads™ Human T-Activator CD3/CD28 for 2 days.

Instrument set up and acquisition

- BD FACSuite™ CS&T Research Beads were used to check QC of instrument performance
- Instruments were set up with optimal voltage application settings or lyse/wash settings for BD FACSLyric™ Cell Analyzer settings. Lyse/wash settings were adjusted to keep PE-Cy7 signal on scale
- Compensation was calculated with single-color control cells.

Data Analysis

- FlowJo™ 10.8.1 Software was used for data analysis and Total Spread Matrix (TSM) table generation.
- Gates were drawn based on FMO controls.
- GraphPad Prism was used for graph generation.

Identification of FoxP3+ Treg subsets

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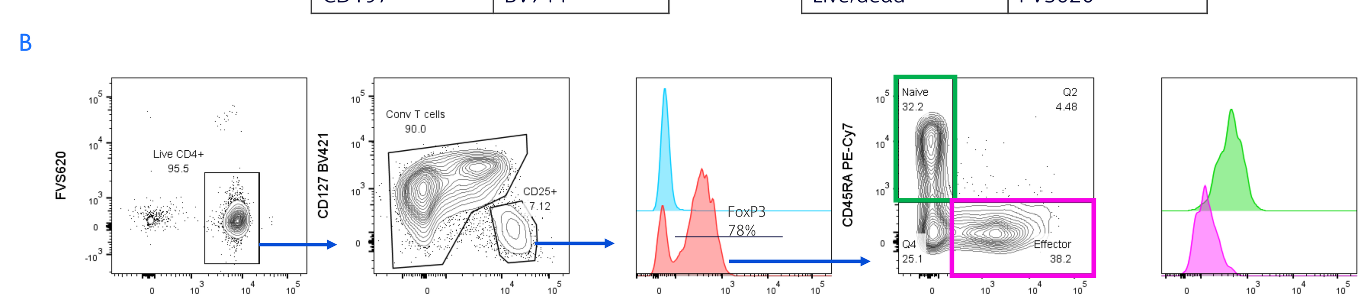
Backbone	Fluorochrome
CD3	BV510
CD4	BV786
CD8	R718
CD45RA	PE-Cy7
CD197	BV711

Drop-ins	Fluorochrome
CD127	BV421
FoxP3	Alexa Fluor™ 488
CD25	RY586
HLA-DR	APC
Live/dead	FVS620



A) The backbone panel was complemented with surface and intracellular markers conventionally used to identify human Treg subsets. B) Representative analysis of fixed and permeabilized PMBCs from N=3 healthy donors. Clear resolution of all the subsets of interest was observed. Data generated on a 4-laser BD FACSymphony™ A1 Flow Cytometer.

Upregulation of inhibitory receptors

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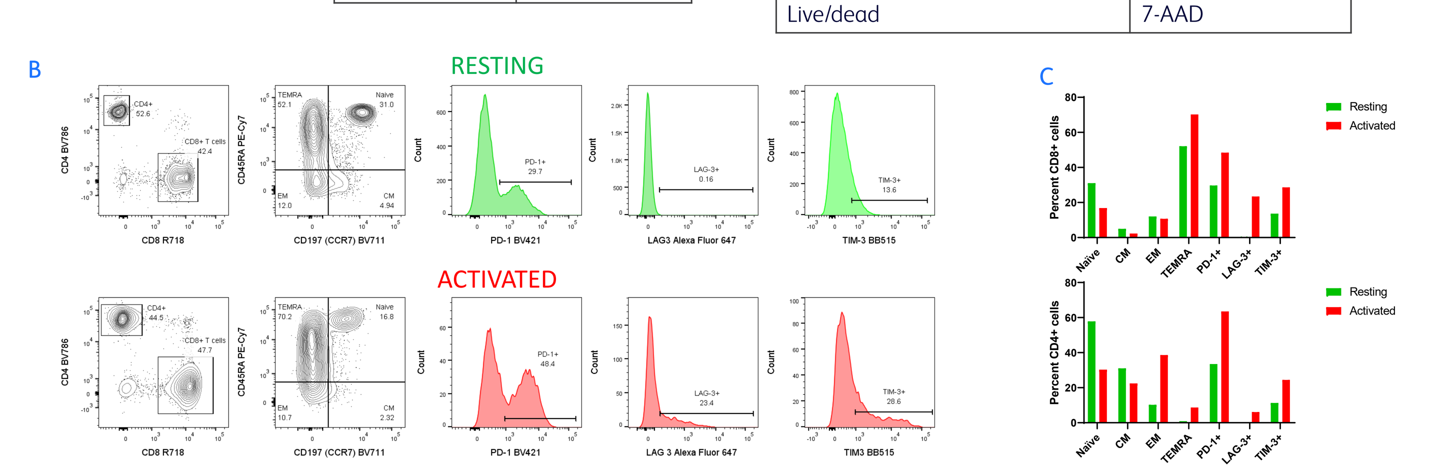
Backbone	Fluorochrome
CD3	BV510
CD4	BV786
CD8	R718
CD45RA	PE-Cy7
CD197	BV711

