Whitepaper: 25-Marker Spectral Flow Panel for Immune Profiling in Fixed Whole Blood

25-Marker Spectral Flow Panel for Immune Profiling in Fixed Whole Blood

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Abstract

Clinical trials rely on high-parameter flow cytometry to quantify immune populations in patient samples before, during, and after treatment. However, inconsistencies in sample collection methods, shipping delays and site-to-site variability lead to loss of roughly ~10-15% of samples and harm data quality. Furthermore, whole blood specimens lose ~30% of live cells after 48 hours and ~50% after 72 hours. While ~70-80% of whole blood specimens are processed live for flow cytometry, the challenges mentioned above along with up to 50% cell loss during sample shipping highlight the need to improve this workflow. Here, we describe a 25-color spectral flow cytometry panel designed for identification of 146 human immune phenotypic and functional marker subpopulations in whole blood collected with sodium heparin and fixed using the TokuKit. The TokuKit uses the Stable-Lyse V2, Stable-Store V2 buffer system distributed by SmartTube (Las Vegas, USA) and enables centrifugation-free whole blood processing in 20 minutes. The ability of the panel to identify immune populations on stabilized, cryopreserved whole blood is highly comparable to whole blood processed live, with a correlation of R=0.97. Furthermore, the cryopreserved blood is stable at -80 degree Celsius for at least one month, with 34/35 (97.1%) of phenotypic populations showing less than 25% change compared to Day 0. We investigated longer term stability via artificially ageing samples to 12 and 24 months. The performance of this panel on stabilized whole blood is highly reproducible with a median %CV of 4.56% (compared to benchmark of 20%) across all measured phenotypic populations when samples are analyzed on different days by different operators.

By enabling investigation of major and minor immune subsets, including T cells, B cells, NK

cells, and myeloid cells, this workflow offers an alternative to live cell processing for global clinical trials.

Introduction

High-parameter flow cytometry enables detailed immune-phenotyping, with 20+ marker panels allowing hundreds of immune subpopulations to be analyzed (representing up to 16X increase in resolution over conventional flow). For clinical trials, this technological progress provides the ability to identify treatment and dose-related effects in participants' immune systems. Coupling insights into immune activation with response data is a powerful way to help guide the decision making process during clinical trials. To date, high-dimensional cytometry in clinical trials suffers from challenges with sample stability, the logistics around sample collection, and the complexity of data analysis. Sample stability is limited since an estimated 70% of whole blood specimens for flow cytometry are processed while the cells are live, restricting the processing window to 24 - 72 hours and requiring a nearby cytometry laboratory. While fixation can preserve cells and extend this processing window, it has been limited in cytometry due to its tendency to disrupt epitopes, preventing some antibodies from recognizing their target[1,2]. Various reagents and blood collection tubes have been developed to overcome limitations in sample stability, such as Cyto-Chex® BCT (Streck, La Vista, USA), Cytodelics® (Cytodelics, Södertälje, Sweden), Transfix® (Cytomark, Buckingham, UK) and SmartTube® (Las Vegas, USA). Many reports have assessed and compared the stabilizing abilities and compatibility with cytometry of these reagents, with varying outcomes[3-5]. Combining previous research as well as our own internal investigation into these options, we developed the TokuKit, a blood specimen stabilization kit to simplify sample collection and extend specimen shelf life. The TokuKit is based on the Stable-Lyse V2, Stable-Store V2 buffer system manufactured by SmartTube®. This kit enables sample storage at -80°C and solves critical challenges in sample stability and associated logistic difficulties by fixing whole blood samples while ensuring compatibility with our high-dimensional cytometry panels. When paired with the Spectral Flow Assay described herein, TokuKit enables profiling of 146 immune cell populations, achieving frequencies comparable to those seen with

live cell staining. This approach is well-suited for large, multi-center clinical trials, enabling long-term (herein tested 1 month as well as 12 and 24 months accelerated ageing) sample storage, batching of samples, and centralized processing.

We adapted this panel from a validated PBMC profiling assay developed by Cytek Biosciences (Fremont, CA, USA) and optimized for use with fixed, cryopreserved whole blood. The design enables analysis of 146 immune populations and functional marker subsets. The panel leverages spectral flow cytometry to resolve overlapping fluorochromes, allowing for high-dimensional analysis with minimal spillover.

The primary objective of this panel is to enable immunoprofiling beyond traditional TBNK profiling with a particular emphasis on T-cell subsets. The panel was optimized to allow whole blood preservation using our TokuKit. TokuKit fixation is a 2-step process, built to stabilize 2 mL of whole blood for long term storage at -80°C, without the requirement of centrifuges or other specialized equipment such as pipettes. This simple, 2-step procedure can be performed at any clinical site with access to either a -80°C freezer or dry ice.

We compared each gated population to live samples to confirm the panel's ability to accurately distinguish all intended subsets on fixed samples. To ensure robustness across biological variability, whole blood samples from multiple donors were processed and analyzed. Additionally, we tested the panel's reproducibility by having different laboratory personnel perform experiments on separate days, demonstrating consistency across operators and time points.

Results

By 72 hours, live storage of blood samples results in ~50% loss of cells and disproportionately affects myeloid cells

Live processing of clinical trial blood specimens for flow cytometry generally comes with a limited stability window of 0 - 72 hours[3]. Given that 72 hours is a prolonged time for blood cells to survive outside of the human body, we first investigated the live cell count of whole blood cells after 0, 24, 48 and 72 hours at room temperature in a sodium heparin vacutainer. We detected a

statistically significant loss of ~16% in cell numbers as early as 24 hours (p= 0.0353). By 72 hours, live cell count reduced by roughly 50% (p=0.001; **Figure 1A**). We next investigated whether this cell loss was equal across major immune populations, and whether phenotypic frequencies change after up to 72 hours of storage at room temperature. We found that, across all three donors, not only the live cell count decreased, but the immune composition changed over time. Relative frequencies for both T- and B-cells were relatively stable over 72 hours, and no significant changes were observed in Natural Killer (NK)-cell frequencies. However, we found that certain myeloid cell frequencies were especially affected over time. At 0 hours, neutrophils constituted around 70 - 80% of total leukocytes, but after 72 hours, their frequency dropped by half to ~40% of total leukocytes. Even more drastic was the loss of monocytes, which started out at approximately 20% of all non-granulocytes. After 24 hours, monocyte frequency was reduced to less than 5%, and by 72 hours there were only 2.7% monocytes present within all non-granulocytes (**Figure 1B**). Dendritic cell (DC) frequencies did not show a significant change over time.

Apart from the logistical challenges live samples impose on clinical trials, the observed statistically significant decline in monocytes as soon as 24 hours after collection underscores the need for immediate whole blood preservation at the clinical site to ensure comparability of cytometry data between patients. This becomes especially important when looking at cells outside of T- or B-cells, and is crucial when studying certain myeloid cell types.

Teiko's TokuKit provides simplified whole blood collection for clinical trials

Teiko's 25-marker spectral flow panel was designed for use on whole blood samples collected from human donors with TokuKit (Teiko, Salt Lake City, USA). The collection process is illustrated in **Figure 2**. The kit contains all materials needed for whole blood processing, no additional equipment with the exception of personal protective equipment (PPE) is needed. After collecting blood into a sodium heparin vacutainer, 2 mL of blood is mixed with "Buffer 1", using the transfer pipette supplied within the kit. The blood-buffer 1 mix is then incubated for 15 minutes at room temperature to fix leukocytes and lyse red blood cells. After incubation, the

blood-buffer mixture is transferred into “Buffer 2”, to quench the fixation reaction and prevent overfixation. From there, the user has 15 minutes to transfer the specimen into a -80°C freezer or dry ice.

This collection method in combination with the panel described herein allows for measurement of frequencies of 40 phenotypic human immune populations, including T cells, B cells, NK cells, and myeloid cells. Additionally, 106 functional marker positive subpopulations can be resolved (e.g. PD-1+ T-cells, CD8+ NK-cells). The list of gated phenotypic populations and functional marker subpopulations can be found in **Figure 4**.

Optimization of the panel and staining protocol is required for fixed blood samples

Fixation has previously been described to hamper target detection for certain epitopes[3-5] and we hypothesize that this has largely inhibited broader adoption of fixation for cytometry samples in clinical trials. On the other hand, many mass cytometry assays are indeed staining fixed cells, which led us to investigate fixation for our flow cytometry panel. We therefore tested each antibody in the panel to ensure their ability to detect epitopes in fixed whole blood. For two antibodies (CD25 and CD127), we tested multiple clones before finding a fixation-compatible option (see **Figure 3A**). Interestingly, we found that the identification of γδT-Cells benefited from adding cell permeabilization despite the target being expressed on the cell surface (**Figure 3B**). This suggests that permeabilization can improve the accessibility of surface targets in fixed samples. However, fixation and mainly permeabilization alter the scatter profile of blood cells, resulting in the need for a neutrophil marker, such as CD66b, for gating as neutrophils can no longer be gated under forward/side scatters.

Gating strategy for 25-color whole blood spectral flow panel

A complete gating strategy can be found in **Figure 4**. Gating begins with the exclusion of neutrophils from all CD45+ cells using CD66b. Basophils are subsequently removed based on the markers HLA-DR- and CD123+. B-cells are identified using CD19 or CD20 and further characterized into subpopulations: B-memory cells (switched and unswitched), B-naive cells,

marginal zone B-cells, and plasmablasts, based on the expression of IgD, IgM, CD27, and CD38. T-cells are identified as $CD3^+$ cells and subclassified into γδ T-cells, NKT cells, CD4+, or CD8+ subsets using γδTCR, CD56, CD4, and CD8. CD4+ and CD8+ T-cell subsets are further delineated into T-central memory, T-naive, T-effector memory, or Effector memory RA-positive (TEMRA) cells by analyzing the expression of CD27 and CD45RA. Among the remaining CD3- CD19- CD20- cells, NK cells are identified by CD56 expression and further classified into cytolytic, non-cytolytic, and cytokine-producing NK cells based on CD16 and CD56 expression levels. Monocytes are identified as HLA-DR+ and categorized into classical monocytes (CD14+ CD16-), intermediate monocytes (CD14+ CD16+), and nonclassical monocytes (CD14- CD16+). Dendritic cells (DCs) are gated from the HLA-DR+ CD16- CD14- population and further characterized into classical DCs, transitional DCs, or plasmacytoid DCs using CD11c and CD123. Classical DCs can be further subdivided into cDC Type 1 and cDC Type 2 using the markers CD1c and CD141.

Apart from phenotypic markers, the panel also includes six functional markers, indicating activation or exhaustion status on specific subsets. The functional markers can be found in **Figure 4B** along with the specific immune cell subsets they were assessed in. Some of these markers serve dual-purpose, such as CD8 or CD25, which is used as a phenotypic marker on T-cells while also representing a functional marker on NK-cells or B-cells, respectively.

Phenotypic immune cell populations are comparable (R=0.97, n=3) between TokuKit processed and live whole blood samples

The performance of the panel was rigorously evaluated through several approaches. First, we determined the effects of fixation. We tested the performance of the panel on whole blood that had been stabilized and cryopreserved and compared the staining patterns to blood cells that had been stained live. **Figure 5A** illustrates dot plots for phenotypic immune populations comparing live staining to samples that were stained after TokuKit processing and cryopreservation for 30 days. After confirming both approaches yield similar results when inspected visually, we compared relative cell frequencies between the two processing methods.

We found the correlation between live and TokuKit samples to be excellent with a Pearson correlation of R=0.97 (**Figure 5B**) across three donors. Individual donor correlations were R= 0.97, 0.99 and 0.96.

We then compared frequencies of functional marker positive subsets (e.g. PD-1+ CD4 T-cells) between samples processed live and with TokuKit, and found the correlation to be very strong as well with R=0.91, across three donors, with individual donor correlations of R=0.9, 0.97, 0.86 (**Figure 5C**).

We assessed specificity of each marker by determining the frequency of marker positive events in samples where the target marker was absent. To do so, we created fluorescence-minus-multiple following the design rationale previously described[6]. We prepared four different FMM cocktails, each missing 5-6 antibodies (see Supplementary Data). All markers were detected at less than 1% frequency of total events when the marker was absent from the panel, confirming high specificity of each marker within the panel. The average marker positive frequency in absence of the actual antibody resulted in 0.08% (refer to Supplementary Data for the full list).

