Flow Cytometry Unveiled:

Expert Perspectives on Multi-Parameter Cell Analysis

Expert Insights

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Contents

Introduction

Flow cytometry is a transformative analytical technique used to measure the properties of cells or particles as they flow in a fluid stream through a beam of light and relies on the use of fluorescent markers, antibodies tagged with fluorophores, that specifically bind to cell-associated molecules [1]. Over the last decade, an increased understanding of the complexities of biological systems has driven technological innovations and an appreciation for high-parameter cytometry [2].

Flow cytometry facilitates multiparametric analysis of single-cell phenotypes, essential for dissecting complex immunological landscapes, cell sorting, viability assays, and cell cycle profiling. It's instrumental in characterizing and elucidating the functional dynamics of heterogeneous immune cell populations [3]. One of the key strengths of flow cytometry is its high-throughput capability, enabling the analysis of thousands of cells per second. This rapid data collection is essential for studying complex biological systems and diseases, such as cancer, where it can provide insights into the heterogeneity of cell populations.

Advances in flow cytometry technology have led to the development of high-parameter flow cytometry, which allows for the simultaneous analysis of many different markers on a single cell. This high-dimensional data is powerful for dissecting the intricate cellular hierarchies and functions within biological samples. Flow cytometry continues to evolve with improvements in instrumentation, reagent development, and data analysis techniques. These advancements are propelling the field forward, offering deeper insights into cellular functions and enabling researchers to uncover new aspects of cellular biology and disease pathology. As a cornerstone of cell analysis, flow cytometry research remains a dynamic and expanding field, integral to both basic biological research and clinical applications.

This Expert Insights eBook begins with a study by Konecny *et al*. [4] introducing OMIP-102, a cutting-edge 50-color spectral flow cytometry panel designed for comprehensive phenotyping of the human immune system, with a focus on T cells and dendritic cells. This panel allows for detailed analysis of immune cells from limited samples, facilitating in-depth studies into immune function and interactions. Optimized for compatibility with multiple platforms, OMIP-102 enables high-throughput, multi-parametric data collection and supports the integration of advanced data analysis techniques, marking a significant progression in immunophenotyping capabilities.

The second paper by Whyte *et al*. [2] explores the benefits of overnight antibody staining in flow cytometry. Extended incubation enhances the detection of antigens, improves specificity, and reduces background noise, leading to more consistent and reproducible data. It also allows for the use of lower antibody concentrations, which significantly reduces the costs associated with high-parameter panel experiments. The article discusses practical considerations for optimizing staining conditions, including antibody titration and fixative choice, while also addressing potential impacts on cell viability and advocates for tailored overnight staining protocols to improve the accuracy and cost-efficiency of high-parameter flow cytometry.

Overall, flow cytometry, leveraging fluorescently conjugated antibodies, offers high-resolution, quantitative analysis of cellular phenotypes and functions, particularly within immunological research. Enhanced by technological innovations, it facilitates the interrogation of intricate immune cell subsets, advancing our comprehension of immunodynamics and informing clinical diagnostics.

Through the methods and applications presented in this Experts Insights eBook, we hope to educate researchers on new technologies and techniques for flow cytometry. To gain a deeper understanding of available options for improving your research, we encourage you to visit Bio-Rad Laboratories.

Andrew Dickinson, Ph.D.
Content Strategist at Wiley

References

[1] Robinson, J.P., *et al*. (2023). Flow cytometry: the next revolution. *Cells*. DOI:10.3390/cells12141875

[2] Whyte, C.E., *et al*. (2022). Do more with less: improving high parameter cytometry through overnight staining. *Current Protocols*. DOI:10.1002/cpz1.589

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[4] Konecny, A.J., *et al*. (2024). OMIP-102: 50-color phenotyping of the human immune system with in-depth assessment of T cells and dendritic cells. *Cytometry Part A*. DOI:10.1002/cyto.a.24841

OMIP-102: 50-color Phenotyping of the Human Immune System with In-depth Assessment of T cells and Dendritic Cells

Adapted from Konecny *et al.*, 2024

This article describes an optimized 50-color spectral flow cytometry panel for in-depth analysis of the immune system in human blood and tissues. The panel was established and tested using peripheral blood mononuclear cells (PBMCs) but also included CD45 to enable analysis of human tissue samples. Lineage markers for all major immune cells were included, as were phenotyping markers focused on the activation and differentiation status of the T cell and dendritic cell (DC) compartment. Simultaneous measurement of this large number of proteins allows a comprehensive study of the immune status in human samples with a limited number of cells. Notably, the panel was designed to be compatible with cell sorting for further downstream applications. Furthermore, to facilitate the implementation of such a panel across different cohorts and samples, a 45-color version was established for use with different spectral cytometry platforms. Finally, new metrics were developed to systematically identify the optimal combination of 50 fluorochromes and evaluate fluorochrome-specific resolution in the context of a 50-color unmixing matrix.

Background

The immune system is the body's defense system against pathogens and is also essential for maintaining steady-state homeostasis in tissues and preventing the development of malignant tumors. Information about the composition and activation status of immune cells can be used to study their differentiation and function. To facilitate data interpretation, an immune cell population is ideally analyzed in the context of other immune cell populations. Thus, to comprehensively study the state of the immune system, it is crucial to capture as much information from as many different cell types as feasible. This is particularly true for assessing immune cell function *in situ*, e.g., in human tissue samples. However, these human tissues are often limited in size and availability, which hampers parallel analysis with multiple panels or applications. The development of an analysis approach that can provide broad and in-depth phenotyping, paired with the ability to preserve cell populations of interest for downstream applications (e.g., single-cell RNA sequencing) is hence of importance.

The interactions between professional antigen-presenting cells (APCs) and different T cell subsets are of particular interest in the context of studying anti-tumor immune responses. Dendritic cells (DCs) are highly specialized APCs and are generally divided into cross-presenting populations that have distinct functions for steering an adaptive immune response.

The panel presented in Figure 1 was designed to comprehensively capture the differentiation and activation status of human T cells and APCs (Table 1), while also measuring B cell, natural killer (NK) cell, and innate lymphoid cell (ILC) phenotypes (list of markers depicted in Fig. 1B and Table 2). Optimization was done on human cryopreserved PBMCs, but the panel includes CD45 as a pan-hematopoietic marker and thus could be used on human tissue-derived leukocytes.

Table 1

Purpose	50-color phenotyping of antigen-presenting cells and T cells
Species	Human
Cell types	PBMCs and non-lymphoid human tissue
Cross-references	OMIP-069, OMIP-044, OMIP-050, OMIP-058, and others

Summary table for application of OMIP 102

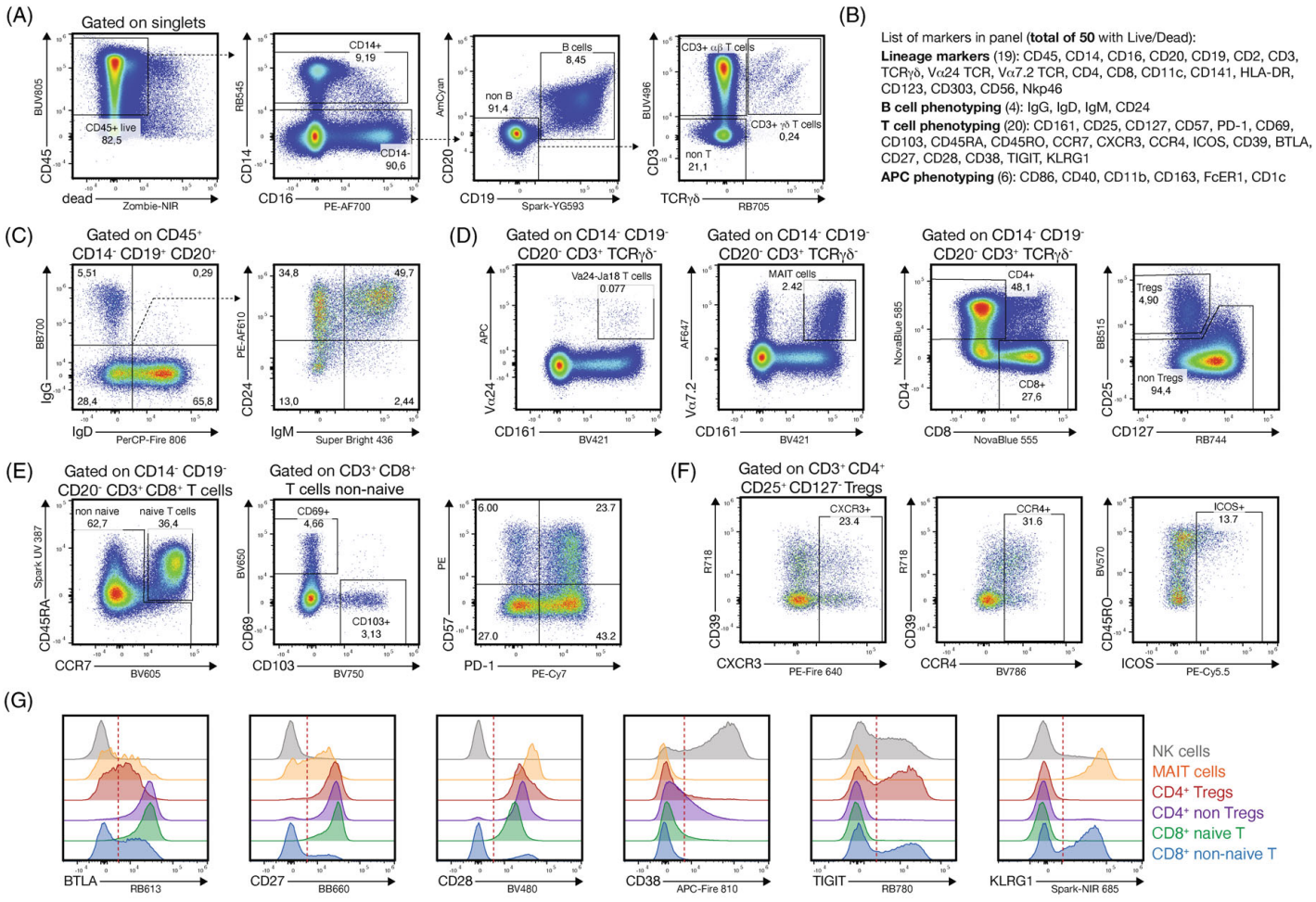

Figure 1. Overview gating of the 50-color panel on cryopreserved PBMCs. PBMCs were obtained from commercial vendors and stained as described in the online materials of this manuscript. The optical configuration of the instrument is also described in online material. Pre-gating of plots is annotated in the figure or indicated by dotted black arrows. For some plots, different donors are shown for clarity. The gating strategy was devised such that the staining pattern for every marker in the panel can be shown at least once on a single A4 page. The raw data has been deposited on the Flow Repository with the identifier FR-FCM-Z73V. Abbreviations: AF, AlexaFluor; APC, allophycocyanin; BB, brilliant blue; BUV, brilliant ultraviolet; BV, brilliant violet; NIR, near-infrared; PE, phycoerythrin; Qdot, quantum dot; RB, RealBlue.

Figure 1 panels: (A) Gating strategy for CD45+ live cells, monocytes, B cells, and γδ and αβ T cells. **(B)** Overview of the 50 target molecules analyzed with this experiment. Some of the markers can be used for phenotyping multiple immune cell lineages. **(C)** Representative plots for the main phenotyping markers in the B cell lineage (IgG, IgD, IgM, and CD24). **(D)** Gating strategy to delineate invariant NKT cells, MAIT cells, CD4+ and CD8+ T cells, as well as CD4+ regulatory T cells (Tregs). **(E)** Representative plots for CD69, CD103, CD57, and PD-1 expression on non-naïve CD8+ cytotoxic T cells. **(F)** Expression pattern for CD39, CXCR3, CCR4, CD45RO, and ICOS on the CD4+ Treg population. **(G)** Histogram overlays for the expression pattern of BTLA, CD27, CD28, CD38, TIGIT, and KLRG1 on NK cells (gray), MAIT cells (orange), CD4+ Tregs (red), CD4+ non-Tregs (purple), CD8+ naïve T cells (green), and CD8+ non-naïve T cells (blue). Dotted red lines indicate positivity cut-offs.

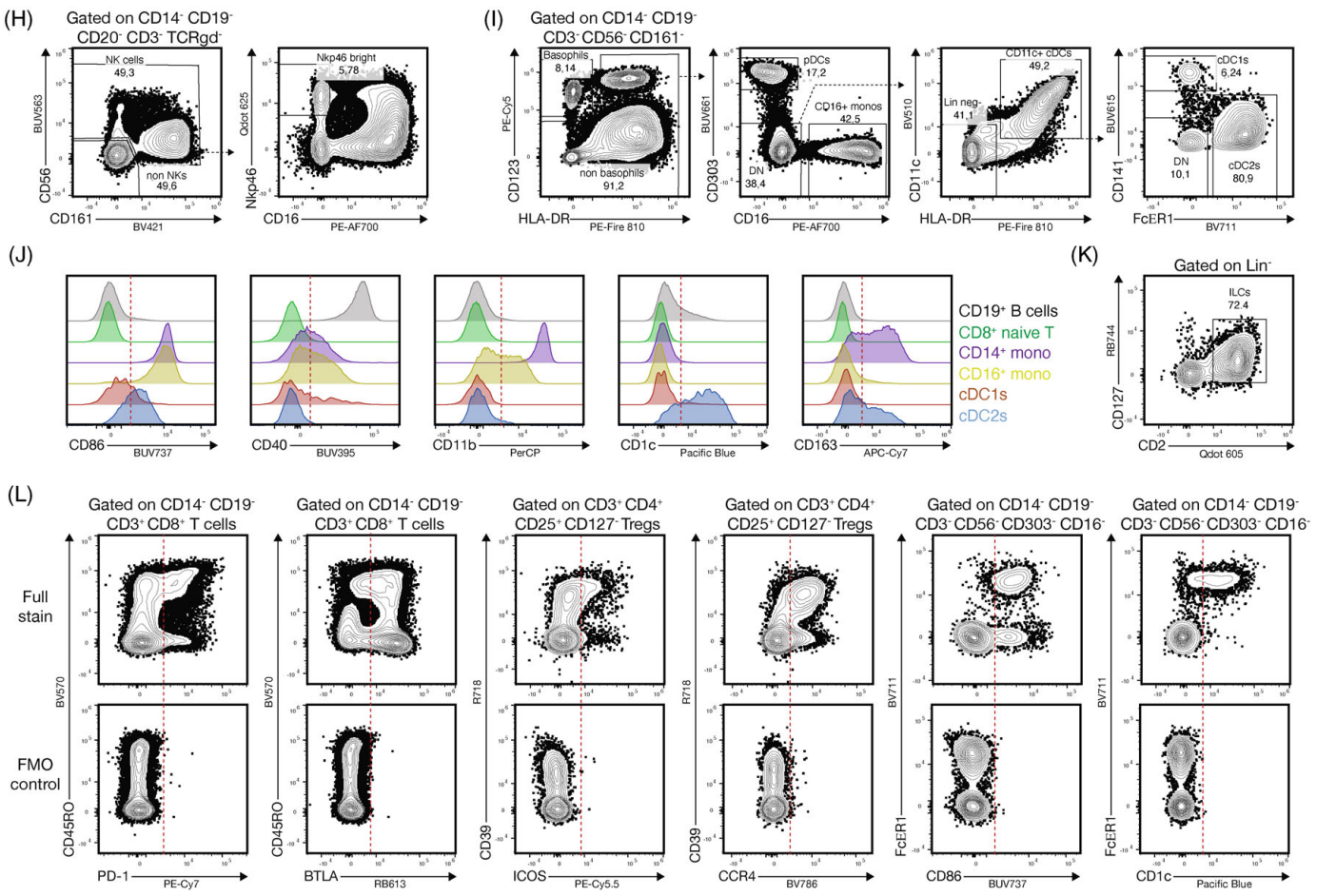

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Figure 1 panels: (H) Gating strategy for NK cells and NK cell subsets based on CD56, CD161, CD16, and NKp46. **(I)** Gating strategy for Basophils (CD123+ FcER1+ HLADR_), plasmacytoid DCs (CD303+ HLA-DR+), pan conventional DCs (CD11c+ HLA-DR+), and the cDC1 (CD141+), and cDC2 (FcER1+) subsets. **(J)** Histogram overlays for the expression pattern of CD86, CD40, CD11b, CD1c, and CD163 on B cells (gray), CD8+ naïve T cells (green, negative control), CD14+ monocytes (purple), CD16+ monocytes (yellow), CD141+ cDC1s (red), and FcER1+ cDC2s (blue). Dotted red lines indicate positivity cut-offs. **(K)** Gating strategy for Lin_ CD2+ CD127+ innate lymphoid cells (ILCs). **(L)** A selection of fluorescence-minus-one (FMO) controls for the indicated markers: PD-1 and BTLA on CD8+ T cells, ICOS and CCR4 on Tregs, and CD86 and CD1c on CD11c+HLA-DR+ cDCs as indicated. Dotted red lines indicate positivity cut-offs. Note that there is no or only negligible spreading error present.