Drop-ins	Fluorochrome
PD-1	BV421
TIM-3	BB515
Open for potential CAR-T or ag-specific T cells detection	Streptavidin-PE (not used in this panel)
LAG-3	Alexa Fluor™ 647
Live/dead	7-AAD



A) The backbone panel was complemented with surface markers conventionally used to identify activated or exhausted T cells . B) Clear resolution of all the subsets of interest was observed on PMBCs from healthy donor rested or activated for 48 hr in culture . C) Expected differentiation and increase in inhibitory receptor-expressing CD4+ and CD8+ T cells was measured. Data generated on a 3-laser 12-color BD FACSLyric™ Cell Analyzer.

Comprehensive T cell differentiation analysis

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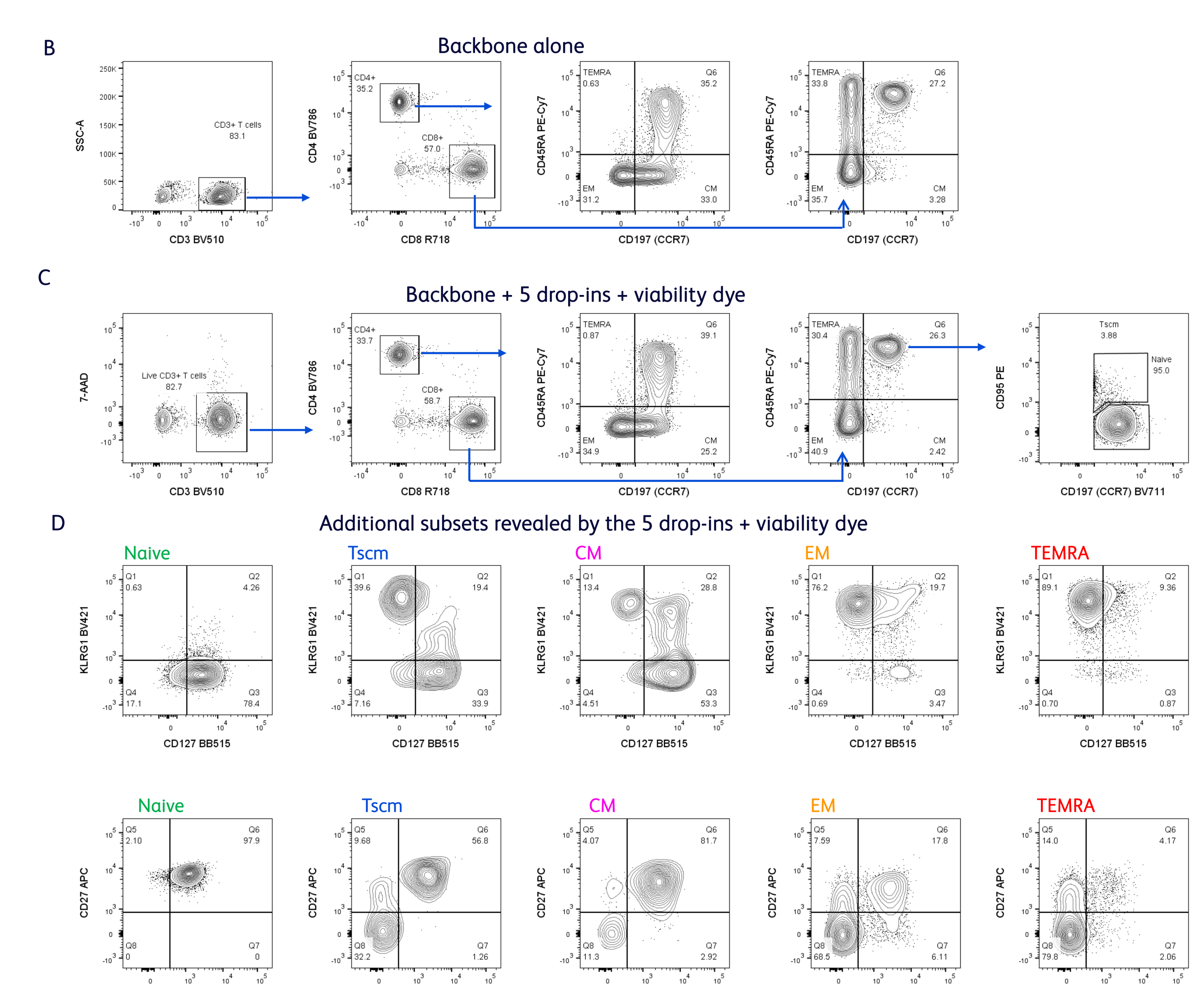
Backbone	Fluorochrome
CD3	BV510
CD4	BV786
CD8	R718
CD45RA	PE-Cy7
CD197	BV711