TokuKit samples remain stable after storage in -80°C

We evaluated post-fixation stability for TokuKit processed whole blood specimens to ensure test results are not affected by storage-related variables. We assessed stability using whole blood specimens from three healthy donors. We collected whole blood, processed with TokuKit, split into replicates for each time point, and stored the aliquots at -80°C. We ran sets of replicates on Day 0 and after 1 month in storage at -80°C. We also performed accelerated stability studies, where samples were artificially aged at higher temperature to represent a 12- or 24-month old specimen. Our results showed that after one month of storage, 97.1% of phenotypic populations experienced an absolute percent change of less than 25%, with an average absolute median change of 7.88% across all populations analyzed (**Figure 6A**). Only one population showed a greater than 25% change when comparing samples analyzed after 1 month of storage to Day 0, namely the Plasmablasts. Plasmablasts are a rare B-cell population, and median cell counts

within this population were 302 cells. Low frequency populations tend to show higher variation. After 12 and 24 months of storage (accelerated), the average absolute median change across all populations was 10.2% and 10.9% respectively, indicating that the phenotypic populations can safely be identified at similar frequencies after storage (**Figure 6C and E**). Two phenotypic populations showed a greater than 25% change in their relative frequency at both the 12 and 24 month accelerated time points, namely cytolytic (CD16 negative) NK cells and unswitched B-memory cells, suggesting that these two populations may be susceptible to a greater amount of variation over time than others. After 24 months of accelerated ageing, the double-negative T-cells (CD4- CD8-) as well as double-positive T-cells (CD4+ CD8+) are just outside of the 25% threshold with relative changes of +27.9% and -26.6% change, respectively, compared to Day 0. Similarly, two monocytic subsets, namely intermediate Monocytes (CD14+ CD16+) and non-classical Monocytes (CD14- CD16+) showed variation of +54.7% and -35.8% change to D0, respectively. Accurately separating different subsets of monocytes can be difficult because the levels of CD14 and CD16 on their surface are not clearly distinct (see **Figure 4C**, Monocyte gate).

Within functional marker populations, we found that 86.6% of populations showed an absolute change of less than 25%, with an average absolute median change of 10.7% (**Figure 6B**). After 12 and 24 months of storage (accelerated), the average absolute median change across all functional marker populations was 12.5% and 21.9% when compared to D0 (**Figure 6D and F**). Interestingly, at both the 12 and 24 month accelerated timepoints, the majority of populations that fell outside the set threshold of 25% change showed an increase in relative population frequency compared to Day 0. This potentially indicates that changes over time were largely influenced by gating thresholds, rather than biological samples losing marker expression.

TokuKit samples maintain single-digit inter- and intra-run precision

We next examined the performance of the panel by testing its reproducibility. To do so, we performed intra-run and inter-run studies. In our intra-run studies, the same biological sample was split into three aliquots and processed by the same operator on the same day, while the

inter-run study compared running aliquots of the same biological specimens on different days, processed by different operators. Precision analyses were performed with specimens from three whole blood donors. An overview of all precision testing results can be found in Table 2. Precision for the intra-run study resulted in a median %CV of 3.38% for phenotypic populations. Two out of three donors each had one population with a CV. For functional marker subsets, the median %CV was 5.50%. For precision studies, we also compared the variance of marker expression levels for functional markers by looking at median fluorescence intensity (MFI) between runs in marker-positive subpopulations. Intra-run comparisons showed a median %CV of 3.04% for MFI variance.

The inter-run precision, where a sample was split into aliquots and processed on different days by different operators, resulted in a median %CV of 4.56% for phenotypic populations and a median %CV of 7.28% for functional marker subsets. Variance for functional marker expression assessed via MFI showed a median %CV of 4.08%.

Table 2: Precision results

		Median CV [%]
Intra-run (n=3)	Phenotypic Immune Cell Frequency	3.38
	Functional Marker Frequency - Frequency of Parent	5.50
	Functional Marker Frequency - MFI	3.04
Inter-run (n=3)	Phenotypic Immune Cell Frequency	4.56
	Functional Marker Frequency - Frequency of Parent	7.28
	Functional Marker Frequency - MFI	4.08

Discussion

The development of a high-parameter spectral flow cytometry panel compatible with stabilized whole blood represents a significant advancement for immune monitoring in clinical trials. Clinical trials have relied on fresh whole blood for ~70% of specimens processed in flow cytometry, limiting sample stability and imposing logistical constraints on multi-site studies. Here, we demonstrate that our 25-marker spectral flow panel, when paired with the TokuKit stabilization system, enables reliable immune profiling from cryopreserved whole blood while maintaining high concordance with live-cell staining.

Our findings confirm that live whole blood specimens exhibit significant cell loss over time, with a 50% reduction in live cell count after 72 hours. This reduction, combined with site-to-site variability in processing, contributes to the high (~10-15%; Source: private discussions with translational scientists) sample attrition rates observed in clinical trials. By stabilizing whole blood using the TokuKit, these challenges are mitigated, preserving immune cell frequencies and functional marker expression profiles for extended periods. A critical aspect of panel optimization was ensuring compatibility with fixation, as fixation has been known to alter epitope recognition for certain markers. To address this, we rigorously tested antibody clones and staining protocols. Additionally, careful clone selection was necessary to ensure accurate detection post-fixation. These optimizations highlight the importance of validation when adapting live-cell protocols for use with fixed specimens.

Another challenge introduced by fixation is the alteration of light scatter properties, which can obscure traditional gating strategies. To circumvent this, we incorporated CD66b as a neutrophil exclusion marker, allowing for precise immune population identification. Our gating strategy effectively mimicked standard immune phenotyping approaches, enabling robust classification of lymphoid and myeloid subsets, including T-cell memory populations, B-cell subsets, dendritic cells, and monocytes.

Ultimately, stabilized samples showed a strong correlation with live-cell processing (R=0.97 for

phenotypic markers and R=0.91 for functional markers), confirming that fixation does not introduce significant artifacts.

Beyond phenotypic accuracy, the stability and reproducibility of our panel were assessed over time and across multiple operators. Stability studies revealed that 97.1% of phenotypic populations exhibited less than 25% change after one month at -80°C, with an average absolute median change of 7.88%. Functional marker subsets showed slightly greater variation, with 86.6% remaining within 25% of baseline values. These findings support the feasibility of long-term sample storage and centralized batch analysis. Our analysis of artificially aged samples, representing either 12 month or 24 month storage at -80C (91% and 83% of populations with <25% change compared to Day 0, respectively), suggest that phenotypic stability might extend well beyond the one month naturally aged samples tested herein. As time progresses, further timepoints are needed to determine the maximum shelf life of TokuKit processed whole blood.

Intra-run and inter-run precision studies demonstrated low variability, with single-digit median CVs (3.38% and 4.56% for phenotypic populations), respectively. This high level of reproducibility underscores the panel's suitability for multi-center clinical trials.

In summary, the TokuKit-enabled spectral flow panel provides a robust solution for immune monitoring in clinical trials, offering a streamlined workflow that overcomes the limitations of live-cell processing. By ensuring compatibility with fixed whole blood, maintaining strong correlation with live samples, and enabling long-term storage with minimal loss of data integrity, it is ideal for large-scale, multi-site immune profiling with improved logistical feasibility.

Materials and Methods

Table 1. Reagents

Marker	Fluorochrome	Clone	Purpose

CD66b	Spark UV™ 387	G10F5	Neutrophils
CD25	BUV™ 661	M-A251	Regulatory T cells (Treg), B-cell activation (functional marker)
CD127	BUV™ 737	eBioRDR5	Regulatory T cells (Treg)
CD45RA	cFluor® V450	HI100	T cell memory subsets
CD20	cFluor® V547	2H7	B cells
CD141	cFluor® B515	M80	Dendritic cells (cDC1)
CD8	cFluor® B532	SK1	Cytotoxic T cells
CD14	cFluor® B548	63D3	Monocytes
HLA-DR	cFluor® B690	L243	Monocytes, Activated T cells (functional marker)
CD4	cFluor® YG584	SK3	T helper cells
CD16	cFluor® BYG610	3G8	Natural Killer cells (NK), Monocytes, Neutrophils
IgD	cFluor® BYG667	IA6-2	Naive B cells

TCRγδ	cFluor® BYG710	B1	γδ T cells
CD11c	cFluor® BYG781	3.9	Dendritic cells (cDC)
CD1c	cFluor® R668	L161	Dendritic cells (cDC2)
CD19	cFluor® R685	HIB19	B cells
CD123	cFluor® R720	6H6	Plasmacytoid dendritic cells (pDC)
CD45	cFluor® R780	2D1	Leukocytes
CD27	cFluor® R840	QA17A18	Memory B cells, Naive T cells, Central Memory T cells
IgM	Brilliant™ Violet 510	MHM-88	B cell subsets
CD3	Brilliant™ Violet 570	UCHT1	T cells
CD28	Brilliant™ Violet 650	CD28.2	T cell activation
CD38	Brilliant™ Violet 711	HIT2	Plasma cells, Activated T cells (functional marker)
CD56	Brilliant™ Violet 750	5.1H11	Natural Killer cells (NK)

PD-1	Brilliant™ Violet 785	EH12.2H7	Exhausted T cells (functional marker)

Sample collection and TokuKit processing

We collected whole blood specimens from consented, deidentified healthy volunteers into a sodium heparin vacutainer. We split the samples into one arm for live cell processing and a second arm using the TokuKit processing according to manufacturers' instructions. Briefly, we transferred 2mL of blood to Buffer 1 and incubated for 15 minutes at room temperature. After incubation, we transferred the entire mixture from buffer 1 into buffer 2. We then stored the samples at -80°C until analysis.

General Staining protocol

For samples processed live, we processed the blood on the day of collection. We lysed red blood cells (RBC) using Ammonium-Chloride-Potassium (ACK) lysis buffer (made in house).
For TokuKit processed samples, we retrieved the samples from -80°C, thawed them in a 37C water bath until only a small piece of ice was left and inverted several times until the sample was completely thawed.
We pelleted cells by centrifugation for 5 minutes at 4C. We next removed the supernatant and washed in an equal volume of BD Pharmingen™ Stain Buffer (BD stain buffer). After counting, we aliquoted 3 million cells per sample to receive multicolor staining and collected 0.3 million cells per sample as an unstained control.

Live cell staining protocol

After aliquoting, we washed the live cells in Phosphate Buffered Saline (PBS) twice before adding a viability dye to stain dead cells for 15 minutes at room temperature (Viadye, Cytek Biosciences). We next washed the cells twice in BD stain buffer and blocked Fc receptors for 10 minutes with Human TruStain FcX™ (Biolegend) before adding 5uL of cFluor BYG710 TCR gd (Cytek). We mixed well by pipetting, and incubated at room temperature for 20 minutes with

gentle agitation. After incubation, we added the rest of the antibody cocktail, containing 24 additional antibodies (see Table 1) at 5ul per test in BD Horizon™ Brilliant stain buffer plus (BD Biosciences), and incubated again for 20 minutes with gentle agitation at room temperature. We next removed unbound antibodies by washing the cells twice in BD stain, before fixing the cells with 1.6% paraformaldehyde (PFA) for 20 minutes at room temperature. After fixation, we washed the cells twice in BD stain buffer.

TokuKit staining protocol

Next, we washed cells with 1mL of 1X eBiosciences permeabilization buffer (Thermo Fisher) diluted in type 1 water. After one wash, we added an additional 1mL of 1X eBiosciences permeabilization buffer to the samples and incubated for 10 minutes at room temperature with gentle agitation to permeabilize the cells. Afterwards, we pelleted cells and resuspended in BD stain buffer. We blocked Fc receptors for 10 minutes with Human TruStain FcX™ (Biolegend). Next, we added 5uL of cFluor BYG710 TCR gd (Cytek) to the cell suspension, mixed well by pipetting, and incubated at room temperature for 20 minutes with gentle agitation. We added the remaining antibodies (Table 1) at 5ul/test in BD Horizon™ Brilliant stain buffer plus (BD Biosciences) to the samples and incubated for 20 minutes protected from light with gentle agitation. After incubation, we washed samples twice in BD stain buffer to remove unbound FABs.

Preparation of single color reference control samples

We prepared single color reference controls for each run, following the same staining protocol as described above. We generated individual sets of reference controls for either live cell processing or TokuKit fixed samples.

Spectral flow cytometry analysis

We conducted spectral flow cytometry analysis using a Cytek Aurora. Prior to each experiment, we calibrated the instrument using the SpectroFlo® QC Beads (Cytek Biosciences) to ensure optimal sensitivity and alignment of the detectors.

We acquired the cells in a 96-well plate format or in individual test tubes, depending on the sample size, using SpectroFlo. We processed the cells at a flow rate of 1,000-3,000 events per

second at low speed.

We performed spectral unmixing using the SpectroFlo® software which employs a linear unmixing algorithm. We gated FSC files using Cell Engine. We analyzed cell subsets of interest using the gating strategy in **Figure 4C**, and we performed quantitative analysis to calculate the frequency and fluorescence intensity of populations.

Artificial ageing of samples

To produce specimens that reflect long term storage of whole blood samples, we artificially aged samples using recommendations from the Accelerated Ageing Calculator available through Packaging Compliance Labs (link). In order to produce a specimen resembling storage of 12 months at -80°C, we placed the samples in -30°C for 12 consecutive days before analysis. For samples mimicking 24 months of storage at -80°C, we placed them at -30°C for 23 consecutive days.

Statistical analysis

We performed all analyses in Python (v3.9), using SciPy v1.11.3 for Pearson correlation and regression analyses. To evaluate how well stabilized whole blood preserved immune subset distributions, we computed Pearson correlation coefficients (R) between TokuKit-fixed and live-processed samples. We performed regression analyses to quantify systematic shifts, using identity plots (y = x) to visualize deviations. We flagged populations with significant variation beyond the ±25% threshold as falling out of specification.