Table 2 Reagents used for OMIP-102

Specificity	Alternative Name	Clone	Fluorochrome	Purpose
CD45	Protein tyrosine phosphatase, receptor type, C, PTPRC	HI30	BUV805	Pan-Hematopoietic marker
CD3	Part of the TCR complex	UCHT1	BUV496	Lineage marker for pan T cells
CD4	NA	SK3	NovaFluor Blue 585	Lineage marker for CD4+ T cells
CD8	NA	OKT8	NovaFluor Blue 555	Lineage marker for CD8+ T cells
TCRgd	Gamma delta T cell receptor	B1	RB705	Lineage marker for gd T cells
Va24-JA18	TCR chains	6B11	APC	Marker for invariant NKT cells
Va7.2	V alpha 7.2 TCR chain	3C10	Alexa Fluor 647	Marker for MAIT cells
CD19	NA	SJ25C1	AmCyan	Lineage marker of B cells
CD20	NA	2H7	Spark YG 593	Lineage marker of B cells
CD14	Lipopolysaccharide receptor	M5E2	RB545	Lineage marker of monocytes
CD16	Fc gamma receptor, FcγRIII	3G8	PE-Alexa Fluor 700	Lineage marker of monocytes, phenotyping of NK cells
CD11c	Integrin alpha X, ITGAX	S-HCL-3	BV510	Lineage marker of conventional DCs
CD303	Clec4c	V24-785	BUV661	Lineage marker of pDCs
CD123	Interleukin-3 receptor, IL3RA	9F5	PE-Cy5	Lineage marker of Basophils and pDCs
CD141	BDCA-3, or Thrombomodulin	1A4	BUV615	Lineage marker for cDC1s
FcER1	High-affinity IgE receptor	AER-37 (CRA-1)	BV711	Lineage marker of cDC2s and basophils
CD56	Neural cell adhesion molecule 1, NCAM1	NCAM16.1	BUV563	NK cells
HLA-DR	MHC class II	L243	PE-Fire 810	MHC class II, antigen presentation
IgM	Immunoglobulin M	SA-DA4	Super Bright 436	Phenotyping of B cells, differentiation
CD24	Signal transducer CD24	SN3	PE-Alexa Fluor 610	Phenotyping of B cells, differentiation
IgD	Immunoglobulin D	W18340F	PerCP-Fire 806	Phenotyping of B cells, differentiation
IgG	Immunoglobulin G	G18-145	BB700	Phenotyping of B cells, differentiation
CD40	TNFRSF5	5C3	BUV395	Phenotyping of B cells and DCs, activation
CD1c	NA	L161	Pacific Blue	Phenotyping of DCs and monocytes
CD86	B7-2	FUN-1	BUV737	Phenotyping of DCs and monocytes, activation
CD11b	Integrin alpha M, ITGAM	M1/70	PerCP	Phenotyping of DCs and monocytes

Specificity	Alternative Name	Clone	Fluorochrome	Purpose
CD163	Scavenger receptor for hemoglobin	GHI/61	APC-Cy7	Phenotyping of DCs and monocytes, marker for DC3s
NKp46	CD335, Natural cytotoxicity triggering receptor 1, NCR1	NA	Qdot 625	Phenotyping of NK cells
CD28	NA	CD28.2	BV480	Phenotyping of T cells
CD57	Human natural killer-1, HNK-1	NK-1	PE	Phenotyping of T cells and NK cells
TIGIT	T cell immunoreceptor with Ig and ITIM domains, VSIG9	TgMab-2	RB780	Phenotyping of T cells and NK cells
CD38	Cyclic ADP ribose hydrolase	HB-7	APC-Fire 810	Phenotyping of T cells and NK cells, activation
CD39	Ectonucleoside triphosphate diphosphohydrolase-1, ENTPD1	A1	R718	Phenotyping of T cells and NK cells, activation
KLRG1	Killer cell lectin-like receptor subfamily G member 1	SA231A2	Spark NIR 685	Phenotyping of T cells and NK cells, activation
CD161	Killer cell lectin-like receptor subfamily B member 1, KLRB1	DX12	BV421	Phenotyping of T cells and NK cells, MAIT marker
CD27	TNFRSF7	M-T271	BB660	Phenotyping of T cells, activation
CD272	B- and T-lymphocyte attenuator, BTLA	J168-540	RB613	Phenotyping of T cells, activation
CD278	Inducible T-cell costimulator, ICOS	ISA-3	PE-Cy5.5	Phenotyping of T cells, activation
CD279	PD-1, Programmed Death 1	EH12.1	PE-Cy7	Phenotyping of T cells, activation and exhaustion
CD183	CXCR3, CX chemokine receptor 3	G025H7	PE-Fire 640	Phenotyping of T cells, migration
CD194	CCR4, chemokine receptor 4	1G1	BV786	Phenotyping of T cells, migration
CD197	CCR7, chemokine receptor 7	G043H7	BV605	Phenotyping of T cells, naive versus memory
CD45RA	Isoform of CD45	HI100	Spark UV 387	Phenotyping of T cells, naive versus memory
CD45RO	Isoform of CD45	UCHL1	BV570	Phenotyping of T cells, naive versus memory
CD103	Integrin alpha E, ITGAE	Ber-ACT8	BV750	Phenotyping of T cells, tissue residency marker
CD69	NA	FN50	BV650	Phenotyping of T cells, tissue residency marker, activation
CD25	Interleukin-2 receptor alpha chain, IL2RA	BC96	BB515	Phenotyping of T cells, Treg identification
CD127	Interleukin-7 receptor subunit alpha, IL7RA	HIL7R-M21.1	RB744	Phenotyping of T cells, Treg identification
CD2	LFA-2, lymphocyte function-associated-2	S5.5	Qdot 605	Identification of ILCs, NK cell phenotyping
Live/Dead	NA	Amine Reactive	Zombie-NIR	Live/Dead cell discrimination

Abbreviations: AF, AlexaFluor; APC, allophycocyanin; BB, brilliant blue; BUV, brilliant ultraviolet; BV, brilliant violet; FITC, fluorescein; NA, not applicable; NIR, near-infrared; PE, phycoerythrin; Qdot, quantum dot; RB, RealBlue.

A representative gating tree is shown in Figure 1, including some fluorescence-minus-one (FMO) controls. These FMOs highlight that by using systematic panel design there is negligible spreading error (SE) for the displayed populations and markers of high interest.

The panel development strategy was based on established best practices as well as multiple novel approaches. First, for identifying a list of potential fluorochromes, a combination of the similarity index (online Fig. S3), the fluorochrome brightness (online Fig. S4), and a newly developed algorithm for automated fluorochrome selection was used. Briefly, 40 fluorochromes were manually selected and then the algorithm-based approach was used to identify the best feasible combinations that allowed expansion to 50 colors (for more detail, see the Strategy for Panel Development section of the online supplementary material). Second, a new metric was developed to evaluate unmixing-dependent spreading error that occurs in highly complex spectral flow cytometry panels. Briefly, unmixing-dependent spreading error increases the noise level for all measured cells on a per-fluorochrome basis, irrespective of the presence of positively stained cells. The practical impact on staining index and overall resolution per fluorochrome is shown in online Figures S4 and S5, together with a more detailed discussion of this phenomenon. Finally, the instrument-specific spillover-spreading matrix and the total spread matrix were utilized for the optimal assignment of fluorochromes based on the biological co-expression of markers (see online Fig. S6).

The panel was developed on two full-spectrum cytometers in parallel: a 7-laser instrument with a total of 186 detectors and a 5-laser instrument with a total of 78 detectors. The final and fully optimized panel as shown in Figure 1 was acquired on the BD FACSDiscover S8 (instrument configuration and setup details are listed in the online materials), together with the FMO control samples. This instrument also allowed cell sorting, highlighting that 50-parameter sorting is feasible to allow very fine-grained isolation of any immune population of interest. Furthermore, a trimmed-down panel version of 45 colors was established to be cross-platform compatible. To the best of the authors' knowledge, this is the first report of a high-dimensional 40-color+ panel that is usable across multiple independent spectral cytometry platforms.

Overall, the data show that this panel can serve as a widely usable and powerful immunophenotyping resource for comprehensive analysis of human immune cells. The opportunity to reliably analyze 50 different target molecules (with the option to perform parallel cell sorting) in a high-throughput fashion is likely to enable previously impossible avenues to study the human immune system. Bio-Rad carries several families of dye-antibody conjugates for flow cytometry applications. Their StarBright Dyes are fluorescent nanoparticles conjugated to extensively validated antibodies. These dyes have exceptional brightness, narrow excitation and emission, and stability with high lot-to-lot reproducibility. Other Bio-Rad dye conjugate families include Alexa Fluor and DyLight.

The development of a comprehensive panel on a standardized instrument may enable the consistent use of a single panel suitable for multiple different studies. Together with the deposition of these data into publicly accessible databases, such a consistent use would facilitate subsequent cross-study analyses with machine learning approaches such as FAUST or other suitable computational techniques.

Similarity to published OMIPs

The most similar OMIP to OMIP-102 described here is OMIP-069 (the first 40-color OMIP to be reported [1]) and OMIP-044 (the first 28-color OMIP reported [2]). There is some overlap with published 28-color OMIPs focusing on T cell phenotyping (e.g., OMIP-050 and OMIP-058 [3]) and several other lower dimensional OMIPs focused on T cells, but there is currently no OMIP that reports the use of 50 different fluorochromes allowing such in-depth phenotyping of T cells and APCs.

References:

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Do More with Less: Improving High Parameter Cytometry Through Overnight Staining

Adapted from Whyte *et al.*, 2022

Advances in flow cytometry enable high-dimensional biological characterization, yet standard short staining protocols often ignore antigen and fluorophore properties. We show that extending antibody incubation times significantly enhances sensitivity and specificity, reduces background, and lowers costs for high-parameter panels. Overnight staining also minimizes interexperimental variability, aiding in consistent data pooling and improving resolution, repeatability, and cost-effectiveness. Optimizing conditions and fixation strategies enhances epitope accessibility.

Introduction

Flow cytometry, a crucial technique for single-cell analysis, has evolved to handle increasing biological complexity through innovations like spectral cytometry and enhanced compensation. High-parameter flow panels demand meticulous design to address antigen localization, expression levels, and fluorophore characteristics, balancing antigen detection with minimal spectral overlap. Challenges include limited antibody-fluorophore conjugates, antibody affinity, and biological barriers such as antigen downregulation upon cell stimulation. These factors complicate antigen resolution, often necessitating compromises. Standardized staining conditions, typically involving brief antibody incubations, can lead to inaccurate cell and marker quantitation, but enhanced staining protocols, particularly overnight antibody incubation, improve data sensitivity, accuracy, reproducibility, and flexibility. This article explores the benefits and constraints of optimizing staining, especially overnight techniques, for more precise flow cytometry outcomes.

Improving flow staining with increased incubation times

Optimizing antibody incubation time is crucial for enhancing resolution in flow cytometry. Antibody-antigen binding, driven by non-covalent interactions, reaches equilibrium when binding and dissociation rates equalize. Conventional incubation (15-60 min) uses high antibody concentrations, achieving substantial binding quickly. However, extended incubation allows similar binding with significantly lower antibody concentrations, reducing variability and improving consistency across experiments. Extended (e.g., overnight) incubation also offers significant advantages in high-parameter panel optimization. Despite its benefits, overnight staining remains underutilized in many flow cytometry labs, representing an opportunity for improved resolution and reproducibility.

Improved sensitivity can be achieved by overnight antibody staining

Fluorophore-conjugated antibody binding to antigens in single-cell suspensions is rapid, but sufficient fluorescence levels are needed for proper antigen expression analysis. Extended antibody incubation enhances fluorescence intensity, achieving similar median fluorescence intensity (MFI) with 10-fold less antibody over 16–20 hours compared to shorter times (Fig. 1). This is critical for resolving difficult antigens, especially those detected by low-affinity antibodies or with limited accessibility. For instance, overnight staining improves the resolution of regulatory T cells (Treg) and intracellular proteins (Fig. 2). It facilitates detecting internalized proteins, such as CCR7 (Fig. 3), by allowing both surface and intracellular protein detection, optimizing flow cytometry staining based on antibody concentration, incubation time, and fixation.

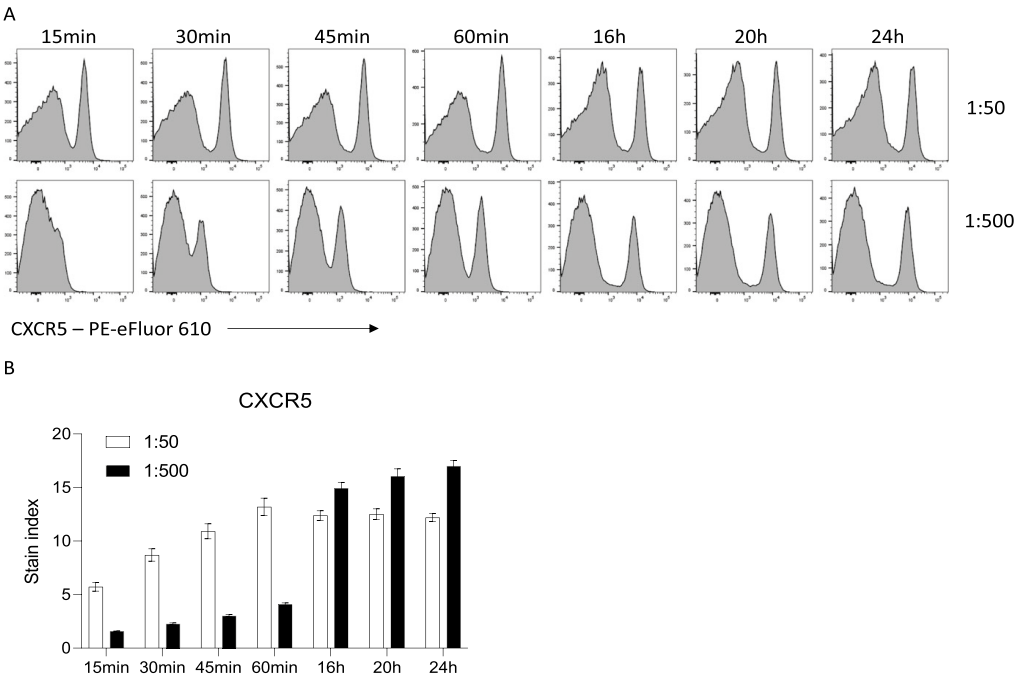

Figure 1. Influence of time and antibody concentration on mouse CXCR5 staining.
(A) Representative histograms of CXCR5 staining on viable C57BL/6 mouse splenocytes after the indicated incubation times. **(B)** Stain index (n = 5, mean ± SD).

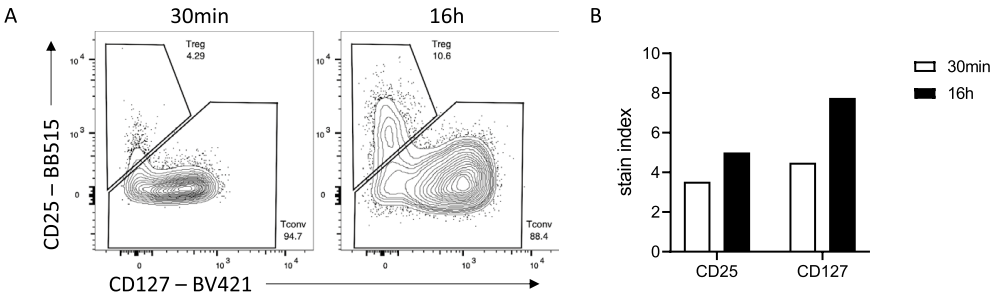

Figure 2. Superior discrimination of human Treg by overnight staining.
(A) Representative flow staining of CD4+ CD3+ cells stained for 30 min or 16 hr. **(B)** Stain indices of CD25 and CD127 stained for 30 min or 16 hr.