Drop-ins	Fluorochrome
KLRG1	BV421
CD127	BB515
CD95	PE
CD27	APC
CD28	BUV395
Live/dead	7-AAD



A) The backbone panel was complemented with surface markers to comprehensively identify different subsets of differentiated T cells. The BUV395 fluorochrome was included as an additional clean drop-in. B) Representative resolution of PBMCs from N=2 healthy donors stained with the backbone alone. C) Addition of six drop-ins does not alter resolution of the backbone markers, while allowing for further identification of Tscm. D) Drop-in addition enabled identification of more T cell subsets. Data generated on a BD FACSymphony ™ A5 Cell Analyzer. Similar results were obtained on a 5-laser BD LSRFortessa™ X-20 Cell Analyzer equipped with a 355-nm UV laser.

Conclusions

- The human T cell backbone panel meets the criteria for a truly flexible and easy-to-expand flow cytometry panel:
  - ✓ Good resolution of major human T cell subsets
  - ✓ The backbone does not impact resolution of the recommended drop-in fluorochromes
  - ✓ The recommended drop-in fluorochromes do not impact the backbone resolution
  - ✓ The recommended drop-in fluorochromes do not impact each other's resolution
- The human T cell backbone panel is compatible with:
  - ✓ Several human sample types: lysed whole blood and fresh, cultured and/or activated PBMCs
  - ✓ Surface and intracellular stain protocols
  - ✓ DNA-binding dyes and Fixable Viability Stain for dead cell exclusion
  - ✓ Use with fluorescent protein GFP and streptavidin-PE, commonly used for detection of antigen-specific T cells or chimeric antigen receptors (CARs)
- The human T cell backbone panel was tested on different flow cytometers with different configurations (3, 4 and 5 lasers) and demonstrated consistent intra- and inter-instrument performance
- Up to five markers plus a viability dye can be added with minimal panel design effort without impact to population resolution and quantification, depending on instrument configuration
  - ✓ No redesign of the core backbone required in order to add new markers
  - ✓ No concerns about spillover, spread, compensation and co-expression when using the recommended drop-ins
  - ✓ Fluorochromes with appropriate brightness still need to be paired with markers based on antigen expression (bright-low, dim-high)
  - ✓ Fluorochrome with different brightness can be chosen for a given detector (e.g., dim FITC or bright BD Horizon Brilliant™ Blue 515 (BB515), dim V450 or bright BV421) thus providing further flexibility in panel design
- The human T cell backbone panel is strategically and prospectively designed to simplify the transition from five up to eleven color flow cytometry panels, leading to increased efficiency and biological insight
  - ✓ Double the number of markers analyzed in a single tube
  - ✓ Dive deeper into T cell biology through more comprehensive immunophenotypic and functional analyses