We assessed reproducibility through intra-run and inter-run precision analyses. To determine intra-run precision, we processed aliquots of the same donor sample on the same day. For inter-run precision, we analyzed samples processed on different days or by different operators. We calculated the coefficient of variation (CV) for each immune subset as:

$$CV(\%) = \left(\frac{\sigma}{\mu}\right) \times 100$$

where σ represents the standard deviation and μ is the mean frequency of the population across replicates. We excluded populations with fewer than 100 cells in two or more subjects from CV calculations. To summarize variability, we computed the median %CV across all populations,

applying an acceptance threshold of 20% for intra-run and 25% for inter-run. We visualized these results using CV scatter plots, plotting population size against %CV with acceptance thresholds overlaid.

To evaluate the stability of TokuKit-fixed samples, we compared immune subset frequencies at baseline (Day 0) to post-storage time points at –80°C. We calculated the percent change relative to baseline using:

$$\%\ Change\ = \left(\frac{Frequency\ at\ time\ point - Frequency\ at\ baseline}{Frequency\ at\ baseline}\right) \times\ 100$$

We set an acceptance threshold of ≤25% absolute percent change. To quantify variability across donors, we calculated the standard error of the mean (SEM) for each subset. We visualized stability trends using scatter plots, mapping population size versus % change with acceptance thresholds labeled.

Data availability

All measurements used to calculate correlation between live and TokuKit samples, stability of TokuKit samples, as well as intra-run and inter-run precision measurements can be found within the Supplementary Data tables.

Figure Legends

Figure 1. Live storage of blood samples results in loss of cell number and immune cell population frequencies. (A) Graph of cell count of whole blood samples stored at room temperature (25C) for 0, 24, 48, and 72 hours. Significance values are indicated. **(B)** Immune cell frequencies calculated as percent of non-granulocytes (for B cell, T cell, NK cell, Monocyte, and Dendritic cell populations) or leukocytes (for Neutrophil) for three whole blood donors (Donor 1, Donor 2, Donor 3) after 0, 24, 28, and 72 hours of storage at room temperature.

Figure 2. Teiko's Tokukit provides simplified whole blood collection for clinical trials. (A) Schematic of the whole blood collection process using the TokuKit (1.). Blood samples are collected in a vacutainer at the clinical site (2.). After blood collection, the vacutainer is inverted 10 times (3.) and 2mL of blood is transferred to the stable lyse buffer, buffer 1, and incubated for 15 minutes at room temperature (4.). The entire mixture from buffer 1 into the stable store buffer, buffer 2 (5.). Samples can then be stored at -80°C or shipped on dry ice.

Figure 3. Optimization of the panel and staining protocol is required for fixed blood samples. (A) Density plots comparing CD127 staining in live samples versus TokuKit samples with and without permeabilization and change in antibody clone. **(B)** Density plots comparing γδTCR staining in live samples versus TokuKit samples with and without permeabilization.

Figure 4. Gating strategy for 25-color whole blood spectral flow panel. (A) Gating tree illustrating the immune populations gated and number of functional markers analyzed within respective immune cell subsets. **(B)** Chart of functional markers analyzed for each cell type on Teiko's spectral flow cytometry standard 25-color whole blood panel. **(C)** Gating scheme illustrating the strategy for gating TokuKit-fixed whole blood samples on Teiko's spectral flow cytometry standard 25-color whole blood panel.

Figure 5. Phenotypic immune cell populations are comparable (R=0.97, n=3) between TokuKit processed and live whole blood samples. (A) Density plots of immune cell populations and subsets (B Cells, T Cells, NK Cells, Dendritic Cells, and Monocytes) compared between live and TokuKit-fixed samples. **(B)** Correlation of population frequencies and **(C)** functional marker subset frequencies between TokuKit-fixed and live samples.

Figure 6. TokuKit samples remain stable after storage in -80˚C (A,C,E) Scatter plots of median percent change in population frequency at 1 month, accelerated 12 months, and accelerated 24 months of storage versus 0 months, respectively. **(B,D,F)** Scatter plot of median percent change in functional marker frequency at 1 month, accelerated 12 months, and accelerated 24 months of storage, respectively.

Figure 7. TokuKit samples maintain single-digit inter- and intra-run precision. (A) Scatter plots of intra-run %CV of population frequencies as a percentage of non-granulocytes, **(B)** %CV of functional markers as a percentage of parent populations, and **(C)** %CV for median fluorescence intensity (MFI) of functional markers for three donors (Donor 1, Donor 2, Donor 3). **(D)** Scatter plots of inter-run %CV of population frequencies as a percentage of non-granulocytes, **(E)** %CV of functional markers as a percentage of parent populations, and **(F)** %CV for median fluorescence intensity (MFI) of functional markers for three donors (Donor 1, Donor 2, Donor 3).

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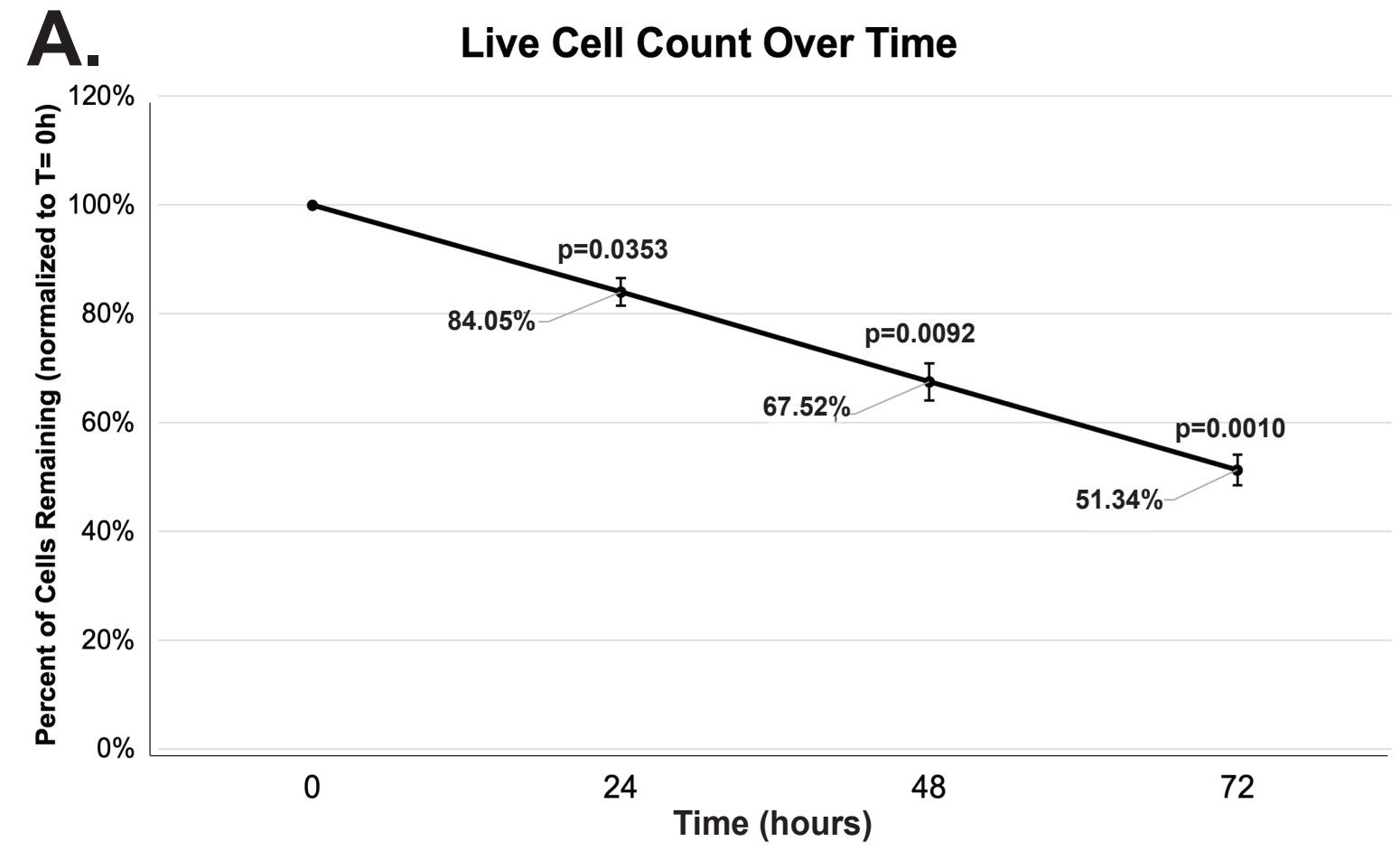
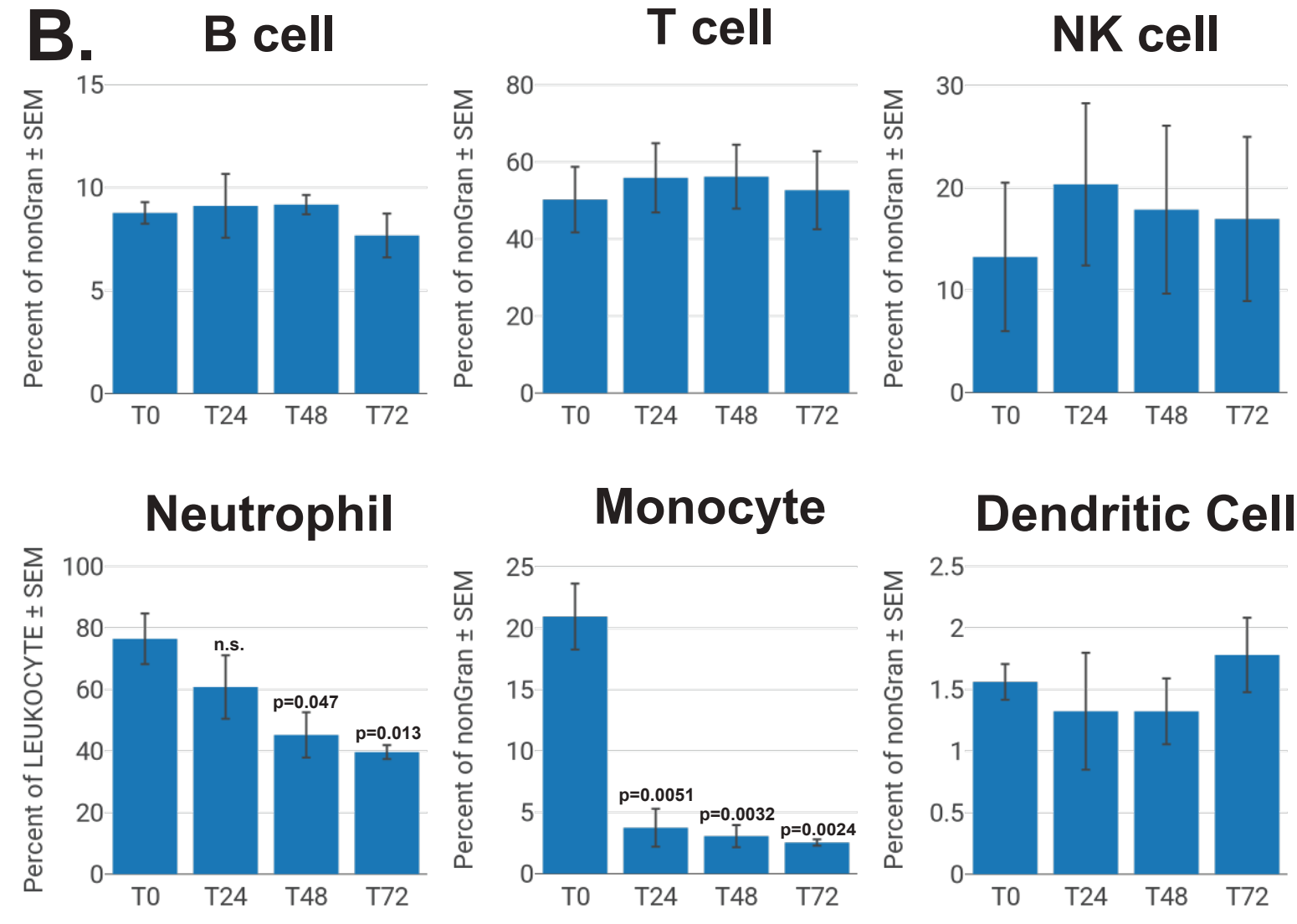
Figure 1


A.
Live Cell Count Over Time
Percent of Cells Remaining (normalized to T= 0h)
120%
100%
80%
60%
40%
20%
0%
p=0.0353
84.05%
p=0.0092
67.52%
p=0.0010
51.34%
0
24
48
72
Time (hours)