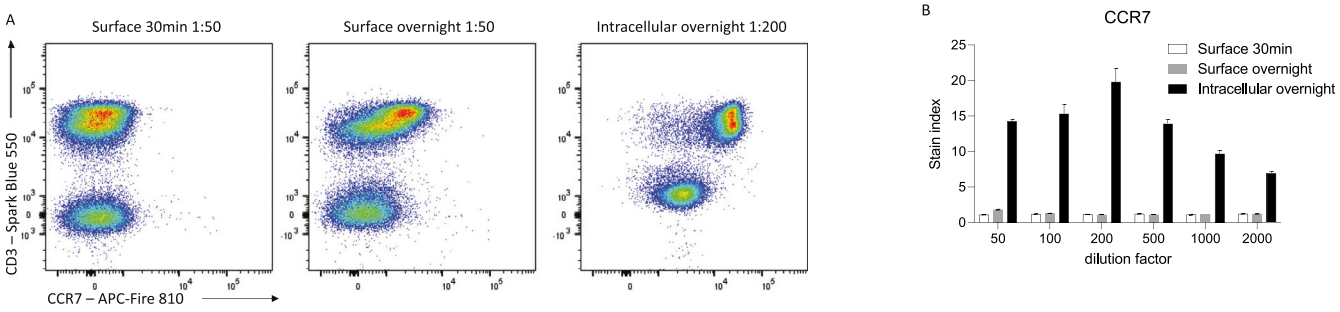

Figure 3. Improved detection of CCR7 with overnight intracellular staining. (A) Representative histograms of CCR7 staining on viable C57BL/6 mouse splenocytes after the indicated incubation times. **(B)** Stain index of CCR7 on CD3+ T cells (n = 3, mean ± SD).

Extended incubation times can reduce interexperimental variability and batch effects

Extended antibody incubation reduces variability between experiments by allowing the antigen-antibody binding reaction to approach equilibrium. Short (15-30 min) incubations yield variable MFIs, impacting data reproducibility due to the binding reaction's exponential phase. Overnight (16-20 hr) incubations stabilize antibody-antigen complexes, leading to consistent MFIs and reducing batch effects.

This was demonstrated with cryopreserved blood stained on different occasions, showing diminished variability and improved antigen resolution with overnight staining compared to 30-min staining (Fig. 4). Standardized overnight staining protocols allow for pooling of data from independent experiments, enhancing reproducibility in longitudinal analyses.

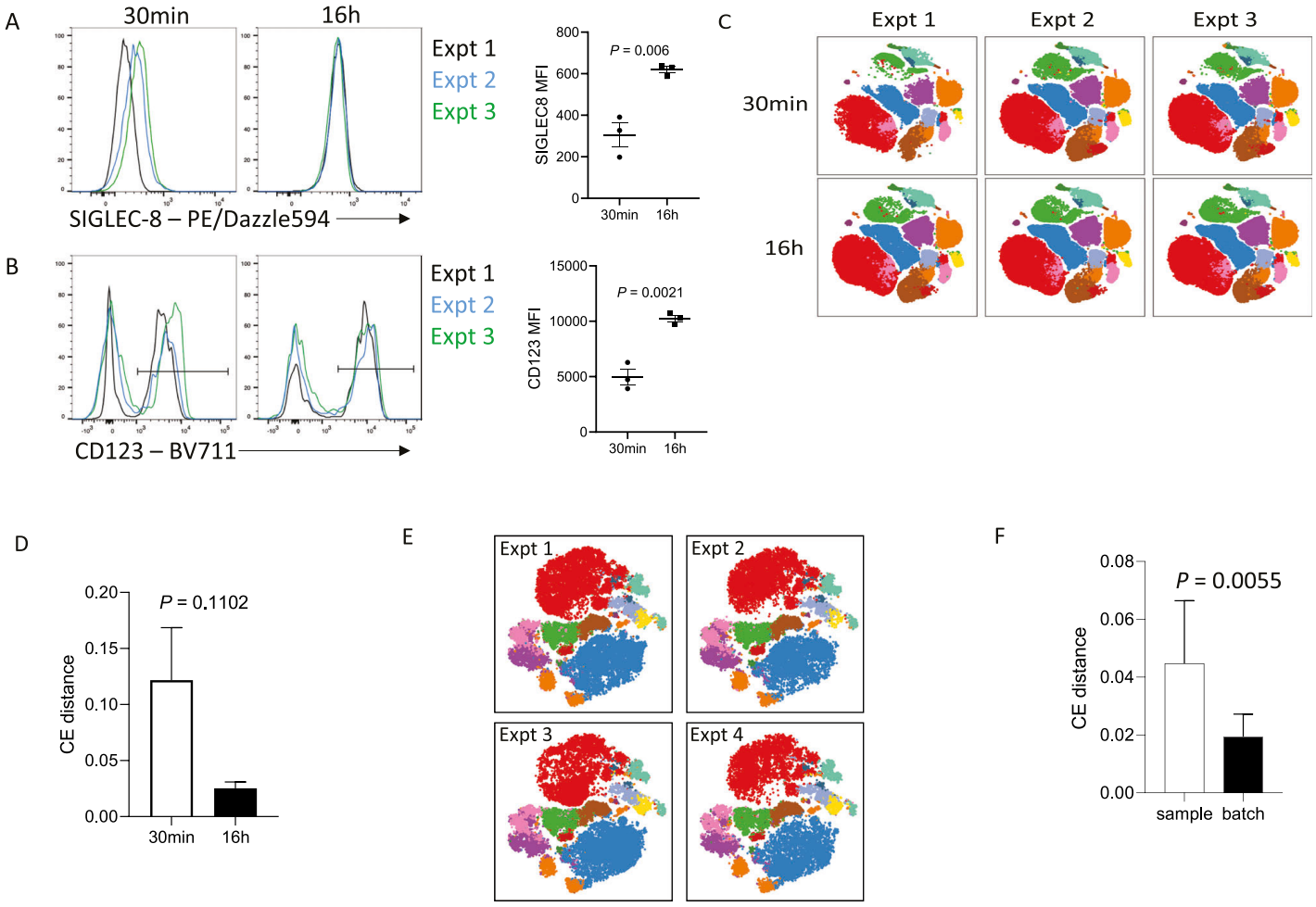

Figure 4. Increasing incubation time reduces batch effects. (A) Representative staining and MFI of SIGLEC-8 on CD45+ SSChi CD16- cells from the same donor over 3 independent experiments. **(B)** Representative staining and MFI of CD123 on CD45+ SSClo CD3− CD19− CD14− CD16− cells from the same donor over 3 independent experiments. **(C)** Human whole blood immunophenotyping data from the same donor over three independent experiments, stained for 30 min or 16 hr. tSNE plots were generated using the parameters CD45, SSC-A, CD4, CD8, CD127, CD16, CD19, CD3, CD123, CD20, CD25, Fcer1a, CD11c, SIGLEC-8, CD56, CD14, and HLA-DR. FlowSOM clusters are shown in a colored overlay. **(D)** Cross entropy distances between samples stained for 30 min or 16 hr. **(E)** Mouse data from 4 experiments over 2 years. Data were acquired on a BD FACSymphony A5 cytometer. tSNE plots were generated using the parameters CD4, CD8, Foxp3, CD103, Neuropilin, CD44, CD62L, Ki67, ICOS, PD-1, CTLA-4, CD25, KLRG1, CD69, ST2, and Helios on CD3+ T cells. Flow- SOM clusters are shown in a colored overlay. **(F)** Cross entropy distances between mouse samples (intra-batch variation) or batches (inter-batch variation). Significance was tested by unpaired *t*-test.

Increased incubation times can reduce costs

Optimal antibody titration balances sufficient signal against the negative background. Insufficient antibody yields weak signals, while excess can cause non-specific binding and positive shifts in negative populations. Empirical determination is required for each experimental setup. Extended (overnight) incubation typically requires 5- to 100-fold less antibody, significantly reducing costs. For high-parameter panels, overnight staining is more cost-effective than 30- to 60-min staining, lowering median antibody costs from £0.21 to £0.04 per antibody and saving £11.03 per sample for panels with 23-50 parameters (Fig. 5).

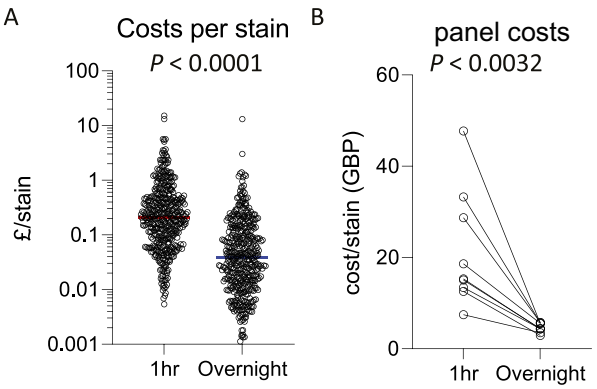

Figure 5. Overnight incubation increases cost-effectiveness. (A) Cost (GBP) per antibody per stain for optimal titration for 1-hr incubation (median cost £0.21) versus overnight incubation time (median cost £0.04). n = 439, Wilcoxon matched-pairs signed-rank test. **(B)** High-parameter (23-50 color) panel costs with 1 hr versus overnight incubation. Paired *t*-test.

Increased flexibility in panel design

Successful high-parameter flow cytometry requires careful panel design, considering each antigen's expression level and co-expression patterns. Low-level antigens are typically paired with bright fluorophores to minimize signal overlap from co-expressed markers. Compromises may become necessary due to antibody-conjugate availability and the need to use sub-optimal dyes to utilize the full fluorescence spectrum in spectral cytometry.

Extended incubation times enhance resolution by allowing more time for antibody-antigen interactions, making lower-concentration and lower-affinity antibody use feasible. This approach improves signal:noise ratios, enabling the use of otherwise suboptimal fluorophore-antigen combinations, as demonstrated with CD3 BV570, Tbet-BV605, and NKp46-PerCP-Cy5.5 (Fig. 6).

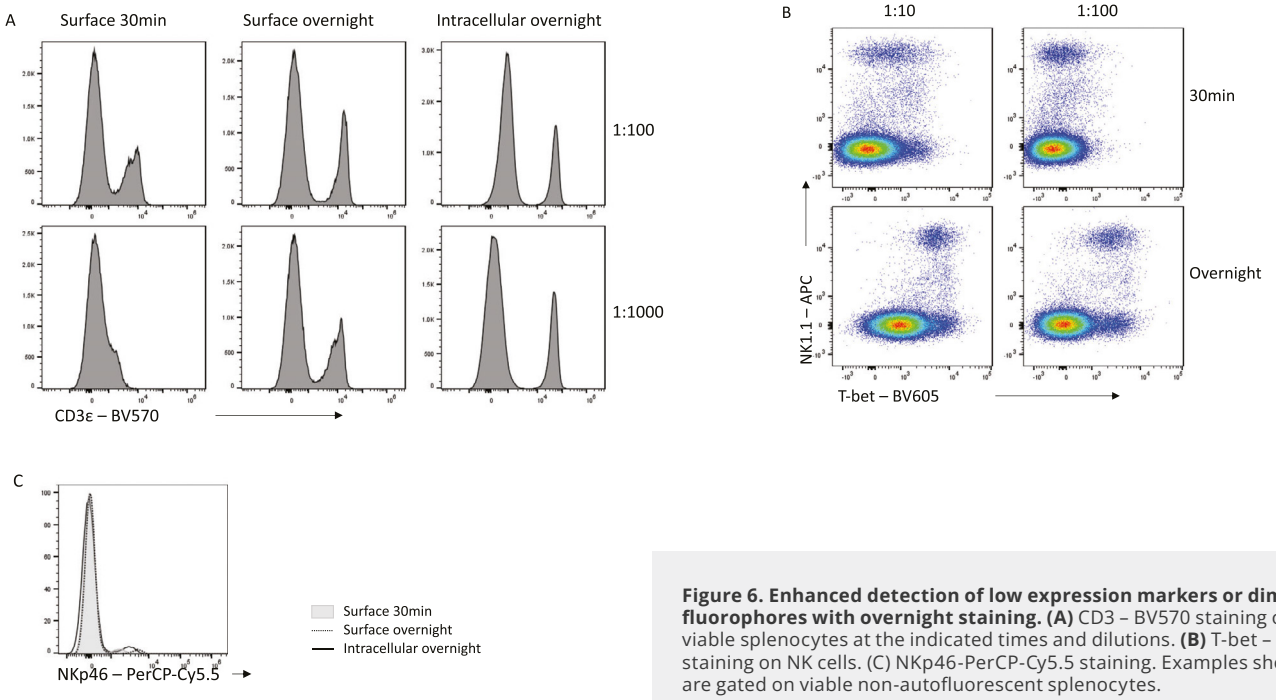

Figure 6. Enhanced detection of low expression markers or dim fluorophores with overnight staining. (A) CD3 – BV570 staining on viable splenocytes at the indicated times and dilutions. **(B)** T-bet – BV605 staining on NK cells. (C) NKp46-PerCP-Cy5.5 staining. Examples shown are gated on viable non-autofluorescent splenocytes.

Reduced interference from unwanted polymer dye and fluorophore interactions

Increasing antibody incubation times reduces the concentration of antibodies needed, minimizing costs and improving data clarity. High-parameter panels often use polymer dyes (e.g., Brilliant Violet, Brilliant Blue), which can aggregate and distort fluorescence signals. Buffers like Brilliant Stain Buffer mitigate this, but lower antibody concentrations further reduce dye aggregation and improve data interpretation (Fig. 7). Moreover, reduced antibody concentration decreases non-specific binding to Fc receptors, particularly with tandem dyes such as Cy5, which can non-specifically bind to CD64 on macrophages. Extended incubation, therefore, enhances resolution and reduces non-specific signals, aiding the use of polymer-based reagents and minimizing false positives in flow cytometry.

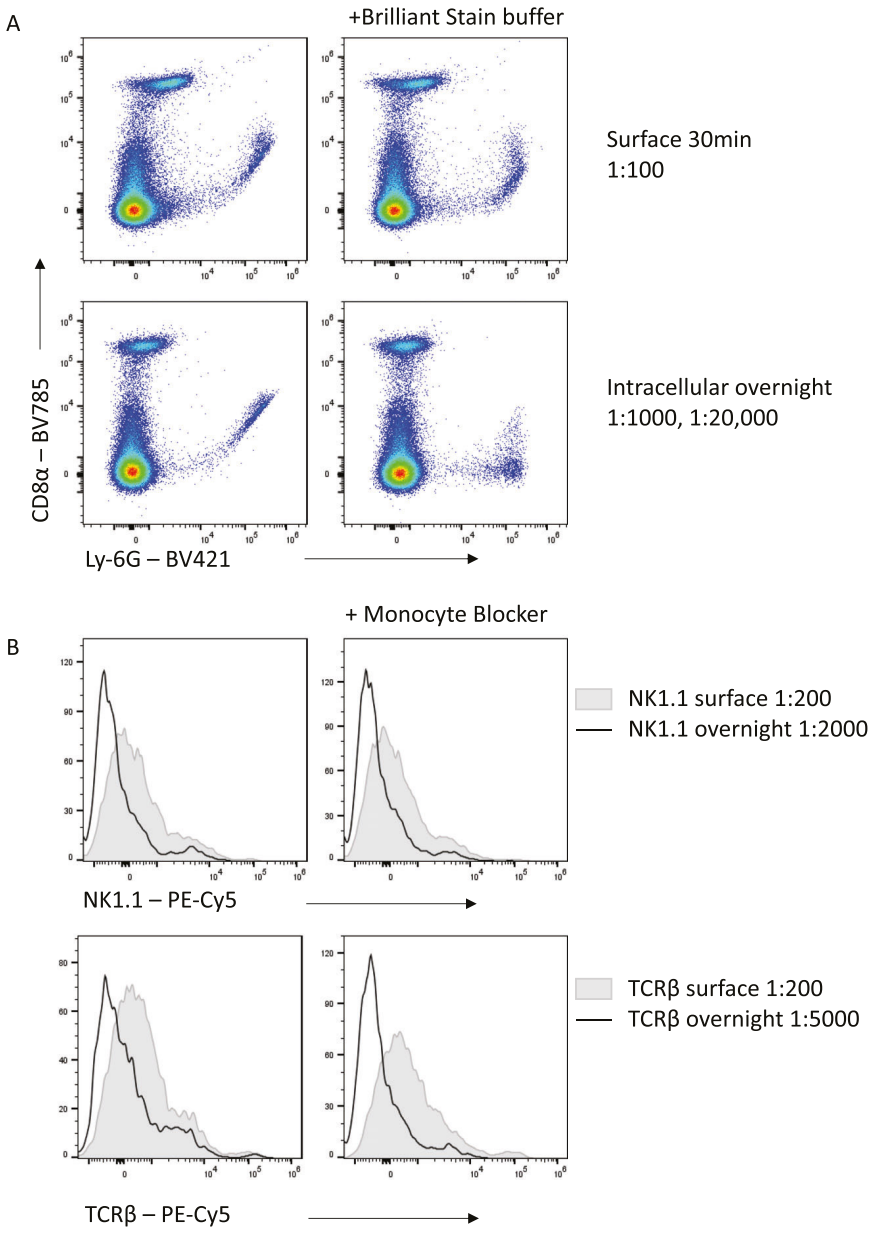

Figure 7. Reduced non-specific binding with lower concentrations of antibody in overnight staining. (A) Brilliant Violet dye interactions in a 30-min stain versus an overnight stain. **(B)** Non-specific binding of PE-Cy5 tandems to macrophages in 30 min versus overnight surface staining. Histograms shown are gated on viable F4/80+ autofluorescent macrophages.

Practical considerations in optimizing staining conditions

Optimizing staining conditions and antibody titration

Following panel design, antibody optimization is crucial for resolving each marker. This involves titrating antibodies to find optimal concentrations, as required amounts vary with conditions such as incubation time, temperature, and sample type. Start by titrating antibodies for 30- to 60-min surface staining, overnight surface staining, and overnight intracellular staining, adjusting based on the experiment's biological context (Fig. 8 and Fig. 9). For example, CD3-SparkBlue550 requires a 1:200 dilution for 30-min surface staining, but a 1:10,000 dilution for overnight staining of fixed cells.

Similarly, overnight intracellular staining with PD-1-BV711 requires 100-fold less antibody than 30-min surface staining. Analyzing the staining index (see equation below) ensures clear positive signals and minimizes background, essential for maximizing antibody resolution.

$$Staining\ index = \left(\frac{MFI_{positive} - MFI_{negative}}{2 \times SD_{negative}}\right)$$

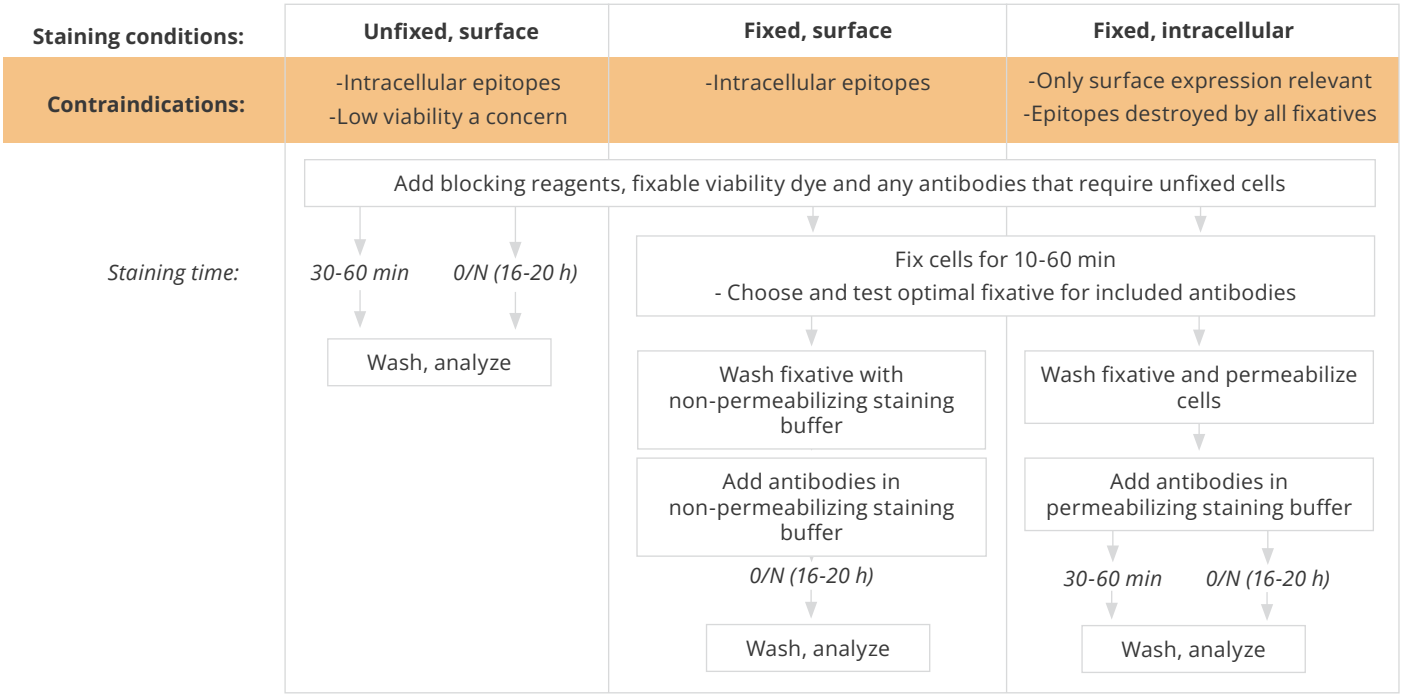

Figure 8. Protocol overview for optimizing staining conditions.

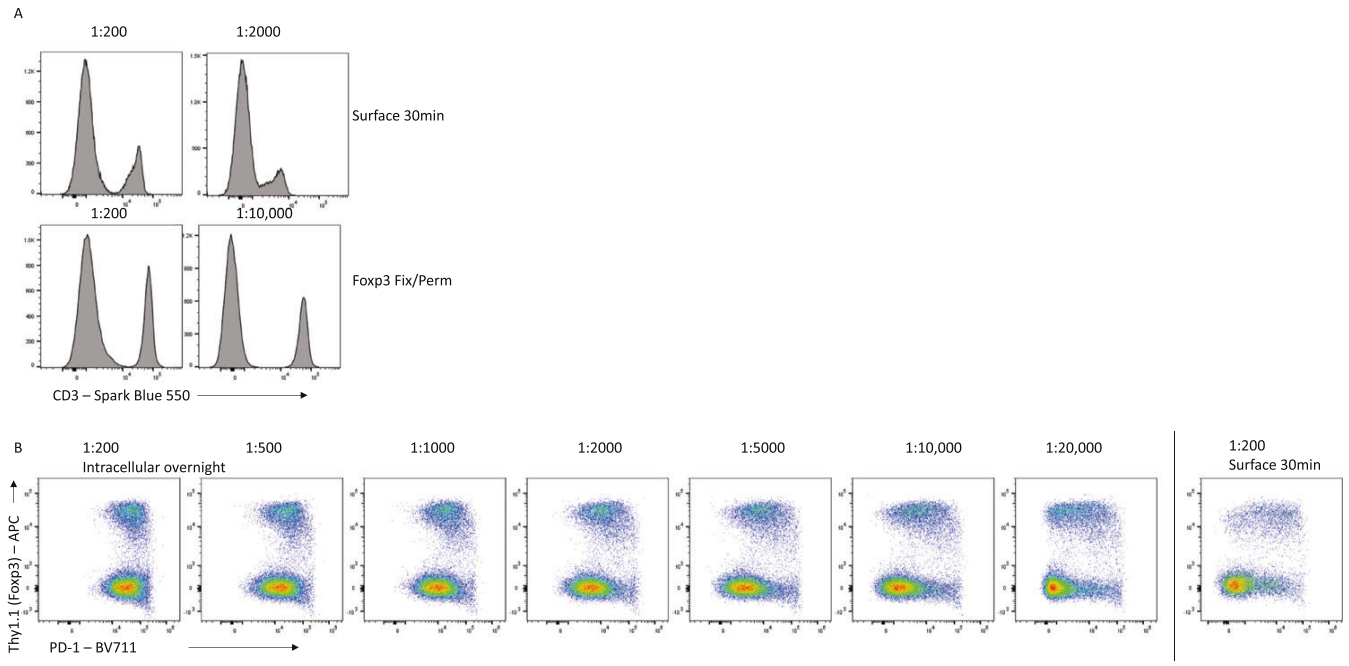

Figure 9. Titration is essential to maximize sensitivity. (A) Titration of CD3-Spark Blue 550. **(B)** Intracellular overnight staining for PD-1 on viable CD4+CD3+ T cells at the indicated dilutions on cells fixed and permeabilized.

Choice of fixative and effects on epitopes

Antibody staining sensitivity often improves post-fixation and permeabilization, but the fixative used affects antibody-antigen binding. Formaldehyde-based fixatives (1-4%) preserve cell structure but may alter epitopes. Permeabilizing agents like methanol and Triton X-100 facilitate antibody access to intracellular targets. Fixation conditions impact antibody performance; for example, CD11b-BV510 and CCR7-BB700 show improved sensitivity with fixation, while CD69 is best stained unfixed (Fig. 10). Antibodies such as CD25-SBV515 require lower concentrations with some fixatives. Optimized fixatives balance preservation and accessibility. Fixation allows co-detection of fluorescent proteins with intracellular targets, although care is needed as tandem dyes can degrade and alter signals post-fixation.

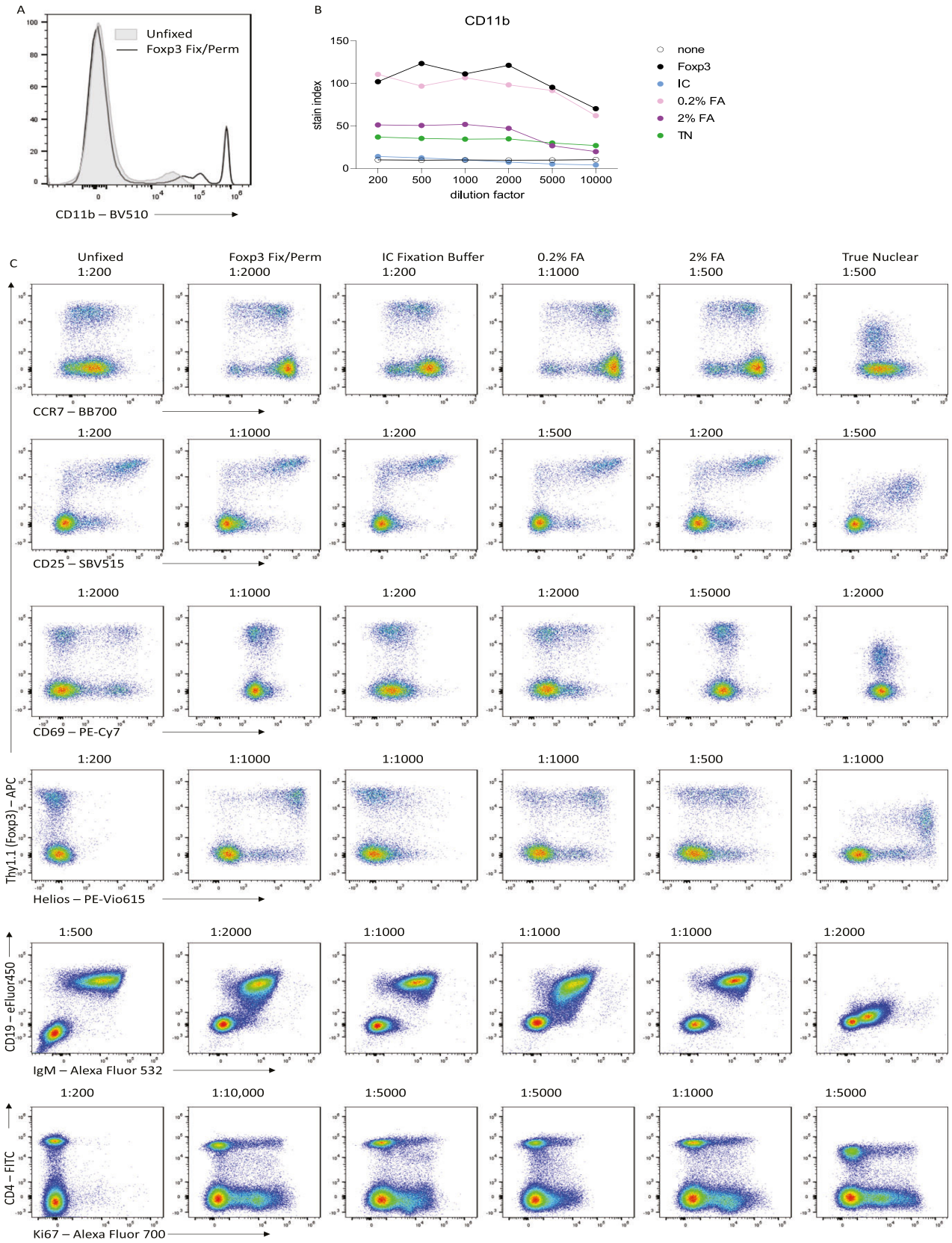

Figure 10. Choice of fixative affects staining intensity and specificity. (A) Representative histograms showing overnight CD11b-BV510 staining on mouse splenocytes either with or without fixation. **(B)** Stain indices for CD11b staining at various dilutions with various fixatives. **(C)** Representative examples of overnight marker staining on murine splenocytes with various fixatives.

Controlling for non-specific staining

Extended incubation times are often misconceived as increasing non-specific staining in flow cytometry. However, non-specific binding affects all staining and should be controlled with proper measures. The ideal control involves staining cells identical to the test sample but lacking the antigen of interest (e.g., using knock-out cells) (Fig. 11). Alternatively, an internal negative control—cells within the sample known not to express the antigen—can be used, ensuring similar autofluorescence profiles. Fluorescence-minus-one (FMO) controls help account for spectral spread. For stimulation-induced signals, unstimulated controls are beneficial.

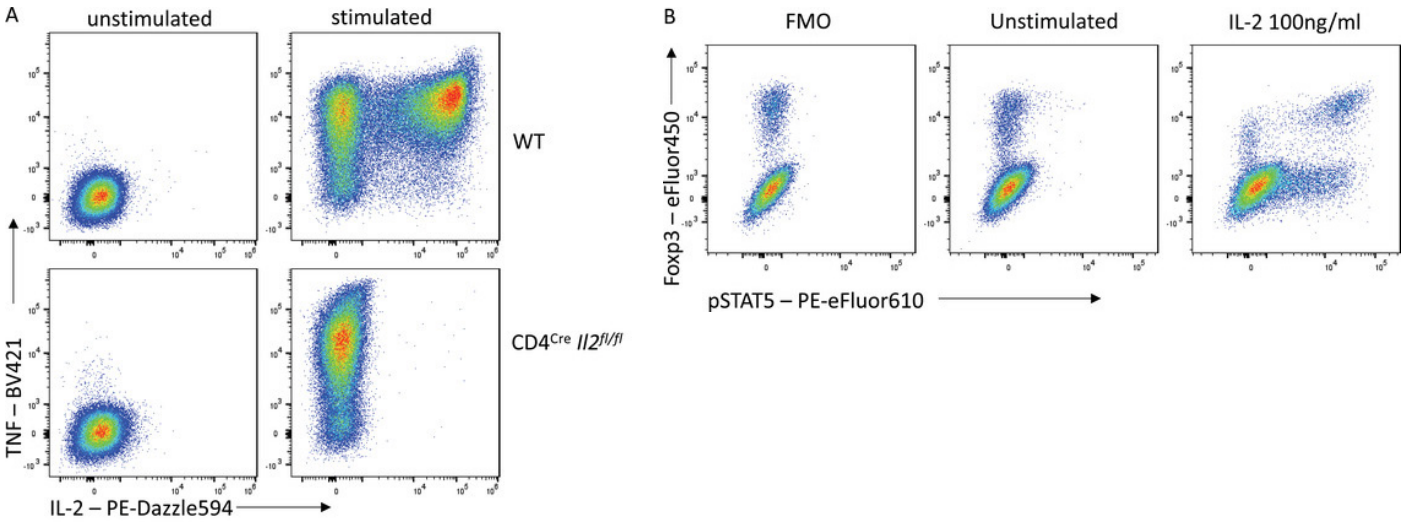

Figure 11. Controls confirm specificity is maintained with overnight staining. (A) IL-2 staining on WT or IL-2-deficient mouse CD4+ T cells. **(B)** pSTAT5 and Foxp3 staining on mouse CD4+ T cells with or without IL-2 stimulation.