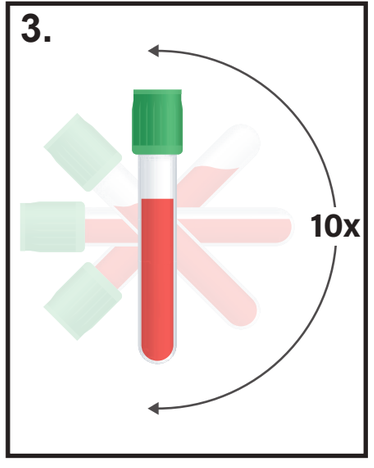

B.
B cell
T cell
NK cell
Percent of nonGran ± SEM
T0
T24
T48
T72

Neutrophil
Monocyte
Dendritic Cell
Percent of LEUKOCYTE ± SEM
Percent of nonGran ± SEM
n.s.
p=0.047
p=0.013
p=0.0051
p=0.0032
p=0.0024
T0
T24
T48
T72

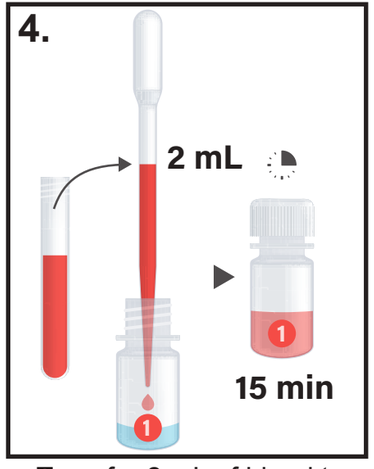
Figure 2

Use Teiko's TokuKit for sample collection and downstream processing

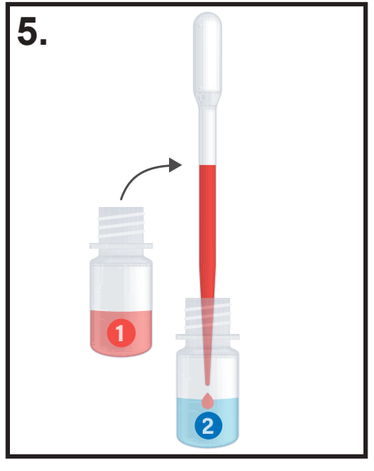
Collect blood in vacutainer at clinical site



Invert vacutainer 10x to mix well



Transfer 2 mL of blood to buffer 1;Incubate for 15 minutes at room temperature

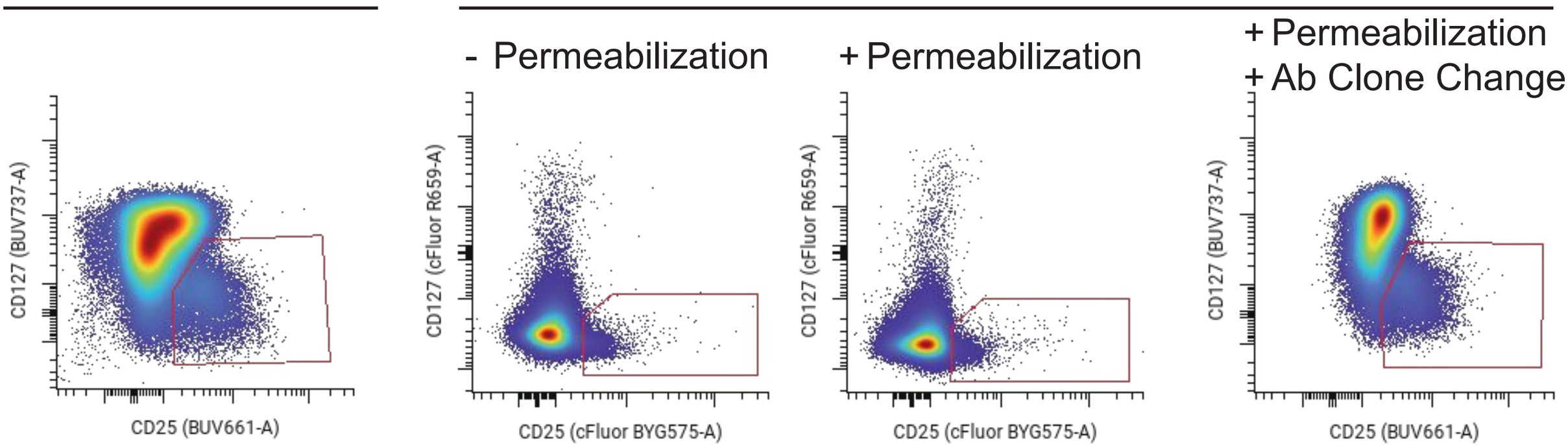


Transfer entire mixture from buffer 1 to buffer 2

Store sample at -80°C or ship on dry ice

Figure 3

A.


Live
TokuKit
- Permeabilization
+ Permeabilization
+ Permeabilization
+ Ab Clone Change
CD127 (BUV737-A)
CD25 (BUV661-A)
CD127 (cFluor R659-A)
CD25 (cFluor BYG575-A)
CD127 (cFluor R659-A)
CD25 (cFluor BYG575-A)
CD127 (BUV737-A)
CD25 (BUV661-A)

B.

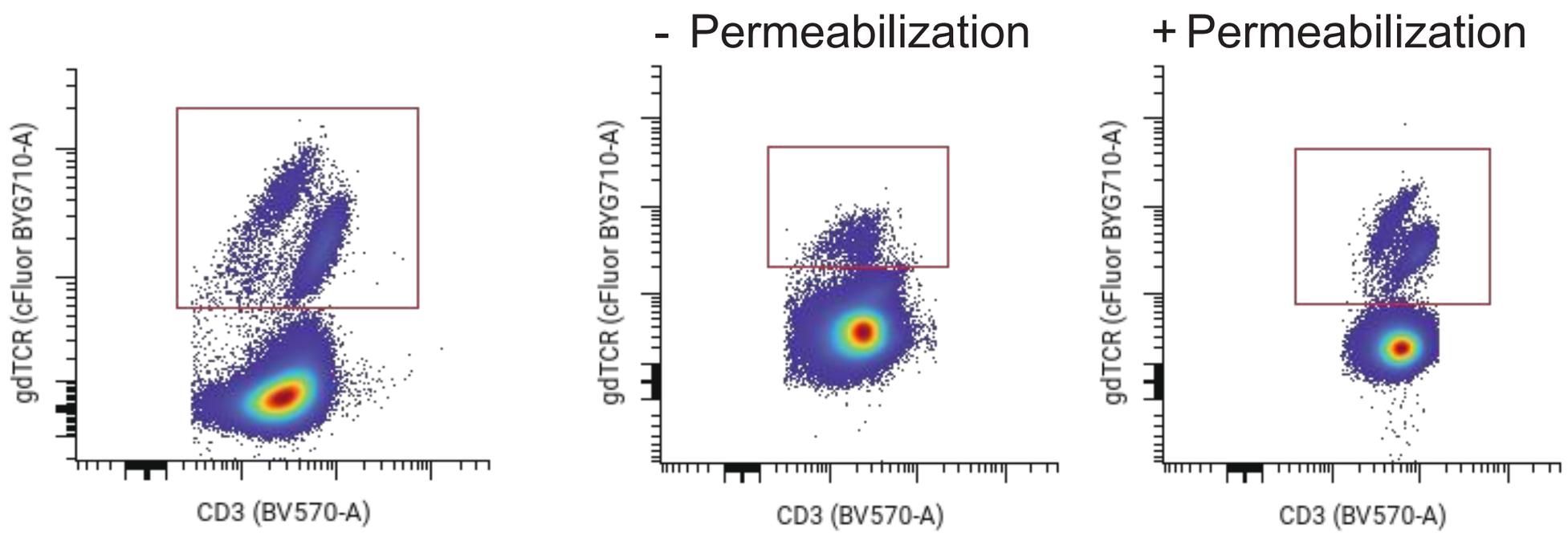
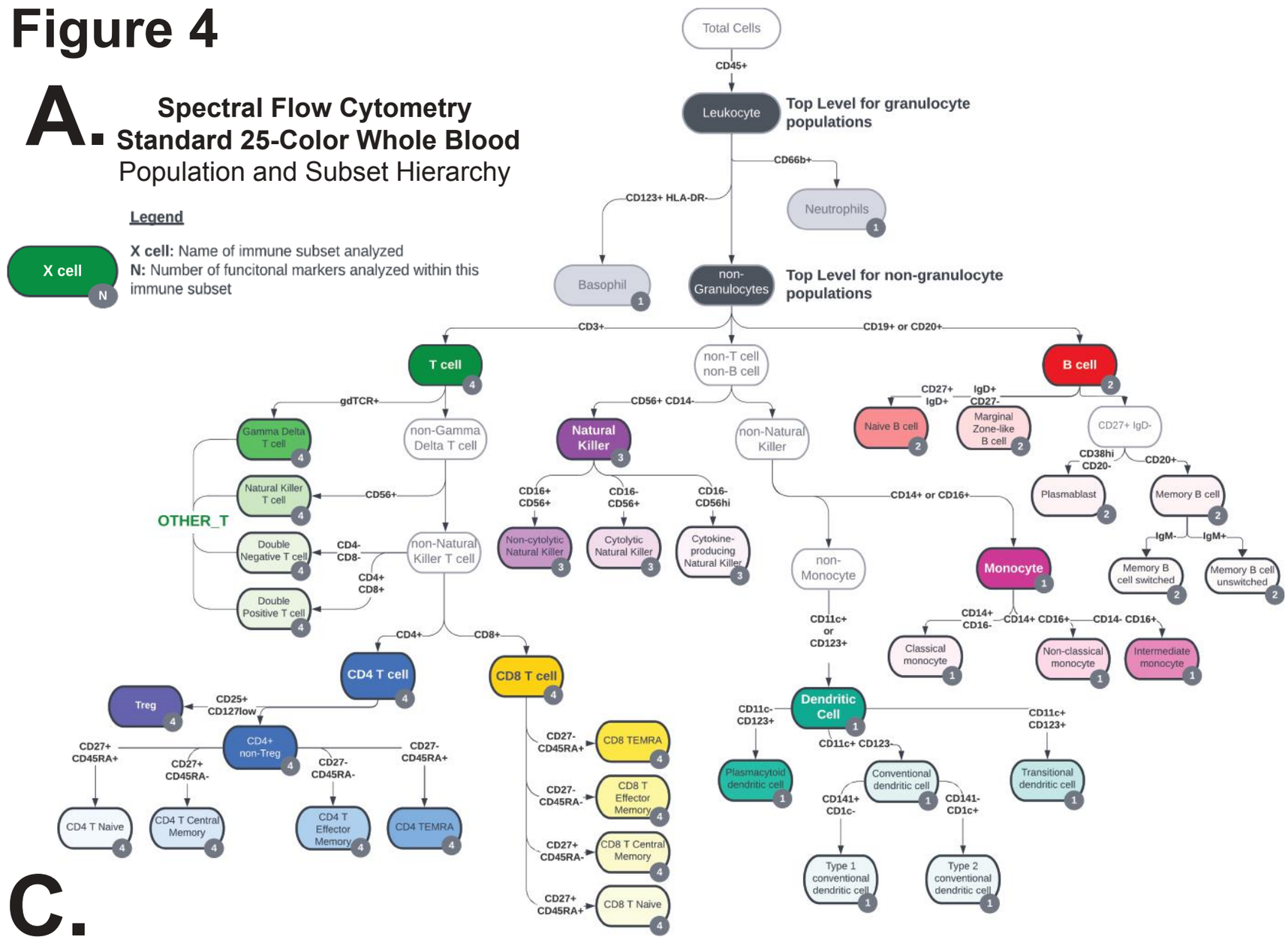

Live
TokuKit
- Permeabilization
+ Permeabilization
gdTCR (cFluor BYG710-A)
CD3 (BV570-A)
gdTCR (cFluor BYG710-A)
CD3 (BV570-A)
gdTCR (cFluor BYG710-A)
CD3 (BV570-A)

Figure 4

A. Spectral Flow Cytometry Standard 25-Color Whole Blood

Population and Subset Hierarchy



B.

Functional Markers	T Cell	B Cell	NK Cell	Monocyte	Dendritic Cell	Granulocyte
PD-1	Y	Y	Y	Y	Y	
HLA-DR	Y		Y			Y
CD38	Y					
CD25		Y				
CD8			Y			
CD28	Y					

C.