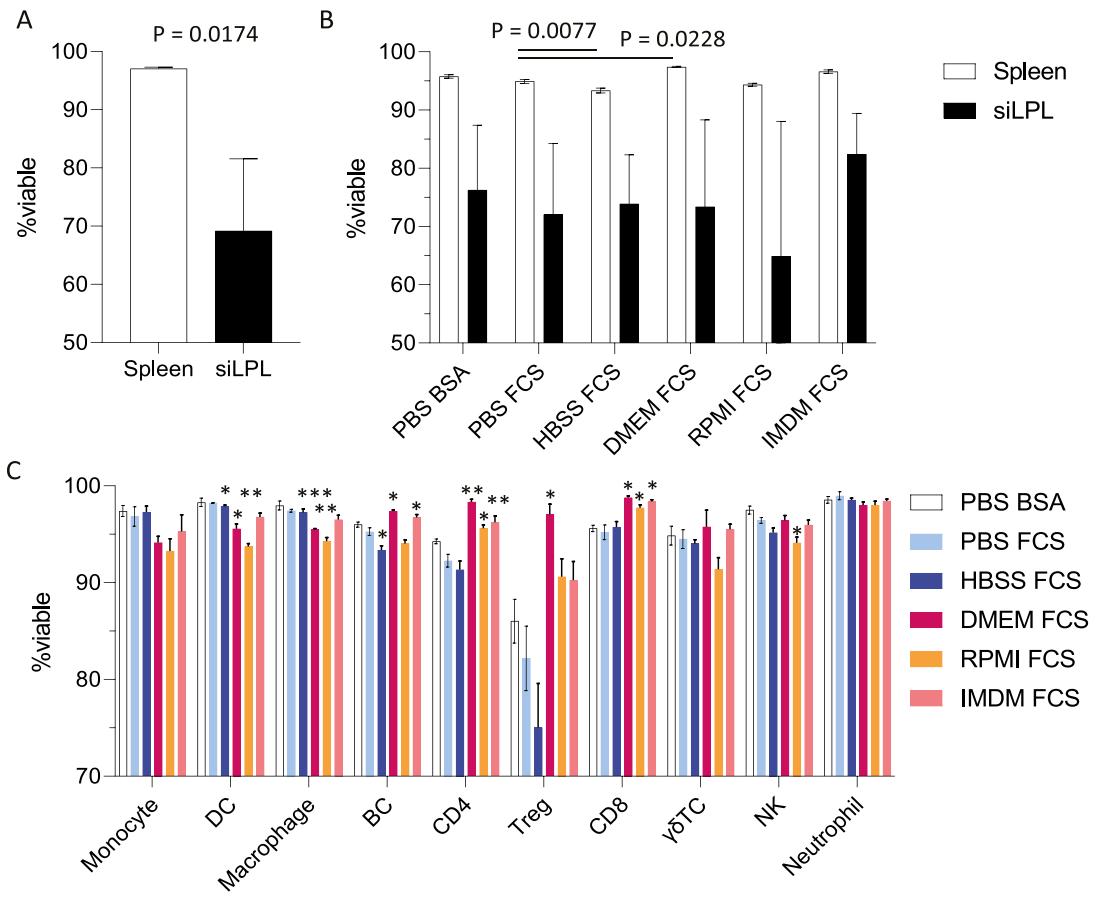

Figure 12. Buffer composition and preparation viability affect cell survival during overnight incubation. (A) Leukocyte (CD45+) viability before overnight incubation (n = 3, mean ± SD) in mouse spleen or small intestinal lamina propria leukocytes. Statistical analysis by unpaired *t*-test. **(B)** Impact of buffer choice on leukocyte viability in overnight incubation. Cellular viability was assessed on single CD45+ leukocytes that were negative for fixable viability dye before overnight staining. **(C)** Viability of various immune cell types from mouse spleen after overnight incubation in different buffers. Statistical analysis for B and C by 2-way ANOVA with Dunnett's multiple comparisons between PBS FCS and all other conditions.

When overnight surface staining is sometimes preferable: Effects on staining and viability

Overnight staining of unfixed cells can yield optimal results for certain surface proteins like CXCR5, which shows an improved signal-to-noise ratio compared to standard or intracellular staining. Viability is crucial when considering this approach; freshly isolated mouse splenocytes maintain viability above 90%, with Tregs at ~80% (Fig. 12). However, cells from prolonged digestion or cryopreserved samples may suffer greater viability loss. Buffer choice also affects viability, with complete media (e.g., IMDM, RPMI) outperforming PBS or HBSS. Thus, while overnight staining enhances resolution, the effects on cell viability should be empirically determined and balanced against improved staining resolution.

Concluding remarks

High-parameter flow cytometry benefits from optimizing staining conditions for each antigen, considering their unique expression and antibody characteristics. Generic, short staining methods often yield suboptimal results, whereas tailored protocols enhance resolution, reduce costs, and improve panel design flexibility. Spending extra time on optimization can enhance marker quantitation and overall data accuracy.

Suggested reading

Andersson, K., Björkelund, H., Malmqvist, M. (2010). Antibody-antigen interactions: What is the required time to equilibrium? *Nature Precedings*. DOI:10.1038/npre.2010.5218.1

Ferrer-Font, L. *et al.* (2021). Panel Optimization for High-Dimensional Immunophenotyping Assays Using Full-Spectrum Flow Cytometry. *Current Protocols*. DOI:10.1002/cpz1.222

From Curiosity to Cutting-Edge: Traversing the Terrain of Immunology and Cytometry

Interview with Dr. Oliver Burton

Website: LinkedIn

Dr Burton is an accomplished immunologist and cytometrist with 17 years of research experience, specializing in identifying therapeutic targets using translational models and multi-parametric flow cytometry. He began his academic career at The University of Cambridge where he obtained his Ph.D.. He founded Colibri Cytometry and has worked as a staff scientist at the University of Cambridge and the Babraham Institute, where he developed innovative flow cytometry panels and conducted extensive research across immunology. Dr. Burton's expertise extends to single-cell technologies and disease models, with numerous keynote speaking engagements at cytometry conferences.

Dr. Burton embarked on his academic path at Williams College in Williamstown, MA, USA, where he pursued a B.A. in Biology from 2002 to 2006. He then progressed to the University of Cambridge in the UK, where, from October 2006 to May 2010, he completed his Ph.D. in Pathology under the mentorship of Prof. Anne Cooke. His doctoral research explored the complexities of the immune system, setting the stage for his future contributions to the field.

Following his Ph.D., Dr. Burton transitioned into the role of a Research Fellow at the Boston Children's Hospital, an affiliate of Harvard Medical School, from October 2010 to 2014. Here, under the supervision of Hans Oettgen, he further refined his research skills and deepened his expertise in immunology. Following this, he served as a Senior Post-Doctoral Scientist at VIB/KU Leuven from April 2017 to December 2018, working with Adrian Liston. This period was marked by significant research achievements and a growing reputation as an expert in his field.

Returning to the UK, Dr. Burton joined the Babraham Institute in Cambridge as a Staff Scientist from January 2019 to April 2023. Under the continued supervision of Adrian Liston, he developed a remarkable 50-color flow cytometry panel, advancing research in key areas such as neuroimmunology, cancer, vaccines, and autoimmune diseases.

In April 2023, Dr. Burton transitioned to his current role at the University of Cambridge as a Staff Scientist. His work involves cutting-edge techniques like *in vivo* CRISPR screening and high-parameter flow cytometry, contributing to the development of a human immune phenotyping platform and broadening the scope of immunological research across all tissues.

Dr. Burton's expertise is not confined to the laboratory, he is also a founder of Colibri Cytometry and an ISAC scholar. His skills in multi-parameter flow cytometry and single-cell technologies are complemented by his proficiency in bioinformatic analysis and cell sorting technologies. He is a recognized leader in the field, often invited as a keynote speaker at prestigious cytometry conferences.

With a career dedicated to the pursuit of knowledge in immunology and cytometry, Dr. Burton's work stands as a testament to his commitment to advancing our understanding of human disease and therapeutic interventions. His academic and professional experiences reflect a deep engagement with the complexities of the immune system and a drive to innovate within his field.

What motivated you to pursue a career in immunology?

I wanted to help people and I thought that was a way of doing it through research. I thought that was something where my skills would be useful and I might be able to contribute to making society better.

It was an interesting field because when I started going into it, it seemed like there was a lot that was unknown. It had a tangible and direct impact on people's lives because it's what we used to make medicines to make people better. That was my main motivation.

Could you share a specific research project that you found particularly exciting or impactful?

Initially, in my Ph.D. and postdoc studies, I was doing work that was closer to the clinic. I was looking at autoimmune diseases and allergies, and it was a bit disappointing in that I felt like the work wasn't really leading to anything.

In the last seven or eight years, I've stepped away from that into a more basic immunology-focused and technical role. That has been a lot more rewarding because I know that when I'm doing leads to new discoveries, even if they're not directly translating into clinical stuff, I know that eventually it will help.

I think that the studies where I felt like I was going to try to make an impact actually didn't and so I'm just happier now that I feel like the stuff I'm doing is productive.

Can you discuss any collaborations or partnerships you've had with other researchers or institutions?

I had some quite nice collaborations when I was in Belgium, we were working at the VIB, which is the Flemish Institute for Biotechnology, and it was a brain disease Research Center. We had the opportunity to work with some really top researchers. We were working with cutting-edge technologies, looking at how to measure things and get more information out of samples. Additionally, we did some really cool biology projects looking at how we make models of human brains so that we can study that better because that's difficult to actually study. That was just a really interesting environment to be in and I enjoyed that quite a bit.

I think, looking back, those are the studies where I was most happy to be collaborating with people because they were bringing in ideas from areas of expertise that I didn't know anything about and doing what seemed like really cool things to me, and I was able to contribute to that because I had a bit of expertise in something that they needed. So we would put those two together and things worked and that's what I like about science. When you can take two people who know, or most people who know, little bits of the puzzle and put them together and get something much bigger out of it.

What techniques or methodologies do you find most valuable in your work?

We do a lot of flow cytometry. That's a really big thing for us because it allows us to get lots of very accurate measurements reliably for not much money and it's relatively easy to do compared to other things. We can turn around the data quickly so we can progress the research more rapidly.

The other thing that is really important for us is mouse research. We do a lot of stuff with animals, we do human work as well, but animal studies are really important for testing a hypothesis about how a medication works. Does it actually work? Is it actually safe? Do you need to go back and refine it? There's a lot of stuff that you just still can't do in a dish. You don't have the whole organism system in a dish, so if you're trying to say we want to treat just the brain we need to make sure that medication is going to the brain and is not doing stuff in all the rest of the body.

That's our specialty: looking at it holistically when we do animal research, taking all the parts of it and not wasting it, and trying to understand if is it targeted and specific the way we want it to be, or are there effects elsewhere that we need to remove?

Could you elaborate on the algorithms you've developed for flow cytometry data analysis? How do these algorithms enhance our understanding of cellular populations?

I haven't done a lot in that area. We've done two things.

One of them isn't really looking at how we understand cells, it's understanding how to make the cytometry data better and more reliable for people. That was a project that I worked on with a mathematician called Carlos

Roca and it was trying to allow people to get consistently better data out of their control samples to be able to then faithfully interpret the data that comes out from the complex samples.

With flow cytometry, you try to label lots of different things. Specifically, each one gets a color label and there can be many, many colors, so telling the machine that reads those colors for you exactly which color it is, and having no contamination between the colors, is a critical part of being able to take that sample where all the paints have been mixed together and tell them how much of each color there is. We came up with a better way of doing that based on my experiences in separating those out and his mathematical skills. That was quite useful for a while, and now the field has moved on.

The other thing that we did, again with Carlos, was looking at a way of separating and differentiating cells when they're analyzed in flow cytometry or single-cell RNA sequencing. A lot of times people try to take all of the information and display it in a way that the human brain can process it because it's a lot of different shapes that are really hard to understand. We can look at things in two dimensions on a piece of paper or maybe three dimensions if it's a shape moving around or rotating on a computer. So those tools are used a lot, especially now in flow cytometry. What we did was work out a way of looking at whether the way that you're showing the data is actually different between two groups, say a healthy group and a disease group, and how different it is to get an overview of statistical analysis of the data. I don't think that technique, to be honest, has been picked up very much or used very much.

In your research on improving cell staining consistency and reducing costs, what novel techniques or strategies have you explored?

A lot of that is just nose to the grindstone testing many, many different conditions. What I'm trying to achieve there is to make things more reproducible so that people, when they do their experiment, will always get the same result and that makes it much easier for them to interpret the data and say, yes, I can compare this from one time to the next.

The other thing that that comes out of it, is that you end up using a lot less reagent to get that reproducibility, which saves medical researchers a lot of money which means that their grants go further so they can get more information. For us, it's meant that we actually end up doing a lot more flow cytometry because it means that the flow cytometry is now better value for money.

As to what goes on in making that happen it's doing what we call titrations, where we take the antibody and use different amounts of it. We take the amount that is supposed to be used, or the maximum amount you can use, and then use half of that and half of that again and half of that again in a series until basically there's nothing left and you're working with homeopathic amounts. I do that for every single antibody that we buy and I test it in that way in two or three different conditions, depending on how we're going to do the staining, whether we're going to be doing it with cells that need to be sorted, whether they need to be fixed, whether they want to be staining inside the cell with different buffers. It's a lot of work and there's not really a lot of shortcuts to it, but what I've been trying to do recently is help people from having to repeat my work by just putting it online in a database because this is the kind of things you don't publish, they're not significant for publication.

How have Bio-Rad's instruments and reagents contributed to your research?

When we first moved to the Babraham Institute, about five years ago now, we were using the ZE5 Cell Analyzer a lot and it was such an amazing experience for me compared to what I had been using, which was primarily beading machines, other cytometers and the CyAn ADP before that, but the Bio-Rad ZE5 Cell Analyzer was fantastic because it had an integrated plate loader and it was rock solid.

What we could do is we could put our samples on it in the morning and walk away from it and it would do all of the work itself, whereas in the past I used to have to sit there and do one tube then the next tube, for hundreds of tubes a day and I would maybe watch a movie or listen to a podcast, but it was pretty boring work, and so that was just fantastic.

More recently, we've benefited from the StarBright Dyes produced by Bio-Rad in our flow cytometry. Because these dyes have been designed with modern flow cytometers in mind, they usually have well-defined, bright colours, so to speak, that allows us to differentiate more dyes and thus get more information out of our data. They also lack some of the problems, such as interactions, instability or inaccuracies when used with compensation beads, that manifest with other families of dyes commonly used in flow cytometry. That just makes them easier to use.

Flow Cytometry

High-Resolution Human Immunophenotyping Panel Incorporating StarBright Dye–Conjugated Antibodies on the ZE5 Cell Analyzer

Sharon Sanderson, Richard Cuthbert, Michael Blundell, and Tracey Long
Bio-Rad Laboratories, Inc., Endeavour House, Kidlington, OX5 1GE, UK

Abstract

Flow cytometry is a powerful technique for collecting vast amounts of information from a single sample. As scientists have increasing demands seeking answers to complex biological questions, flow cytometry panels have progressively increased in size. To achieve high-resolution data from large panels, the panels must be well-designed, with antibodies against markers of interest conjugated to high-quality fluorophores. StarBright Dyes are bright with reduced spillover and are ideal for all flow cytometry experiments regardless of panel size and protocol. Here, using the five-laser (5-L) ZE5 Cell Analyzer flow cytometer from Bio-Rad, we tested StarBright Dyes alongside traditional fluorescent dyes in a large panel. All the instrument's 27 fluorescence channels were utilized, allowing for the simultaneous detection of 27 distinct markers and identification of numerous human peripheral blood subsets. Exceptional quality data with high-resolution of cell populations were obtained in standard and high-throughput mode.

Introduction

Over the last decade, flow cytometry has become more powerful with the ability to answer increasingly complex biological questions, due to the number of antigens and cellular functions that can be measured in a single sample. This increased utility has resulted from advancements in cytometer technology and the development of novel fluorophores that facilitate larger panels.

The StarBright Dye range from Bio-Rad includes fluorophores across all five common laser lines: StarBright UltraViolet (SBUV), StarBright Violet (SBV), StarBright Blue (SBB), StarBright Yellow (SBY), and StarBright Red (SBR) Dyes. The StarBright Dyes are conjugated to validated and highly cited antibody clones, exhibit narrow excitation and emission profiles, and work in all buffers. The benefits of these properties are demonstrated here in a panel that includes 22 StarBright Dyes and reveals their suitability for multiplexing.