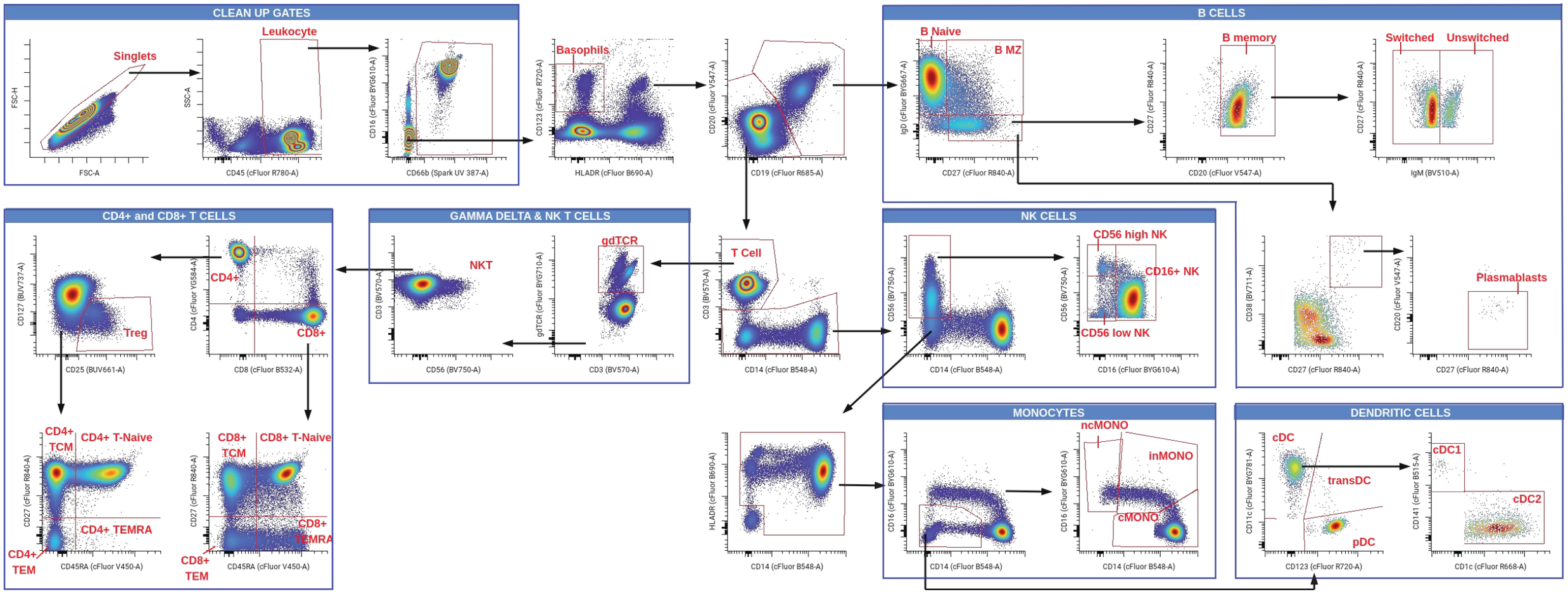
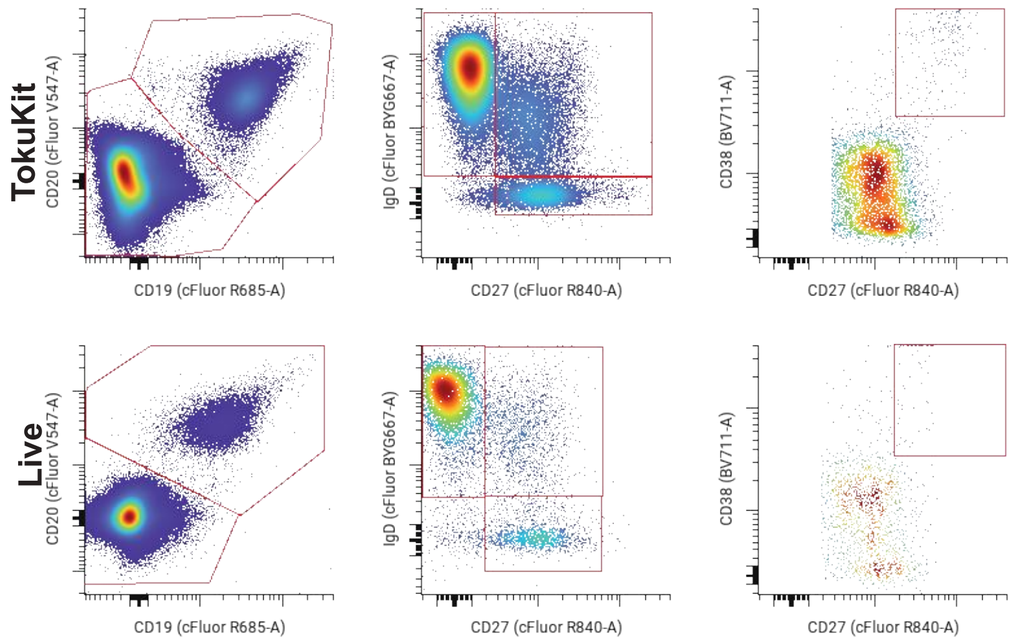


Figure 5

A.

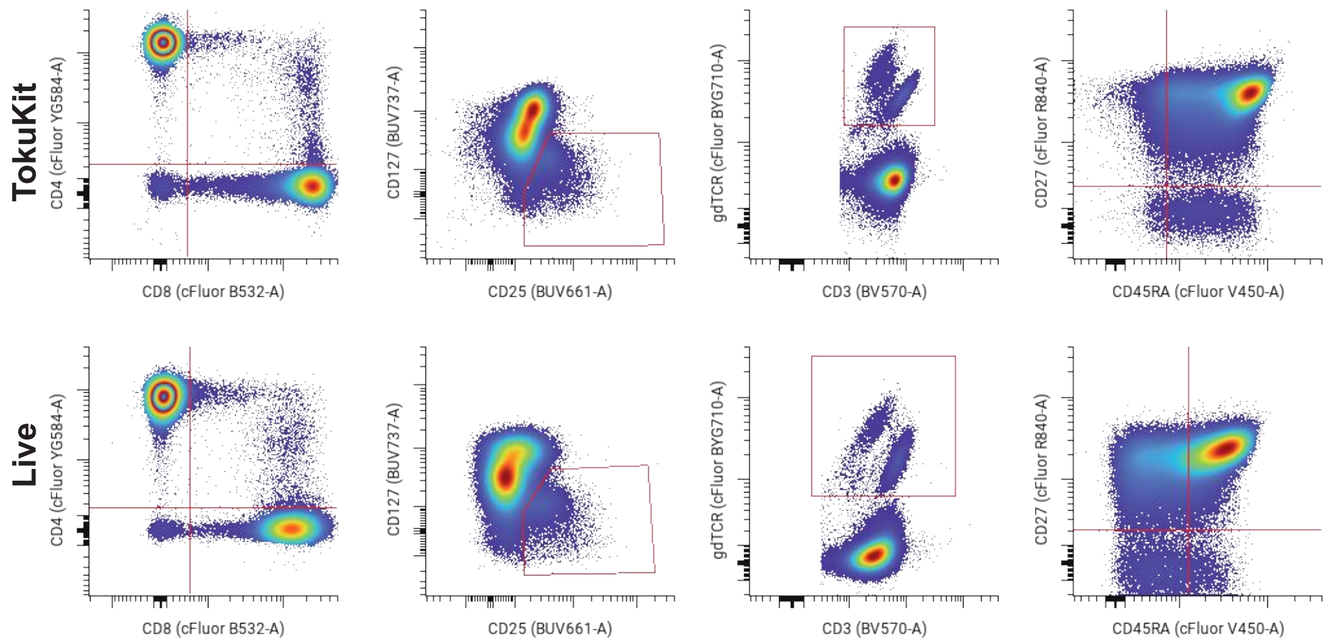
B Cells

Total B Cells | B Cell Subsets | Plasmablasts



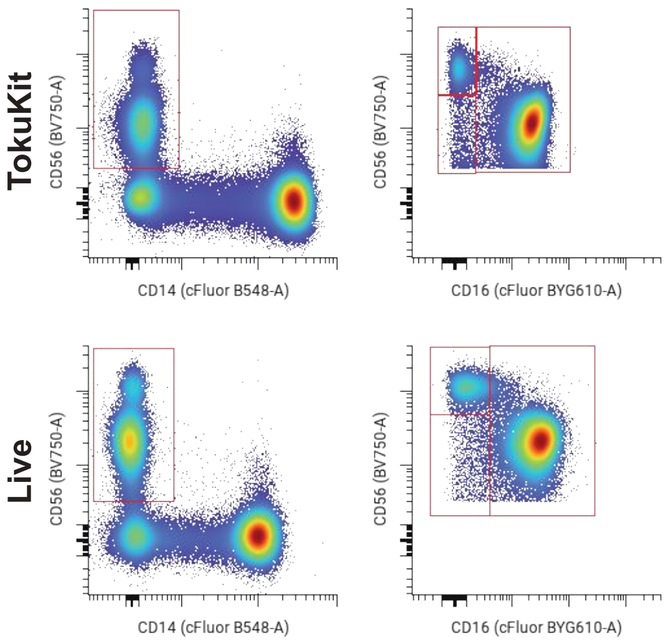
T Cells

CD4⁺ & CD8⁺ | T Regs | Gamma Delta T | CD4⁺ Subsets



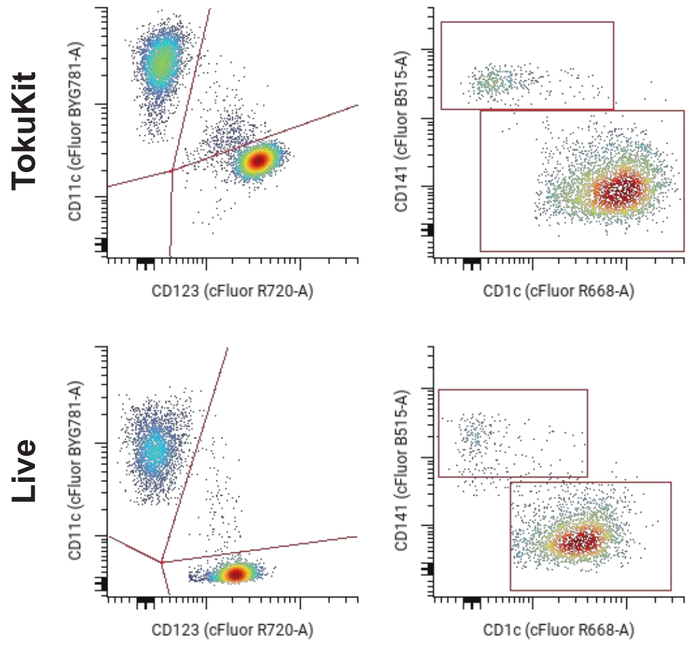
NK Cells

Total NK Cells | NK Cell Subsets



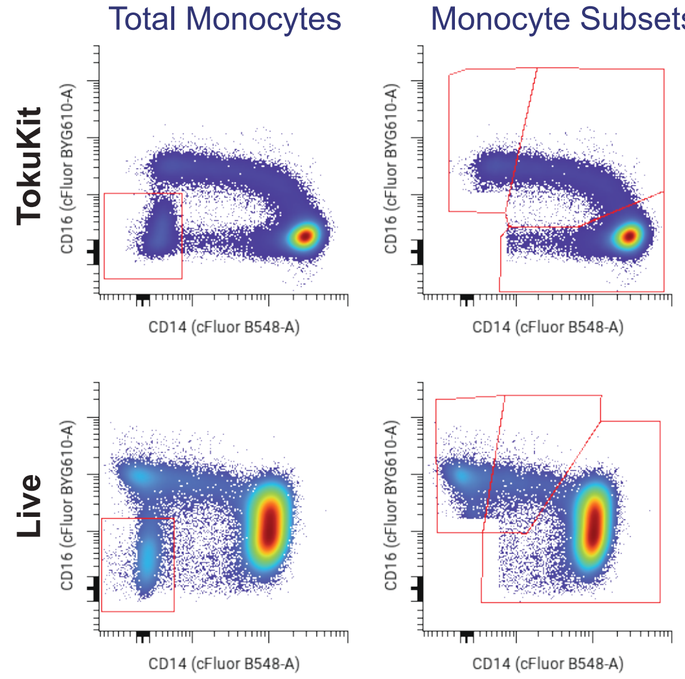
Dendritic Cells (DCs)

DC Subsets | cDC1 and cDC2



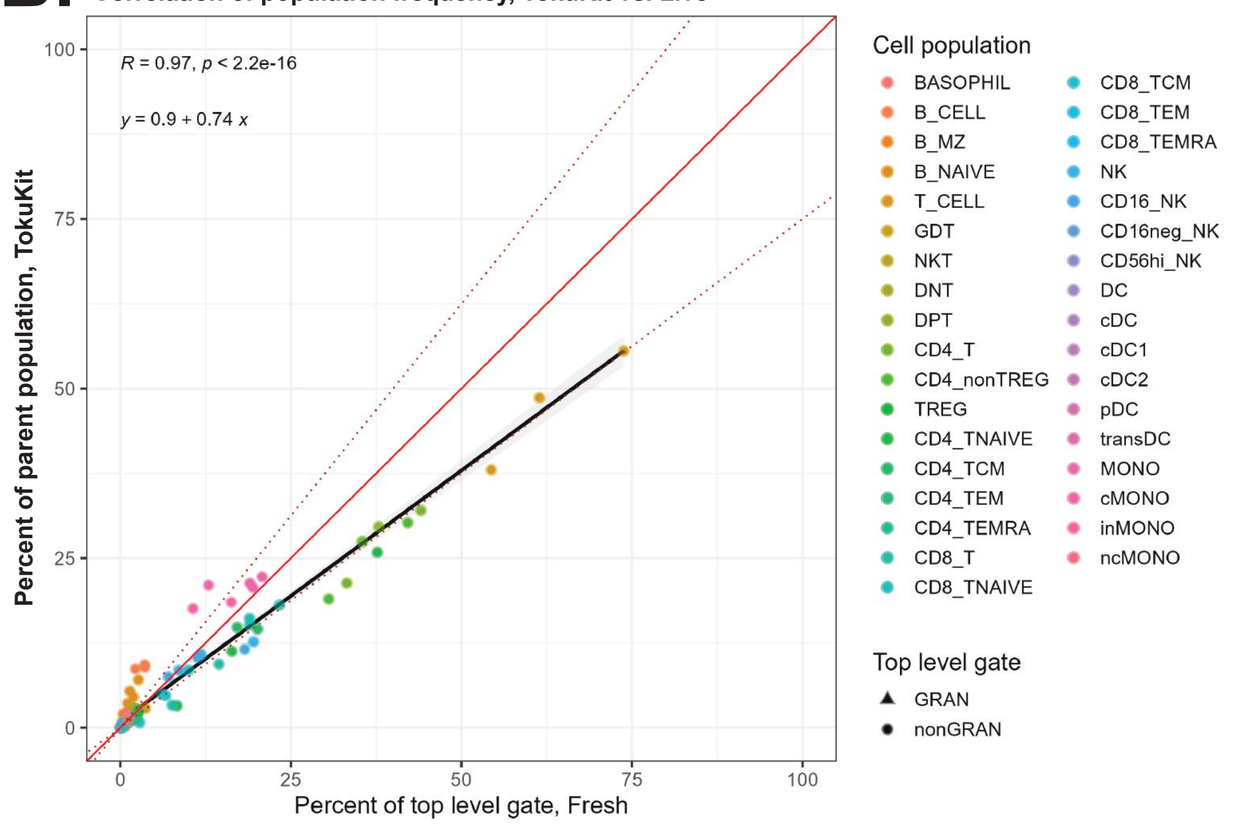
Monocytes

Total Monocytes | Monocyte Subsets

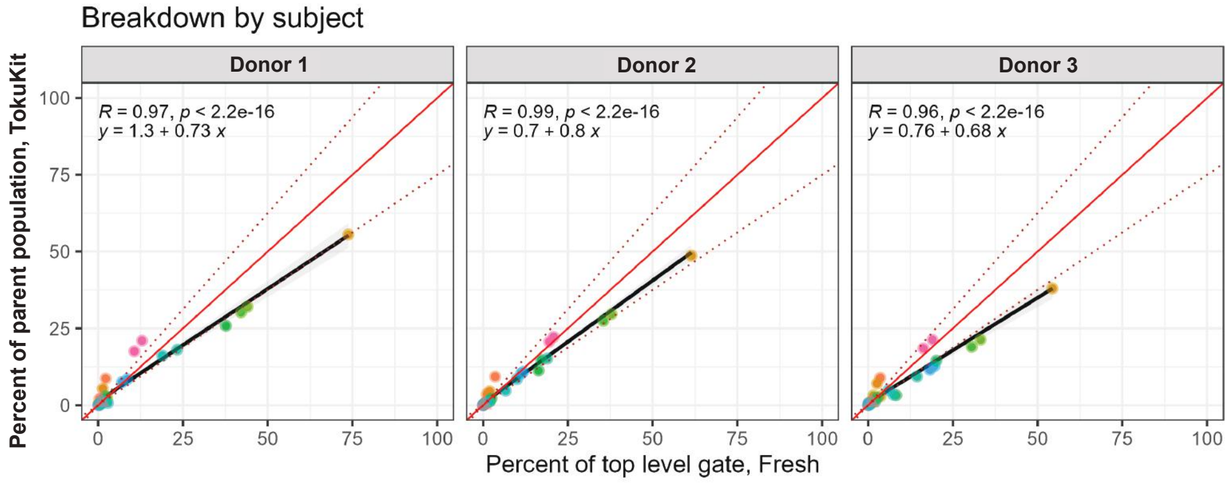


B.

Correlation of population frequency, TokuKit vs. Live

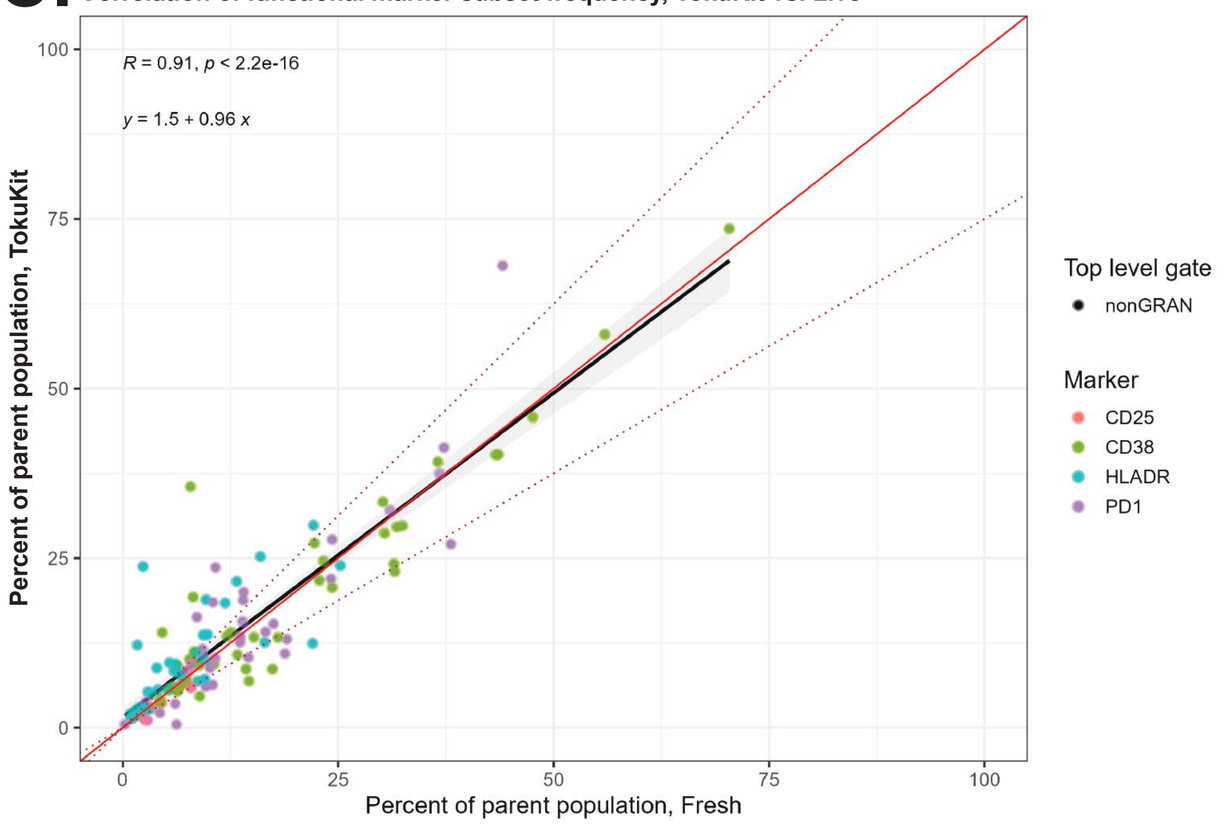


Breakdown by subject



C.