The ZE5 Cell Analyzer has many features that make this cytometer ideal for immunophenotyping. These features include up to five high-quality liquid-cooled lasers and superior optics designed for optimal detection of fluorophores in each laser line. The analyzer can detect up to 27 fluorescence parameters and features a high-throughput mode, where wells are sampled continuously and acquired at a faster flow rate, allowing for rapid sampling even during complex immunophenotyping assays.

Materials and Methods

Staining Protocol

Human peripheral blood was first treated with Red Cell Lysing Buffer (Bio-Rad, catalog #BUF04) to remove red blood cells. The blood samples were then blocked in 10% human serum (Merck, #H4522) for 5 min at room temperature (RT), followed by incubation at RT for 1 hr with fluorescent dye–conjugated monoclonal antibodies, shown in Table 1. Following incubation, samples were washed three times in phosphate buffered saline (PBS) + 1% bovine serum albumin (BSA) (Merck, #A7906) and resuspended in 200 µl (for standard-mode acquisition) or 25 µl (for high-throughput mode acquisition) of PBS /BSA. Propidium iodide (PI) was added five min before acquisition.

For compensation controls, cells were incubated with a single antibody except in the case of antibodies conjugated to SBUV665, BUV421, SBV440, FITC, SBY720, A647, and A700 where UltraComp eBeads Compensation Beads (ThermoFisher Scientific Inc., #01-2222-41) were used. All antibodies were titrated before use and utilized at the optimal dilution.

Panel Design

Bio-Rad's Multicolor Panel Builder and Fluorescence Spectraviewer tools assisted with fluorophore selection in this complex panel. Best practices were followed to generate high-quality data:

- Specific markers were selected that allowed for the biological question to be answered. As the experimental aim was to identify major cell subsets in human peripheral blood, the appropriate markers to detect the major T-cell, B-cell, monocyte, and granulocyte lineages were identified
- The flow cytometer configuration was identified to determine which fluorophores could be detected. The 5-L ZE5 Cell Analyzer with 7 off the UV option A (Bio-Rad, #12014135) was used. Bio-Rads's Multicolor Panel Builder used in conjunction with the Bio-Rad's Multicolor Panel Building Poster identified appropriate fluorophores, based on the optimal laser excitation and emission wavelength
- Where possible, bright fluorophores were paired with markers with a low antigen density. For example, less abundant CD24 was detected with an antibody conjugated to SBV440 (having a relative brightness of 5). Conversely, dim fluorophores were paired with markers with a high antigen density. For example, the more abundant CD8 was detected with an antibody conjugated to SBR815 (having a relative brightness of 3)
- Fluorophore spillover and spread were considered to reduce spreading effects that could complicate analysis. As high spread can make it difficult to identify cells correctly, high-spreading fluorophore pairs were used on mutually exclusive markers (markers not detecting the same cell types). For example, SBR815 was used for CD8 on T cells, whereas SBR775 was utilized for CD19 on B cells. Additionally, as spreading effects are greater with a brighter signal, this impact was minimized by using a dimmer fluorophore, SBY665 (with a brightness of 3), with CD45, which is expressed on all cells of interest
- Rare cells were detected with antibodies conjugated to bright fluorophores. For example, an antibody conjugated to BV421 (with a relative fluorescence of 5) was used to identify less frequent CD56 positive natural killer cells
- A high-quality sample was used. Human peripheral blood was stained the same day the blood was drawn to avoid the presence of a high number of dead cells, which can give false positives. Additionally, PI a live/dead dye, was included to gate out any dead cells that were in the sample during the analysis

The list of antibodies and live/dead dye used in the panel are shown in Table 1.

Data Collection

Samples were acquired on a 5-L ZE5 Cell Analyzer with the UV option A, 355 nm laser upgrade. In standard mode, 300,000 cells were acquired for the multiplex panel and 60,000 cells for the single-stained controls at a rate of 1 µl/sec. For fully stained samples acquired in high-throughput mode, 15 µl sample volumes were acquired at 2 µl/sec.

Table 1. Reagents used.
Antibodies and the live/dead dye used in the multiplex panel.

Target	ZE5 Cell Analyzer Target Laser: Filter	Fluorophore	Antibody Catalog Number*
HLA DP DQ DR	355: 387/11	SBUV400	MCA477SBUV400
CD20	355: 509/24	SBUV510	MCA1710SBUV510
CD33	355: 577/15	SBUV575	MCA1271SBUV575
Live/dead dye	355: 615/24	PI	1351101
CD163	355: 670/30	SBUV665	MCA1853SBUV665
CD28	355: 747/33	SBUV740	MCA709SBUV740
CD62L	355: 780LP	SBUV795	MCA1076SBUV795
CD56	405: 420/10	BV421	318327 (Biolegend)
CD24	405: 460/22	SBV440	MCA1379SBV440
CD45RA	405: 525/50	SBV515	MCA88SBV515
CD45RO	405: 615/24	SBV610	MCA461SBV610
CD40	405: 670/30	SBV670	MCA1590SBV670
CD2	405: 720/50	SBV710	MCA1194SBV710
CD14	405: 750LP	SBV790	MCA1568SBV790
CD57	488: 525/35	FITC	MCA1305F **
CD3	488: 593/52	SBB580	MCA463SBB580
CD11b	488: 692/80	SBB700	MCA551SBB700
HLA ABC	488: 750LP	SBB810	MCA81SBB810
CD10	561: 583/30	SBY575	MCA1556SBY575
CD4	561: 615/24	SBY605	MCA1267SBY605
CD45	561: 670/30	SBY665	MCA87SBY665
CD27	561: 720/60	SBY720	MCA755SBY720
CD38	561: 750/LP	SBY800	MCA1019SBY800
CD16	640: 670/30	A647	MCA5665A647
CD31	640: 720/60	A700	MCA1738A700Di
CD19	640: 775/50	SBR775	MCA1940SBR775
CD8	640: 800LP	SBR815	MCA1226SBR815

*Antibodies are from Bio-Rad unless otherwise marked
**Not currently available for purchase. See #MCA1305GA for purified format.
Axxx, Alexa Fluor; BV421, Brilliant Violet 421; FITC, fluorescein isothiocyanate; PI, propidium iodide; SBB, StarBright Blue; SBR, StarBright Red; SBUV, StarBright UltraViolet; SBV, StarBright Violet; SBY, StarBright Yellow.

Gating Strategy

Analysis was performed using FCS Express 7 Software (De Novo Software by Dotmatics). Dead cells were first excluded from downstream analysis by gating on PI-negative cells. Doublet discrimination was used to identity single cells, followed by gating on CD45+ cells. Lymphocytes, monocytes, and granulocytes — the three major cell populations — were identified based on forward scatter area (FSC-A) and side scatter area (SSC-A) gating (Fig. 1). CD3+ and CD3– cells were then identified. These populations were used for downstream gating strategies to identify subpopulations as shown in Figures 2–5.

Results

An immunophenotyping panel was successfully attained without the use of a special buffer. Major T-cell, B-cell, monocyte, and granulocyte lineages were identified. Various subsets within these lineages were also clearly distinguished. Data in Figures 1–5 were from cells acquired on the ZE5 Cell Analyzer in standard acquisition mode. The spillover and spreading matrices are shown in the Appendix (Supplementary Tables 1 and 2).

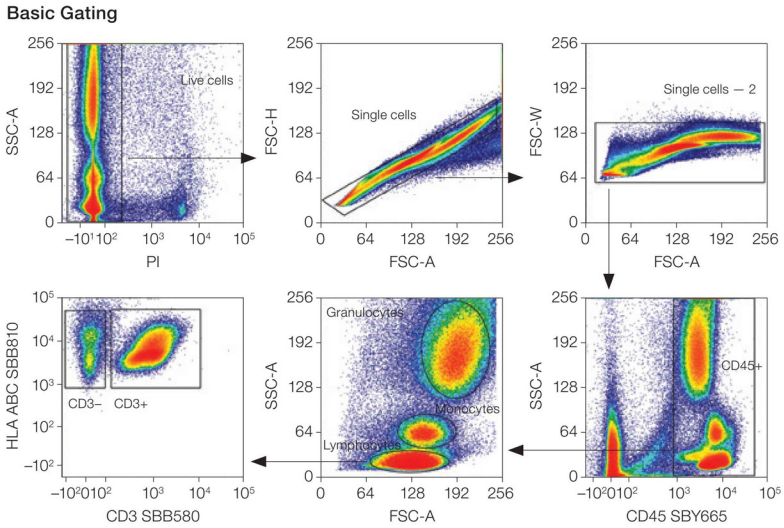

Figure 1. Basic gating strategy. Major lymphocyte (CD3+ and CD3–), monocyte, and granulocyte populations were identified after gating on live, single CD45+ cells. PI, propidium Iodide; SBB, StarBright Blue; SBY, StarBright Yellow.

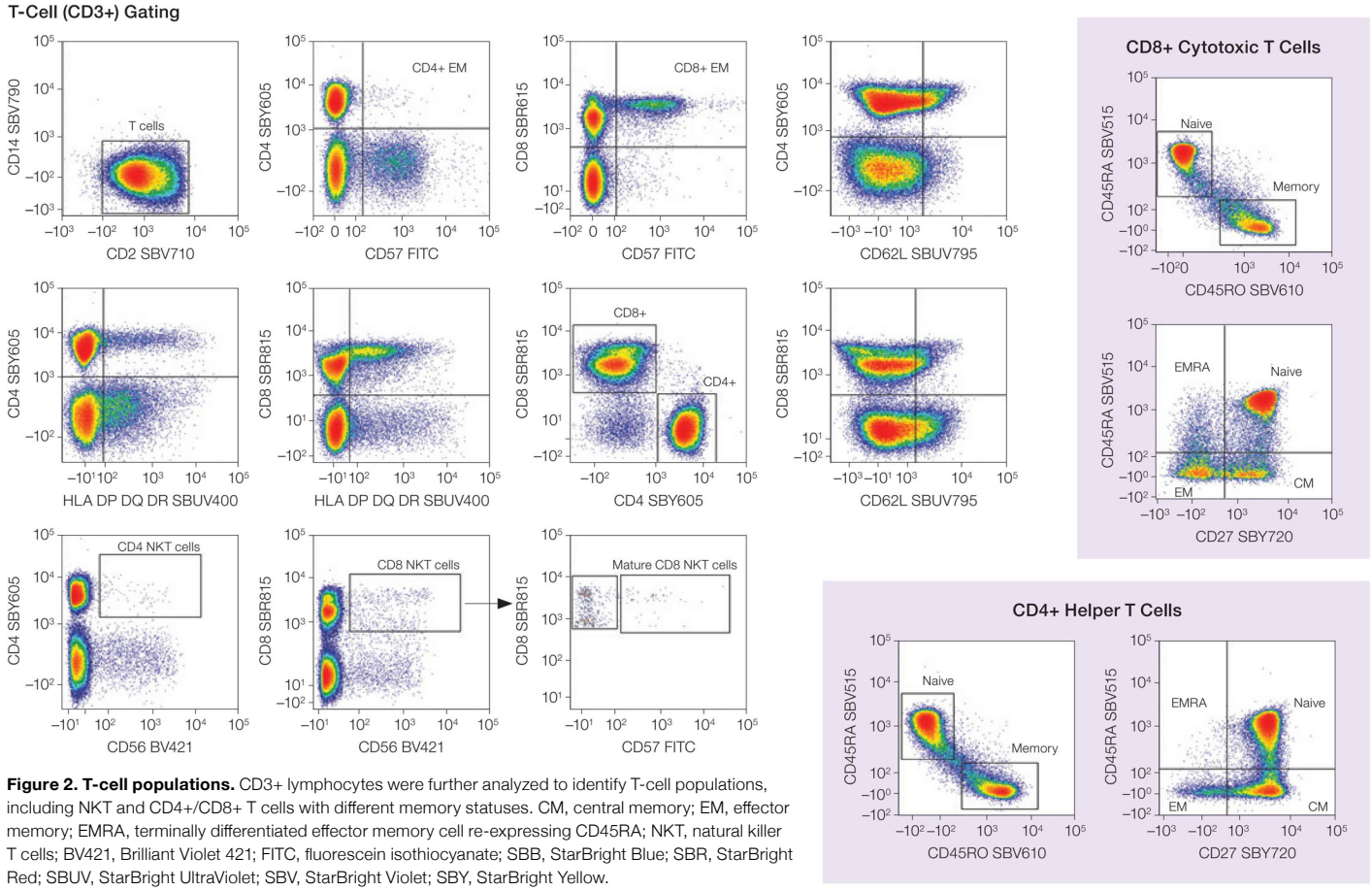

Figure 2. T-cell populations. CD3+ lymphocytes were further analyzed to identify T-cell populations, including NKT and CD4+/CD8+ T cells with different memory statuses. CM, central memory; EM, effector memory; EMRA, terminally differentiated effector memory cell re-expressing CD45RA; NKT, natural killer T cells; BV421, Brilliant Violet 421; FITC, fluorescein isothiocyanate; SBB, StarBright Blue; SBR, StarBright Red; SBUV, StarBright UltraViolet; SBV, StarBright Violet; SBY, StarBright Yellow.

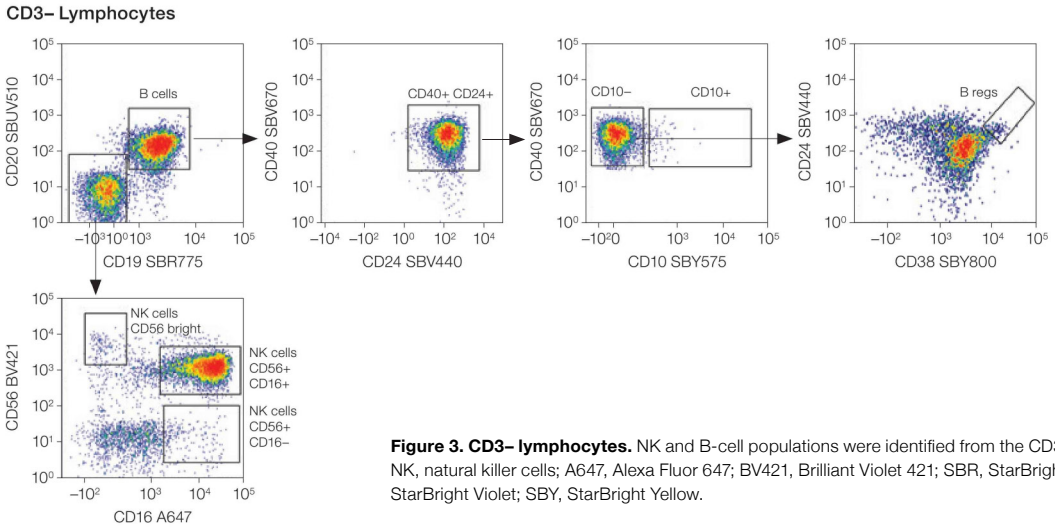

Figure 3. CD3– lymphocytes. NK and B-cell populations were identified from the CD3- lymphocytes. B regs, regulatory B cells; NK, natural killer cells; A647, Alexa Fluor 647; BV421, Brilliant Violet 421; SBR, StarBright Red; SBUV, StarBright UltraViolet; SBV, StarBright Violet; SBY, StarBright Yellow.

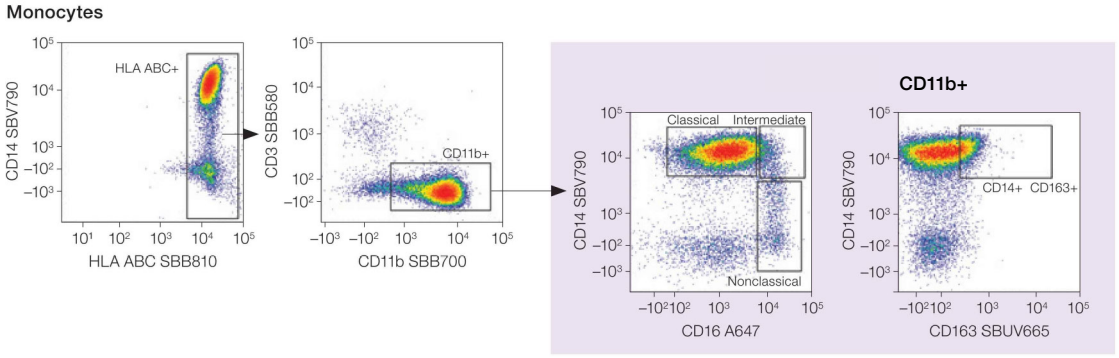

Figure 4. Monocytes. Classical, intermediate, and nonclassical monocyte subpopulations were identified from the monocytes. A647, Alexa Fluor 647; SBB, StarBright Blue; SBUV, StarBright UltraViolet; SBV, StarBright Violet.