Correlation of functional marker subset frequency, TokuKit vs. Live



Breakdown by subject

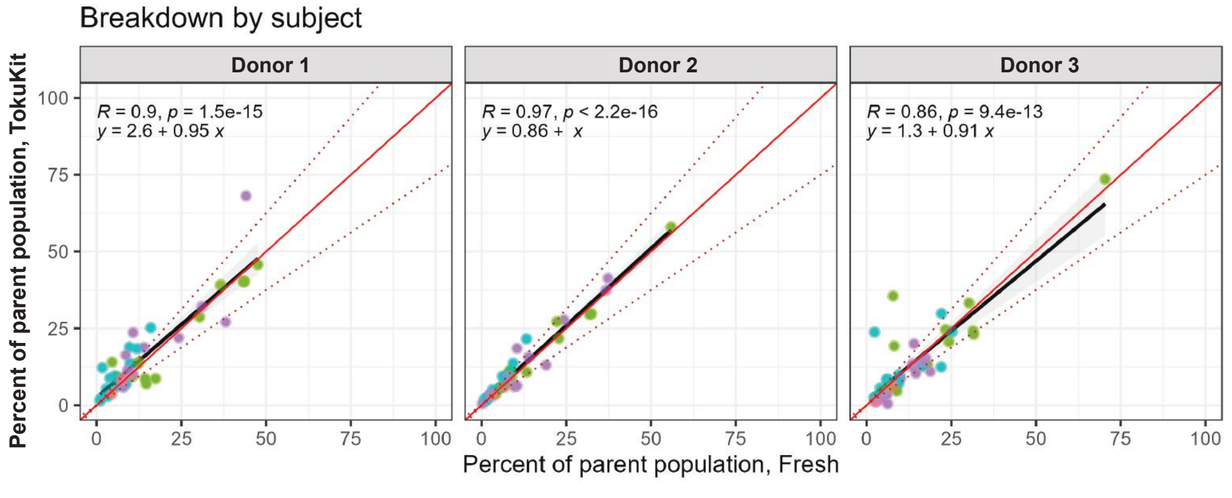
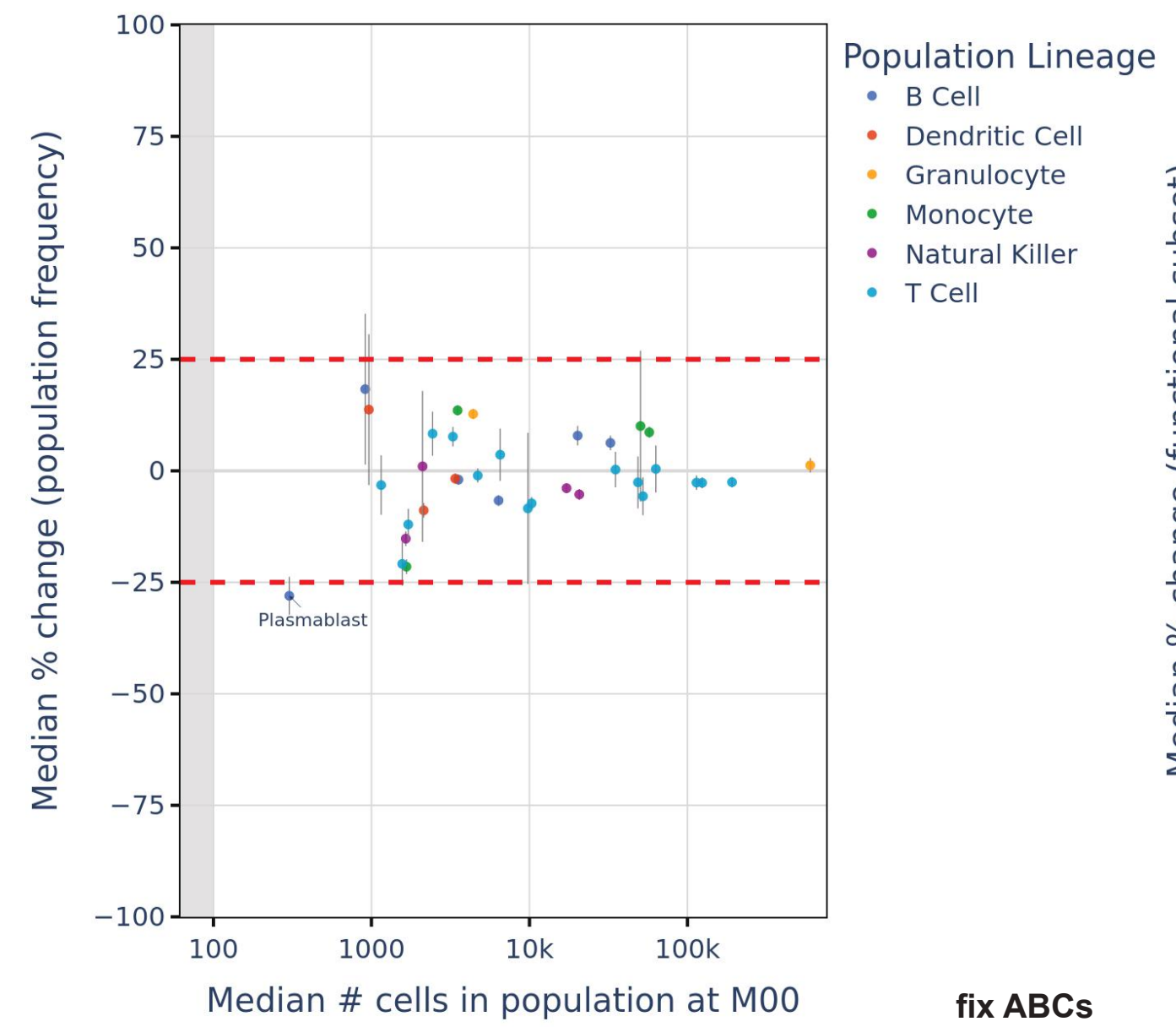
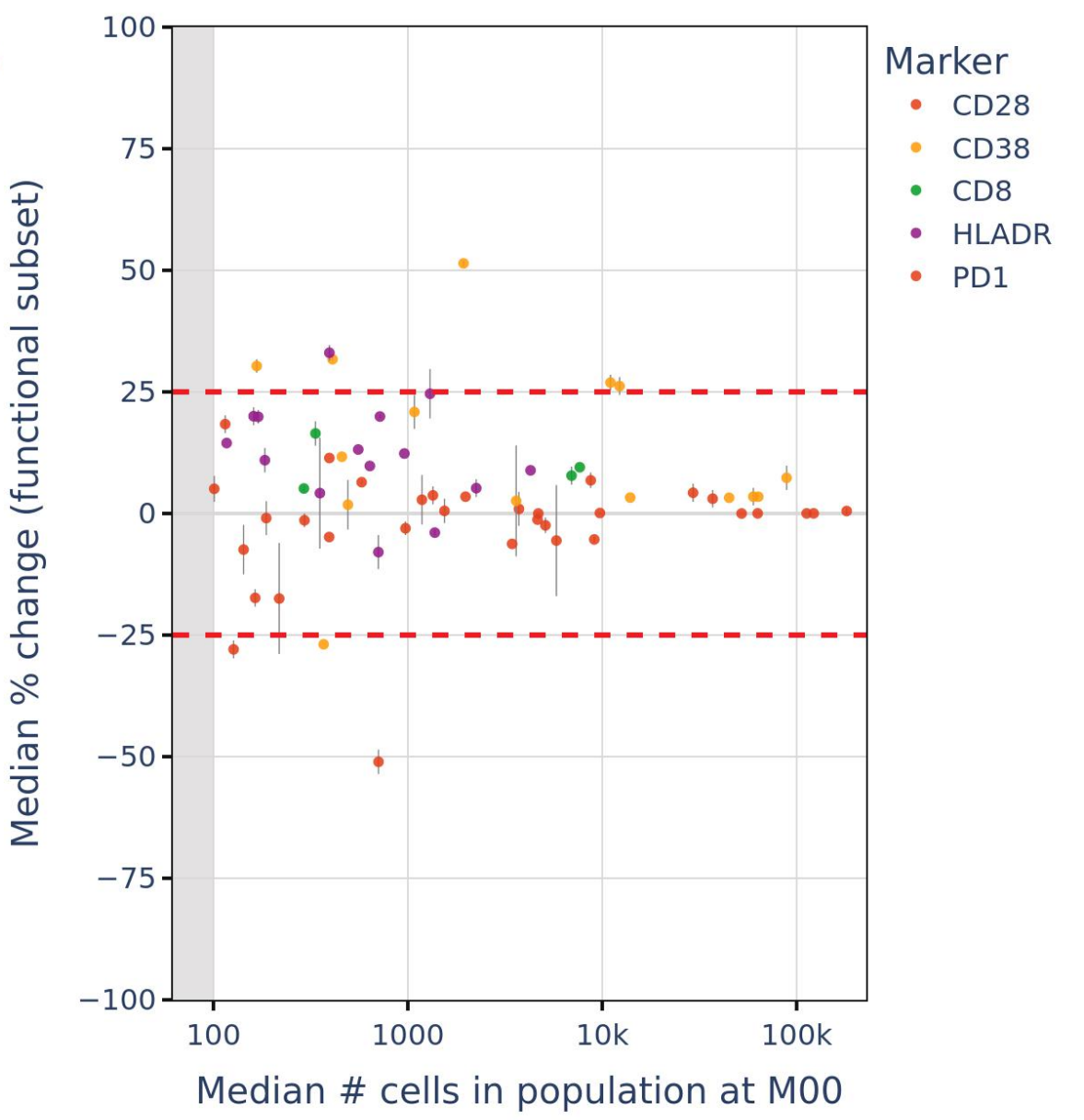
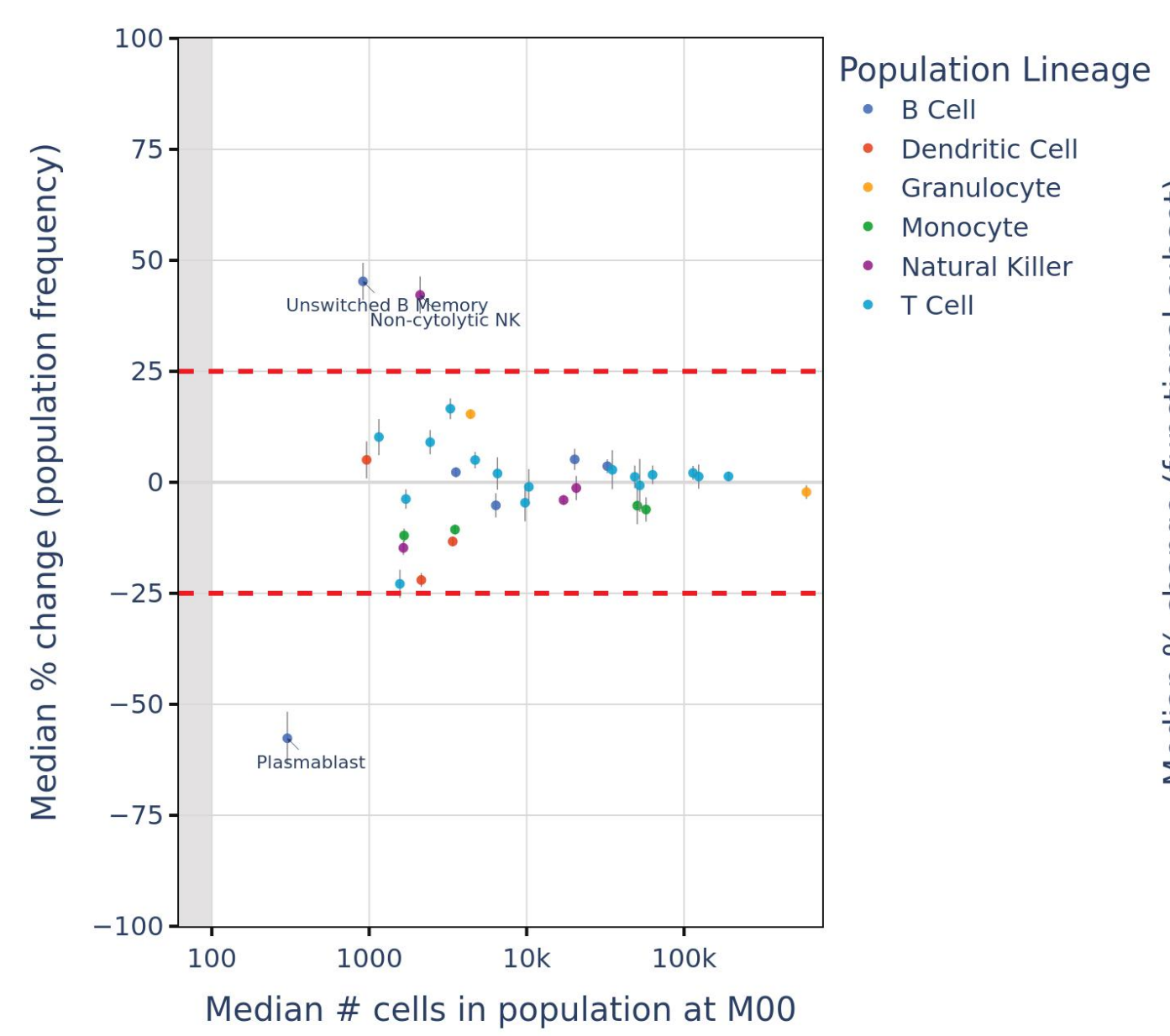