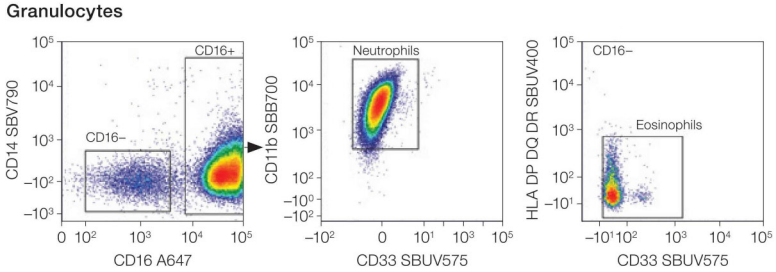

Figure 5. Granulocytes. Neutrophils and eosinophils were identified from the granulocytes. A647, Alexa Fluor 647; SBB, StarBright Blue; SBUV, StarBright UltraViolet; SBV, StarBright Violet

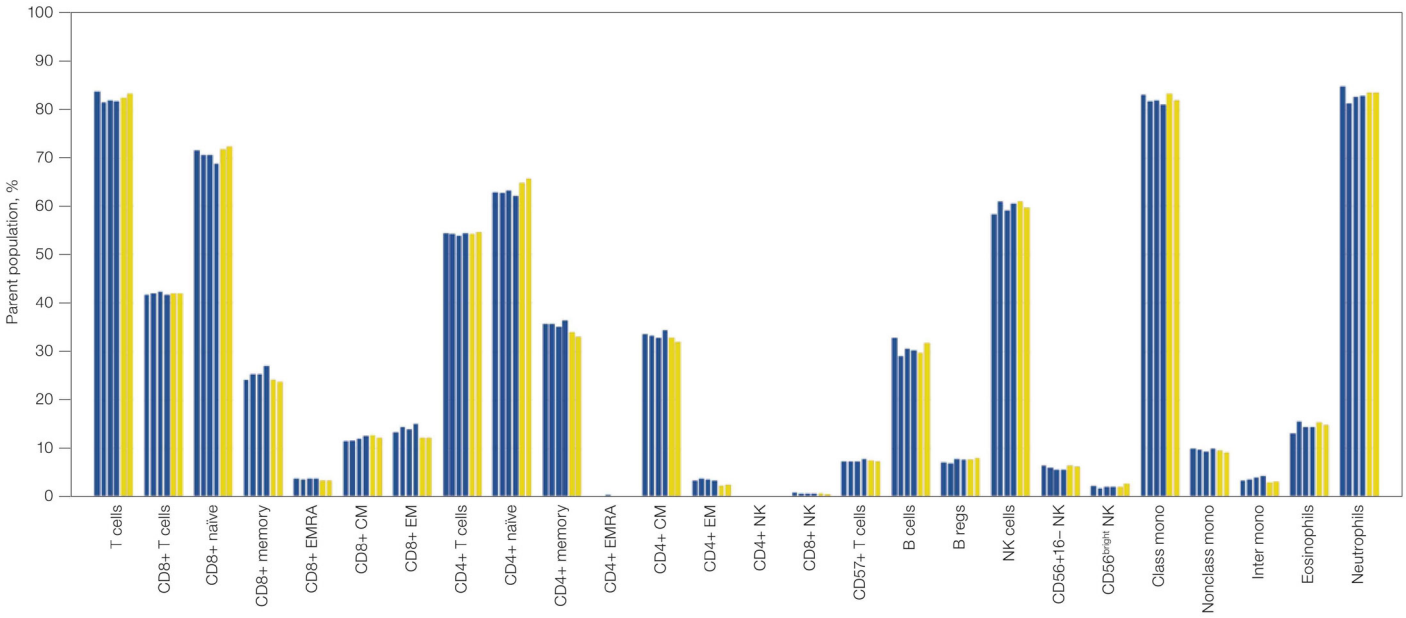

Figure 6. Comparison of data acquired in standard and high-throughput mode on the ZE5 Cell Analyzer. Cell lineages are expressed as a percentage of their parent cell population. Each bar represents an individual replicate collected in high-throughput mode (■ blue bars) and standard mode (■ yellow bars). B regs, regulatory B cells; Class mono, classical monocytes; CM, central memory T cells; EM, effector memory T cells; EMRA, terminally differentiated effector memory cell re-expressing CD45RA T cells; Inter mono, intermediate monocytes; NK, natural killer cells; NKT, natural killer T cells; Non class mono, nonclassical monocytes

Fully stained samples were also acquired in high-throughput mode and the same compensation matrix applied. The five samples were obtained in approximately 8 seconds per sample and data were consistent to that acquired in standard mode (Fig. 6).

Conclusions

A large 27-color immunophenotyping panel was successfully designed. The panel identified multiple cell populations present in human peripheral blood. In addition, high-quality data from the ZE5 Cell Analyzer was produced using standard or high-throughput mode with minimal variations between the two. Major observations included:

- **High cell resolution** — all populations were very clearly identified
- **Bright signals** — all the antibodies with a bimodal signal (distinct positive and negative populations) exhibited clear separation between the two populations
- **No special buffer requirement for multiplexing StarBright Dyes** — some polymer dyes require a special buffer when multiplexing to avoid interactions. StarBright Dyes work in these special buffers, but staining can also be performed in a basic staining buffer (here, PBS + 1% BSA) without interactions
- **Low spillover and spread values** — the use of StarBright Dyes (designed to have reduced signal in parts of the spectrum outside the target filter) resulted in low compensation values for a panel of this size. The highest compensation and spread values were from PI. As PI-positive cells were excluded in the first analysis step, this potential interference had negligible impact on the cell resolution. The highest compensation and spread values between fluorophore pairs were between SBR815 and SBR775. As these values were anticipated at the panel design stage, these fluorophores were used for markers on mutually exclusive cells to minimize effects on the panel
- **Reproducible data at speed** — results in high-throughput mode showed no little differences to those in standard mode across all measured populations, demonstrating that the ZE5 Cell Analyzer can acquire complex immunophenotyping data rapidly and accurately.

StarBright Dyes are an ideal choice for to include in multiplexing panels, as demonstrated on the ZE5 Cell Analyzer in both normal and high-throughput acquisition modes.

Appendix

	FITC	SBB580	SBB810	SBB700	SBUV665	SBUV740	SBUV795	SBUV510	SBUV400	PI	SBUV575	SBV670	SBV710	SBV790	SBV440	BV421	SBV610	SBV515	SBY665	SBY720	SBY800	SBY605	SBY575	A700	SBR775	SBR815	A647	Total
FITC	0.00	0.00	0.00	0.37	0.08	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.20	0.00	0.00	0.27	0.05	0.10	0.00	0.00	0.00	0.00	0.18	0.00	0.00	0.00	0.00	1.34
SBB580	0.28	0.00	0.89	1.68	0.83	0.51	0.46	0.00	0.00	0.42	0.29	0.81	1.36	0.66	0.00	0.00	0.76	0.11	0.85	0.67	0.50	3.14	2.10	0.23	0.31	0.00	0.51	17.37
SBB810	0.12	0.13	0.00	0.35	0.15	0.74	3.18	0.06	0.11	0.07	0.06	0.17	0.38	2.16	0.06	0.04	0.11	0.06	0.17	0.20	1.48	0.48	0.14	0.33	1.81	0.44	0.12	13.12
SBB700	0.16	0.25	2.00	0.00	0.72	1.42	1.54	0.00	0.21	0.12	0.12	0.80	2.44	1.88	0.25	0.24	0.27	0.21	0.74	2.00	2.33	1.46	0.34	1.59	2.35	0.30	0.63	24.37
SBUV665	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SBUV740	0.29	0.14	0.34	0.31	0.21	0.00	2.35	0.00	0.00	0.00	0.00	0.00	0.51	0.79	0.00	0.00	0.00	0.00	0.02	0.85	1.27	0.43	0.00	0.90	2.17	0.38	0.00	10.96
SBUV795	0.00	0.00	0.72	0.00	0.18	1.24	0.00	0.00	0.19	0.05	0.00	0.00	0.11	0.70	0.00	0.00	0.00	0.00	0.14	0.26	1.74	0.50	0.06	0.52	2.19	0.81	0.00	9.41
SBUV510	0.99	0.80	0.94	2.35	1.30	0.92	1.08	0.00	0.49	0.67	0.58	0.69	1.73	0.61	0.22	0.16	0.89	0.57	1.31	0.51	0.47	4.49	1.34	0.35	0.55	0.06	0.71	24.78
SBUV400	0.29	0.33	0.20	0.80	0.37	0.36	0.19	0.20	0.00	0.21	0.18	0.36	0.69	0.17	0.32	0.27	0.25	0.23	0.37	0.10	0.24	1.49	0.28	0.23	0.66	0.00	0.13	8.92
PI	0.00	1.46	3.42	5.60	2.97	2.65	2.45	0.03	0.00	0.00	0.34	1.77	4.30	1.74	0.07	0.26	1.31	0.00	4.58	5.11	4.26	9.02	3.27	1.32	0.96	0.00	1.61	58.50
SBUV575	0.42	0.90	1.34	2.44	1.00	0.41	1.21	0.36	0.20	1.23	0.00	1.29	2.84	0.21	0.58	0.00	1.33	0.11	2.51	1.48	1.06	4.58	2.91	1.21	0.70	0.00	1.97	32.29
SBV670	0.00	0.24	0.61	0.89	1.58	1.20	0.97	0.07	0.11	0.19	0.04	0.00	1.79	1.58	0.18	0.11	0.64	0.07	1.22	0.63	0.90	1.42	0.32	1.06	1.12	0.15	1.09	18.18
SBV710	0.21	0.20	0.69	0.68	0.49	1.49	1.63	0.00	0.09	0.17	0.10	0.80	0.00	1.83	0.15	0.09	0.22	0.11	0.43	0.97	1.18	1.06	0.10	1.20	1.57	0.34	0.40	16.20
SBV790	0.00	0.00	1.21	0.30	0.19	0.60	1.68	0.10	0.00	0.15	0.11	0.00	0.50	0.00	0.00	0.00	0.10	0.11	0.00	0.26	0.79	0.00	0.46	0.62	0.00	0.00	0.00	7.18
SBV440	0.06	0.00	0.12	0.38	0.09	0.00	0.00	0.17	0.00	0.13	0.13	0.13	0.31	0.15	0.00	0.23	0.13	0.24	0.07	0.08	0.00	0.56	0.18	0.00	0.00	0.01	0.00	3.17
BV421	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.01	0.00	0.00	0.06	0.10	0.00	0.00	0.56	0.00	0.00	0.16	0.06	0.00	0.00	0.26	0.00	0.00	0.00	0.00	0.00	1.31
SBV610	0.13	0.71	0.82	2.25	1.11	0.66	0.47	0.06	0.05	0.66	0.14	1.18	1.89	0.74	0.13	0.08	0.00	0.18	1.23	0.56	0.41	4.49	0.82	0.31	0.23	0.00	0.77	20.08
SBV515	0.29	0.27	0.30	0.85	0.39	0.27	0.17	0.16	0.00	0.25	0.19	0.41	0.67	0.34	0.24	0.07	0.41	0.00	0.44	0.20	0.08	1.53	0.46	0.12	0.08	0.04	0.27	8.50
SBY665	0.06	0.13	1.31	2.25	1.66	0.94	0.83	0.05	0.06	0.10	0.04	1.11	1.48	0.92	0.05	0.03	0.17	0.04	0.00	3.30	2.86	0.92	0.34	1.64	1.25	0.23	1.54	23.31
SBY720	0.00	0.04	2.21	1.51	0.15	0.84	0.47	0.02	0.00	0.05	0.00	0.74	0.65	1.17	0.00	0.05	0.00	0.00	0.21	0.00	1.27	0.15	0.25	1.81	2.02	0.21	0.38	14.20
SBY800	0.00	0.00	1.26	0.07	0.10	0.66	1.41	0.00	0.04	0.00	0.00	0.17	0.32	0.99	0.00	0.00	0.00	0.00	0.25	0.47	0.00	0.23	0.06	0.56	2.37	0.68	0.12	9.76
SBY605	0.05	0.31	0.40	0.99	0.50	0.27	0.18	0.00	0.02	0.25	0.05	0.50	0.79	0.31	0.00	0.00	0.49	0.00	0.73	0.45	0.52	0.00	1.24	0.21	0.09	0.00	0.32	8.67
SBY575	0.00	0.29	0.00	1.34	0.25	0.17	0.00	0.00	0.25	0.06	0.36	0.16	0.19	0.00	0.00	0.00	0.57	0.03	0.59	0.36	0.49	2.50	0.00	0.00	0.00	0.00	0.41	8.02
A700	0.00	0.00	0.22	0.08	0.00	0.67	0.94	0.00	0.00	0.00	0.00	0.00	0.32	0.40	0.00	0.00	0.00	0.00	0.00	0.64	0.56	0.00	0.00	0.00	3.95	0.65	0.25	8.68
SBR775	0.00	0.00	0.76	0.22	0.09	0.98	2.28	0.00	0.04	0.04	0.00	0.09	0.26	1.01	0.08	0.00	0.00	0.00	0.15	0.34	1.56	0.00	0.06	1.11	0.00	1.14	0.23	10.44
SBR815	0.11	0.09	2.79	0.31	0.19	3.02	13.36	0.00	0.33	0.05	0.05	0.21	0.45	3.22	0.09	0.06	0.06	0.05	0.18	0.39	4.77	0.34	0.10	2.34	16.23	0.00	0.33	49.12
A647	0.00	0.00	0.11	0.23	0.13	0.28	0.49	0.00	0.00	0.00	0.00	0.16	0.07	0.13	0.00	0.00	0.00	0.00	0.43	0.20	0.38	0.09	0.00	3.48	2.77	0.41	0.00	9.36

Suppl. Table 1. Spillover matrix. Values represent the amount of spillover for each fluorophore. The rows show the fluorophore, whereas the columns display the detector the signal is collected in. Colors progress from green to white to red as more spillover is present. Green indicates no or very low spillover through to white and red as more spillover is present. Axxx, Alexa Fluor; BV421, Brilliant Violet 421; FITC, fluorescein isothiocyanate; PI, propidium iodide; SBB, StarBright Blue; SBR, StarBright Red; SBUV, StarBright UltraViolet; SBV, StarBright Violet; SBY, StarBright Yellow.

	FITC	SBB580	SBB810	SBB700	SBUV665	SBUV740	SBUV795	SBUV510	SBUV400	PI	SBUV575	SBV670	SBV710	SBV790	SBV440	BV421	SBV610	SBV515	SBY665	SBY720	SBY800	SBY605	SBY575	A700	SBR775	SBR815	A647
FITC	1.00	0.17	0.00	0.01	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SBB580	0.10	1.00	0.07	0.37	0.07	0.03	0.01	0.00	0.00	0.13	0.14	0.17	0.09	0.03	0.00	0.00	0.41	0.02	0.00	0.00	0.01	0.33	0.82	0.00	0.00	0.00	0.00
SBB810	0.02	0.02	1.00	0.04	0.00	0.05	0.33	0.00	0.00	0.00	0.00	0.01	0.02	0.47	0.00	0.00	0.01	0.00	0.00	0.00	0.21	0.00	0.00	0.00	0.05	0.01	0.00
SBB700	0.00	0.02	0.72	1.00	0.08	0.29	0.14	0.01	0.01	0.01	0.01	0.17	0.81	0.37	0.01	0.00	0.01	0.01	0.16	0.66	0.59	0.01	0.00	0.43	0.14	0.00	0.05
SBUV665	0.00	0.00	0.06	0.21	1.00	0.53	0.18	0.00	0.01	0.02	0.00	0.28	0.16	0.01	0.00	0.00	0.00	0.00	0.54	0.22	0.20	0.01	0.00	0.44	0.14	0.01	0.61
SBUV740	0.00	0.00	0.01	0.00	0.01	1.00	0.44	0.00	0.03	0.00	0.00	0.00	0.06	0.09	0.00	0.00	0.00	0.00	0.00	0.02	0.06	0.00	0.00	0.13	0.13	0.00	0.00
SBUV795	0.00	0.00	0.07	0.00	0.00	0.21	1.00	0.00	0.04	0.00	0.00	0.00	0.01	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.28	0.00	0.00	0.00	0.10	0.01	0.00
SBUV510	0.84	0.09	0.01	0.03	0.19	0.14	0.10	1.00	0.33	0.36	0.65	0.04	0.02	0.02	0.04	0.00	0.09	0.62	0.01	0.00	0.01	0.08	0.22	0.00	0.00	0.00	0.00
SBUV400	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.03	1.00	0.01	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PI	0.00	1.20	1.51	4.63	1.81	1.20	0.54	0.00	0.00	1.00	0.12	0.40	0.30	0.14	0.00	0.00	0.27	0.00	3.77	2.46	2.24	6.51	2.87	0.00	0.00	0.00	0.00
SBUV575	0.00	0.11	0.00	0.04	0.43	0.18	0.07	0.10	0.12	0.73	1.00	0.07	0.02	0.00	0.02	0.00	0.14	0.02	0.09	0.04	0.01	0.51	1.11	0.01	0.00	0.00	0.01
SBV670	0.00	0.00	0.03	0.11	0.44	0.24	0.08	0.00	0.01	0.03	0.00	1.00	0.52	0.26	0.03	0.00	0.11	0.01	0.18	0.07	0.07	0.03	0.01	0.10	0.03	0.01	0.18
SBV710	0.00	0.00	0.05	0.06	0.06	0.39	0.21	0.00	0.00	0.01	0.00	0.16	1.00	0.50	0.03	0.00	0.02	0.01	0.02	0.16	0.15	0.00	0.00	0.25	0.08	0.00	0.01
SBV790	0.00	0.00	0.05	0.00	0.00	0.14	0.34	0.00	0.00	0.00	0.00	0.01	0.08	1.00	0.03	0.01	0.01	0.01	0.00	0.00	0.16	0.00	0.00	0.01	0.10	0.00	0.00
SBV440	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.01	0.01	0.01	0.00	0.00	1.00	0.12	0.02	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BV421	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.48	1.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SBV610	0.01	0.06	0.01	0.05	0.13	0.03	0.01	0.00	0.00	0.27	0.03	0.36	0.13	0.04	0.02	0.00	1.00	0.06	0.06	0.02	0.01	0.38	0.16	0.00	0.00	0.00	0.00
SBV515	0.10	0.01	0.00	0.00	0.01	0.00	0.00	0.06	0.00	0.03	0.06	0.04	0.02	0.01	0.10	0.00	0.13	1.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
SBY665	0.00	0.01	0.15	0.50	0.25	0.15	0.05	0.00	0.00	0.01	0.09	0.29	0.17	0.08	0.00	0.00	0.01	0.00	1.00	0.44	0.43	0.10	0.02	0.13	0.05	0.00	0.16
SBY720	0.00	0.00	0.32	0.29	0.01	0.24	0.13	0.00	0.00	0.00	0.00	0.02	0.33	0.18	0.00	0.00	0.00	0.00	0.07	1.00	1.07	0.02	0.00	0.47	0.19	0.01	0.04
SBY800	0.00	0.00	0.30	0.01	0.00	0.04	0.12	0.00	0.00	0.00	0.00	0.00	0.01	0.15	0.00	0.00	0.00	0.00	0.02	0.04	1.00	0.00	0.00	0.01	0.13	0.01	0.00
SBY605	0.00	0.02	0.00	0.02	0.03	0.01	0.00	0.00	0.00	0.06	0.00	0.05	0.02	0.01	0.00	0.00	0.14	0.00	0.17	0.05	0.03	1.00	0.43	0.00	0.00	0.00	0.00
SBY575	0.00	0.06	0.00	0.02	0.01	0.00	0.00	0.00	0.00	0.02	0.03	0.01	0.00	0.00	0.00	0.00	0.02	0.00	0.05	0.02	0.01	0.32	1.00	0.00	0.00	0.00	0.00
A700	0.00	0.00	0.01	0.01	0.00	0.04	0.01	0.00	0.00	0.00	0.00	0.00	0.04	0.02	0.00	0.00	0.00	0.00	0.00	0.07	0.06	0.00	0.00	1.00	0.37	0.02	0.01
SBR775	0.00	0.00	0.09	0.00	0.00	0.14	0.26	0.00	0.00	0.00	0.00	0.00	0.02	0.16	0.00	0.00	0.00	0.00	0.00	0.02	0.29	0.00	0.00	0.22	1.00	0.09	0.02
SBR815	0.00	0.00	0.89	0.01	0.00	0.29	2.60	0.00	0.00	0.00	0.00	0.00	0.03	1.17	0.00	0.00	0.00	0.00	0.00	0.03	2.37	0.00	0.00	0.31	4.25	1.00	0.02
A647	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.03	0.02	0.00	0.00	0.88	0.24	0.01	1.00

Suppl. Table 2. Spreading matrix. Values indicate the spillover spreading (SS) amount for each fluorophore into all detectors. The rows show the fluorophore-donated SS, whereas the columns display the detector-collected SS. Colors progress from green to white to red as more spreading is present. Green indicates no or very low spreading and red indicates more spreading. Axxx, Alexa Fluor; BV421, Brilliant Violet 421; FITC, fluorescein isothiocyanate; PI, propidium iodide; SBB, StarBright Blue; SBR, StarBright Red; SBUV, StarBright UltraViolet; SBV, StarBright Violet; SBY, StarBright Yellow.

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Interview with Dr. Theresa Schwaiger

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Dr. Schwaiger is a distinguished biochemist and expert in tumor immunology and infectious disease research. She began her academic career at Ernst-Moritz-Arndt-University Greifswald, where she completed her diploma thesis on T cells in autoimmune pancreatitis. After earning her Ph.D., where she studied tumor rejection and Newcastle disease virus, she worked on African Swine Fever Virus control at the Friedrich Loeffler Institute. Dr. Schwaiger then led a significant project examining pigs as models for coinfections before taking on her current role as Principal Scientist at ViraTherapeutics in 2020, focusing on immune responses to oncolytic virus infections and advancing virotherapy research.

Dr. Schwaiger's academic journey began at the Ernst-Moritz-Arndt-University Greifswald, in Germany, where she studied Biochemistry from October 2006 to June 2011. During her studies, she completed a diploma thesis titled "The Role of T Cells in the Pathogenesis of Autoimmune Pancreatitis in MRL/Mp Mice," in collaboration with the Laboratory for Gastroenterology of the University Medicine of Greifswald.

Following her undergraduate studies, Dr. Schwaiger pursued a Ph.D. at the same university in partnership with the Friedrich Loeffler Institute. From April 2013 to January 2017, she focused on evaluating the mechanisms of tumor rejection in mouse models following infection with the Newcastle disease virus. Her research contributed valuable insights into the intersection of virology and cancer immunotherapy.

After completing her Ph.D., Dr. Schwaiger continued at the Friedrich Loeffler Institute as a Research Associate from December 2016 to March 2017. In this role, she contributed to a biosecurity project funded by the Federal Foreign Office, aimed at controlling the spread of the African Swine Fever Virus (ASFV) in collaboration with the Ukrainian partner institution IVM-NAAS.

Her career progressed as she became a Junior Research Group Leader at the Friedrich Loeffler Institute in Greifswald from April 2017 to April 2020. She led the "KoInfekt" project, a collaborative effort involving 14 laboratories in Mecklenburg-Western Pomerania. The project's goal was to evaluate the pig as a biomedical model for coinfections through clinical, immunological, and infectious parameters.

Since May 2020, Dr. Schwaiger has been serving as the Principal Scientist leading the tumor immunology team at ViraTherapeutics in Innsbruck, Austria. Her work involves evaluating immune responses *in vitro*, in preclinical models, and fresh human tumor samples following oncolytic virus infections. She specializes in designing and conducting flow cytometric analysis, *ex vivo* restimulation assays, and ELISpots to advance the field of oncolytic virotherapy.

Dr. Schwaiger's extensive background in biochemistry, virology, and tumor immunology underscores her commitment to understanding and combating complex diseases through innovative scientific approaches.

What motivated you to pursue a career in immunology and virology?

When I started studying biochemistry, I didn't know in which direction it would go. But already after my first immunology lecture, I was fascinated by the topic. This was also because our professor already discussed many current studies and research approaches with us in the lectures and seminars, so that theory could be quickly linked with current practice. The virology lecture, on the other hand, honestly put me off at the beginning, because it initially included pure taxonomy. What still

fascinates me most is how sophisticated the immune system works and how many molecular alternative pathways exist to make any dysfunctions in the entire system seem irrelevant. Classical/molecular virology has never been the main component of my work, it is always the interaction with the immune system.

Could you share a specific research project that you found particularly exciting or impactful?

I worked for three years as a junior group leader in a big collaboration project which included several labs at the Friedrich Loeffler Institute, the University of Greifswald, and the University of Rostock to study the co-infections of influenza A viruses with bacterial pathogens of a secondary infection (*Streptococci* and *Staphylococci*). The aim was to elucidate the pathogen-host interactions, the course of the disease, and the immune response of the host to identify new strategies for control and prevention. As these co-infections occur not only in humans but also in pigs, we worked on establishing the pig as a biomedical infection model for the co-infections. For me, it was particularly exciting to use a completely different animal model and even more, the fact that all laboratories involved in the project pulled together to collect and make the best use of the generated samples. Thus, many different methods, as well as a wide variety of starting materials were used to obtain information (including flow cytometry, imaging, Multi-omics approaches in various organs, but also trace gas analysis in the breath or the influence on the coagulation system). Not only a very exciting project thematically but also the excellent cooperation in so many different disciplines impressed and inspired me.

Can you discuss any collaborations or partnerships you've had with other researchers or institutions?

Almost all the projects I was working on were collaboration projects involving at least two research institutions but I would like to highlight the following two:

a. The KoInfekt project included an extensive and productive collaboration with the following researchers and their team members [1–6]: Prof. Uwe Völker (Functional Genome Research), Prof. Barbara M. Bröker (Immunology), Prof. Michael Lalk (Biochemistry), Prof. Katharina Riedel (Microbiology), Prof. Dörte Becher (Microbiology), and Prof. Ulrike Seifert (Medical Microbiology) from the University Medical Center Greifswald; Prof. Bernd Kreikemeyer (Medical Microbiology, Virology, and Hygiene), Prof. Brigitte Müller-Hilke (Immunology), and Prof. Jochen Schubert (Anesthesiology and Intensive Therapy) from the University Medical Center Rostock; and Prof. Tim Urich (Microbiology) and Prof. Lars Kaderali (Bioinformatics) from the University of Greifswald, collaborated on the project.

b. My doctoral thesis was done in collaboration with the Laboratory for Gastroenterology under the direction of Prof. Julia Mayerle and Markus M. Lerch and the Laboratory for Immunology led by Ulrike Blohm at the Friedrich Loeffler Institute [7–9].

What inspired you to investigate the oncolytic properties of Newcastle disease virus (NDV) in pancreatic cancer?

In the beginning, I slipped into this project more through my expertise. There was already a collaboration between the Laboratory of Gastroenterology at the University Medical Center Greifswald, where I started as a doctoral student and the laboratory of Dr. Ulrike Blohm at the Friedrich Loeffler Institute. In the first experiment, mice with an experimentally induced pancreatic tumor were treated with NDV (since it is an animal disease virus, it was only possible to work at this site). At that time, Ulrike Blohm's laboratory needed hands-on help in carrying out and evaluating this first experiment and I already had experience in flow cytometry and animal experiments through my diploma thesis. This first trial was very promising and showed a sharp decline in tumor burden in the treated animals. Both Ulrike Blohm's and my enthusiasm were aroused and the curiosity to find out why and in what way this rejection was conveyed strengthened the cooperation. In the end, fortunately, this project became my doctoral thesis [7]. The discrepancy that this virus can be fatal to poultry on the one hand, but on the other hand can destroy malignant mammalian cells and spare healthy tissue in the process, is fascinating and the result of numerous interlocking mechanisms of the immune system. Thanks to the good collaboration with Ulrike Blohm, we continued to scientifically supervise students together who were working on this project while I was junior research group leader in the Coinfect project. The NDV project never let me go and was also the reason why I ultimately applied to ViraTherapeutics GmbH. My curiosity for the immunological processes involved in virotherapy remains unabated.

How do you envision oncolytic viruses like VSV-GP being integrated into current cancer treatment regimens?

I believe that oncolytic viruses, and in particular veterinary viruses, have a great potential to reactivate the immune system that has become blind or restrained by the tumor without letting it overshoot since healthy human cells are not suitable host cells for these viruses. Virus-induced cell death is significantly more immunogenic and tumor-specific proteins presented in the context of a viral infection (and thus in a pro-inflammatory milieu) have a greater potential to generate an effective adaptive immune response. In my opinion, the combination of OVs with e.g., checkpoint inhibitors (PD-1/PD1-L) is particularly promising, as they not only promote the activation of the immune system but also temporarily remove the inhibitory component from the system. Therefore, I consider OVs to be very suitable in combination therapies, as the complexity of the immune response is joined by the complex mechanisms of the tumor environment.

In your study on influenza A virus infections in pigs, what were the key similarities and differences you found compared to human infections?

Similarities:

- The overall course and the absence of clinical symptoms are comparable to a mild influenza infection
- Transient decrease of lymphocytes and increase of monocytes in blood with faster recovery after the second infection
- Increase of counts and expression of cytotoxic molecules in ab and gd T cells in mucosa

Differences:

- Several gd T cells are not at all comparable, all effects observed in this population cannot be compared to humans
- No change in blood neutrophil number in pigs
- Increase in dendritic cell populations in the blood [1]

Your work on autoimmune pancreatitis (AIP) in mice suggests potential alternative treatments. How soon do you think these findings could translate into clinical practice?

As mice are not humans and it is always a long road from preclinical studies in rodents to using these agents in clinical practice, I find it very difficult to estimate the time frame. The differences between the mouse models (and not only concerning the species but also because of the different disease induction) and the human disease are sometimes enormous. Especially because the studies carried out in patients even tend to favor azathioprine (which was less promising in our mouse studies) [10–14].

How have Bio-Rad's instruments and reagents contributed to your research?

Bio-Rad's ZE5 Cell Analyzer has significantly simplified my daily work and that of my team and has led to the generation of data from preclinical studies being reliable and timely. Three points impressed us the most or that made the biggest difference for us in terms of workload and time:

1. The setting on the device that prevents air from entering the system
2. The method and intensity with which the device resuspends the samples (by shaking the whole plate and NOT sucking them up, which always leads to clogging)
3. The high event rate, which is not accompanied by reduced separation, so that we can measure even highly concentrated samples quickly and yet reliably

As far as Bio-Rad's reagents are concerned, it is the StarBright Dyes. For us, especially because of its stability (also in master mixes), we can prepare master mix in advance for colleagues who are unfamiliar with flow cytometry. This massively minimizes our own hands-on time.

What techniques or methodologies do you find most valuable in your work?

Definitely flow cytometry (be it conventional or spectral) and in particular the use of tetramers/dextramers, which enables the analysis of specific T cells. Especially in combination with Single cell RNA Seq.

Could you elaborate on any recent breakthroughs or discoveries in your field?

I find it difficult to limit this to one result or one discovery here. As you can see in many recent preclinical and clinical studies, that show promising results, the approaches are very diverse [15–18]. But I believe that this is exactly where the strength of this field lies. What they generally have in common is that most of them are combined approaches (as already mentioned in point 5). In the end, it is likely that only the combined knowledge of why some approaches work and others do not will lead to the development of an optimal therapeutic approach. I have doubts that there is a universal agent that is effective in all tumor entities. However, I am confident that the better we understand the WHY (why some approaches are effective in some entities and why others are not), the more likely it is that we will find suitable therapies for individual diseases.

How do you prioritize research directions in a field as broad as cancer immunotherapy or virology?

The decision on which research direction to focus is not mine alone, but the results we generate in the laboratory and in preclinical studies have a considerable influence on strategic decisions at Boehringer Ingelheim. Internally at ViraTherapeutics, new ideas and concepts are first discussed and, if the response is positive, the first experiments are carried out. If these are successful, we use the opportunity to draw on the experience and skills of the employees throughout the Boehringer Ingelheim company network.

What advice would you give young scientists interested in pursuing research in virology and immunology?

Don't let yourself be unsettled by the sheer infinite complexity of the systems, but ask yourself an explicit question. And then step by step. Science thrives on curiosity, excitement in discovery, and collaboration.

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