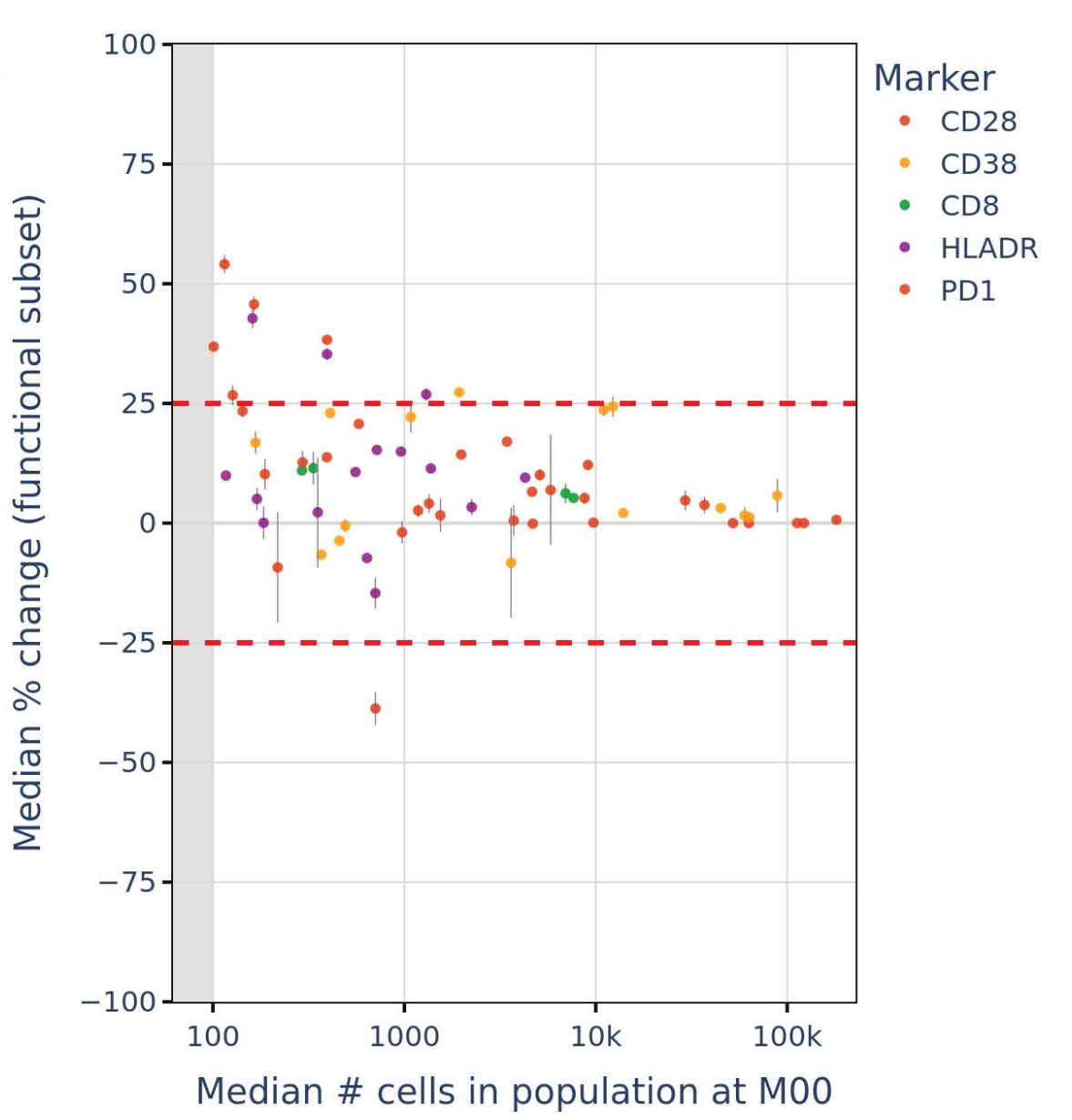
Figure 6

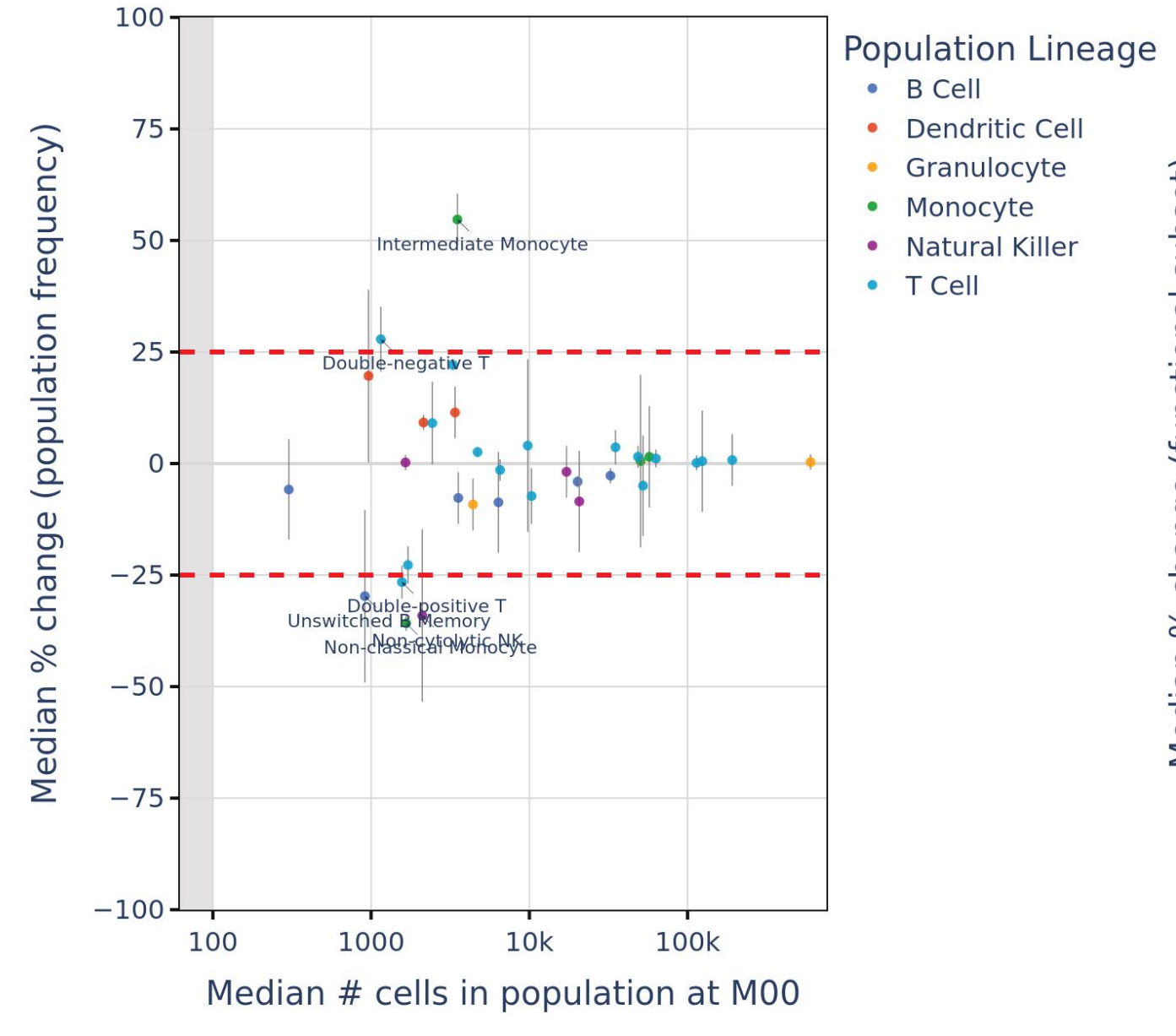




fix ABCs







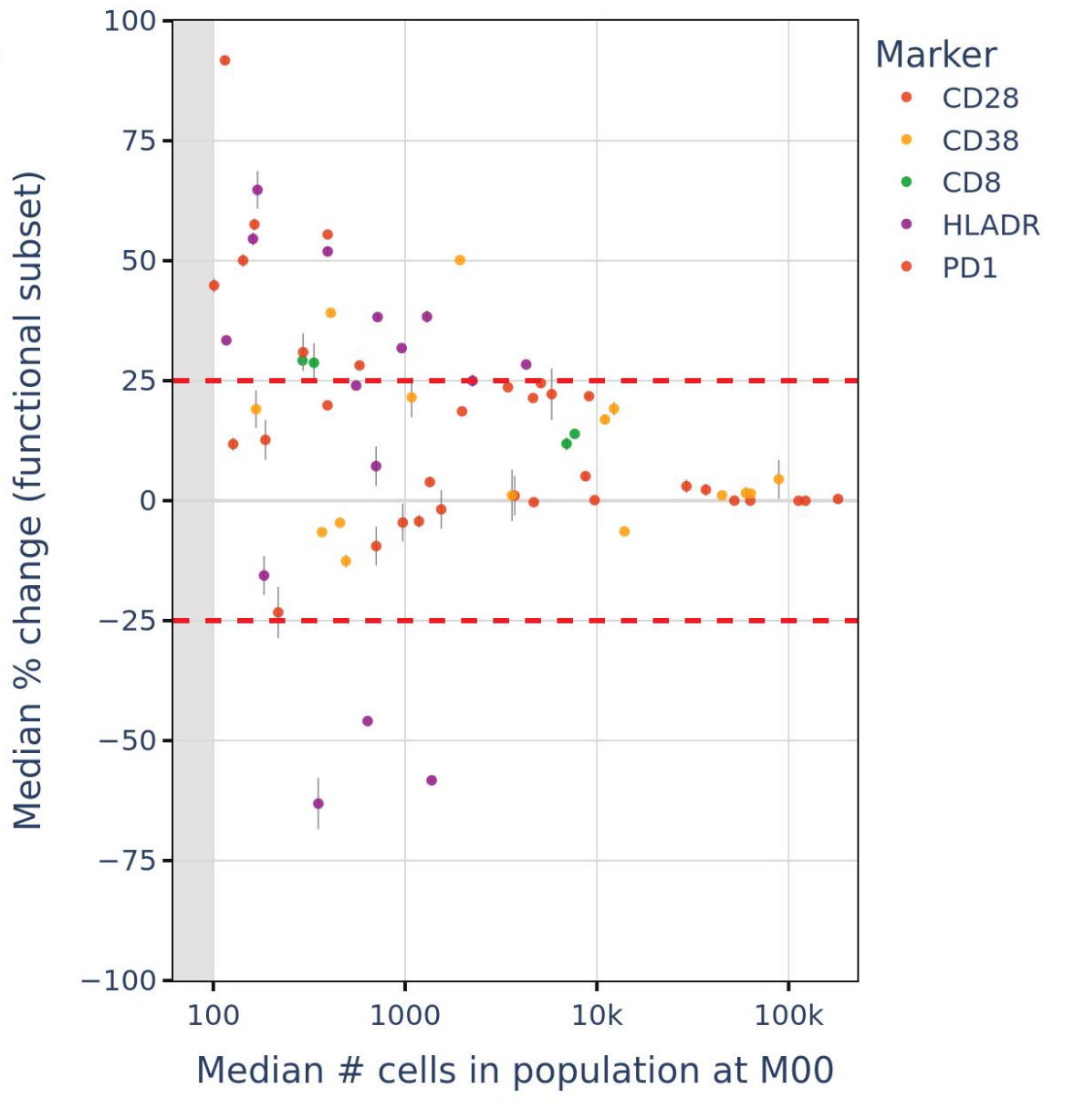
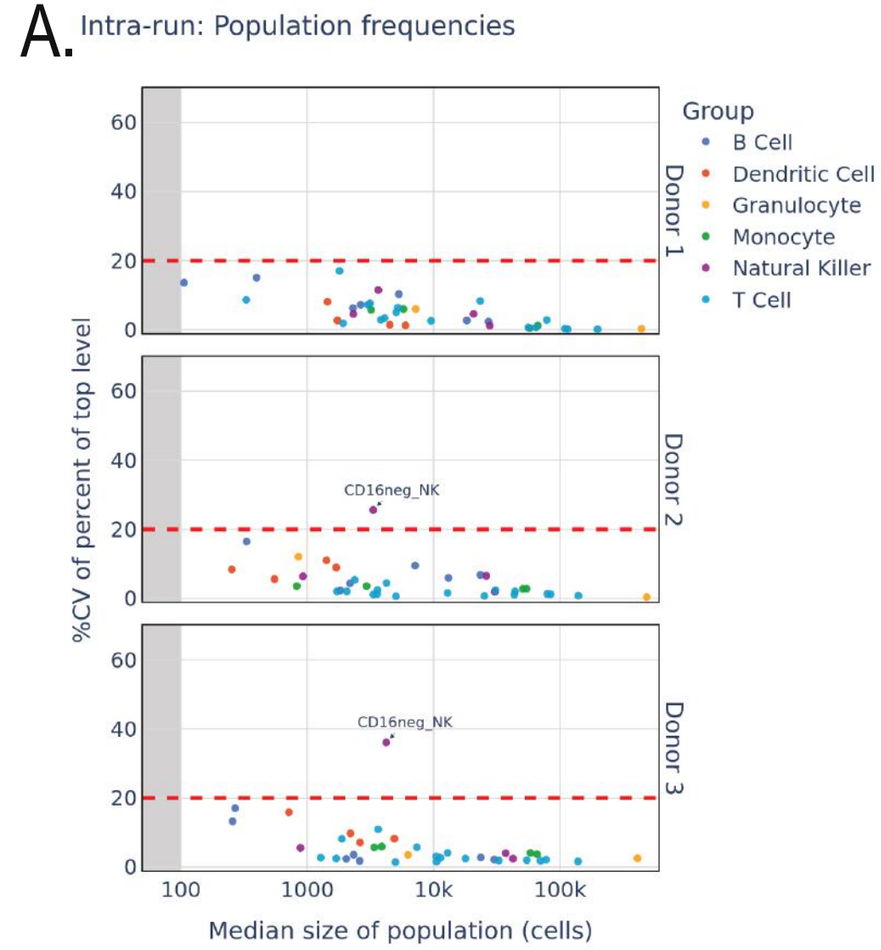
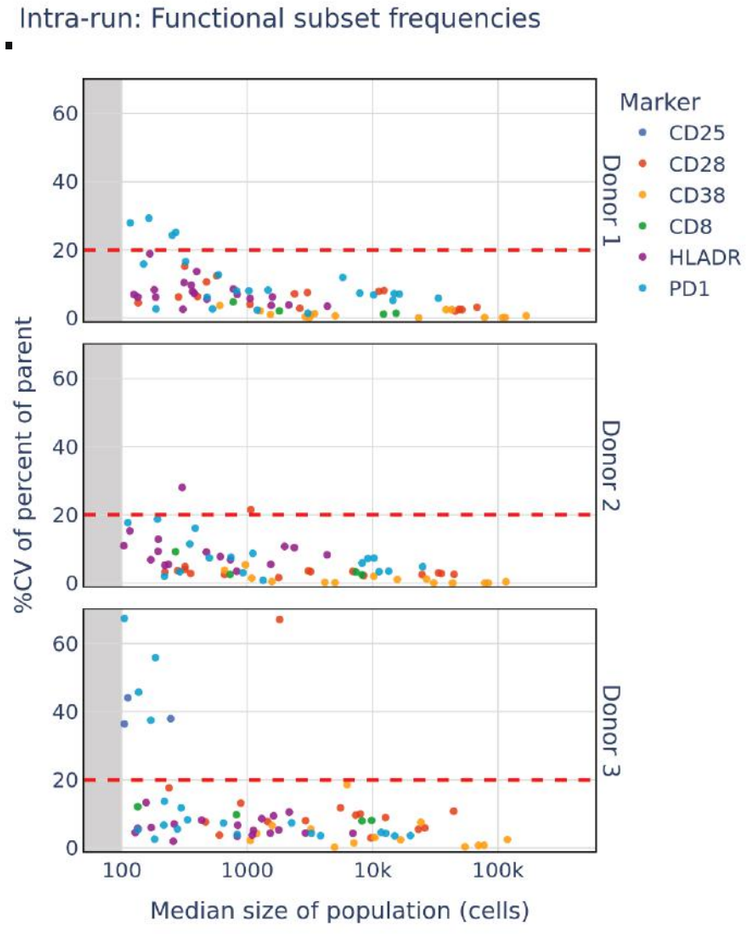
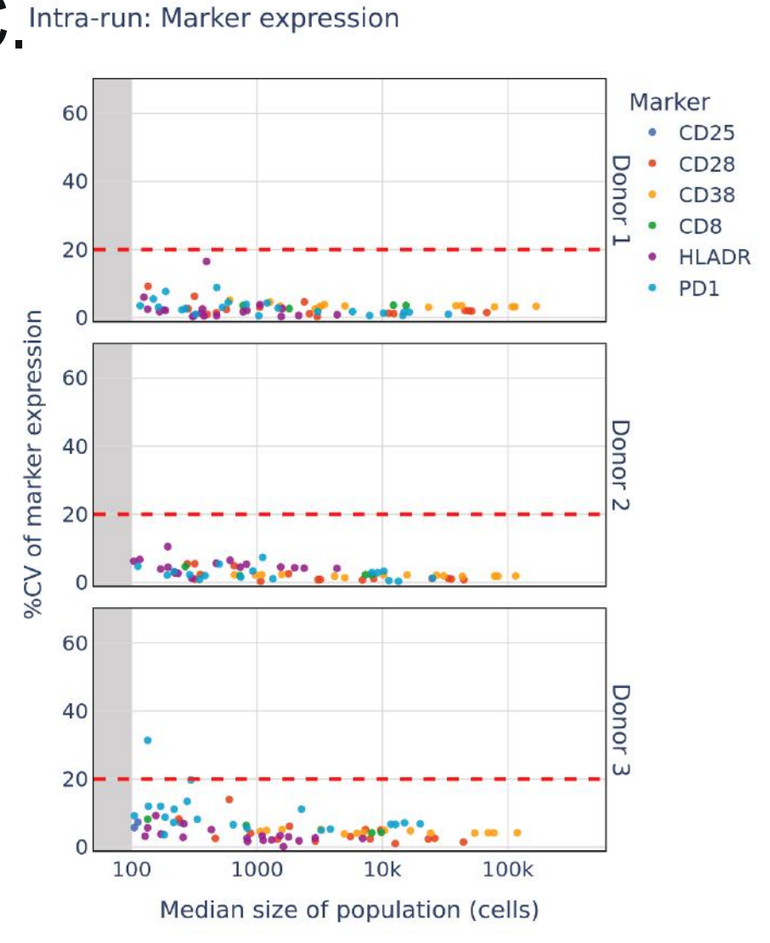
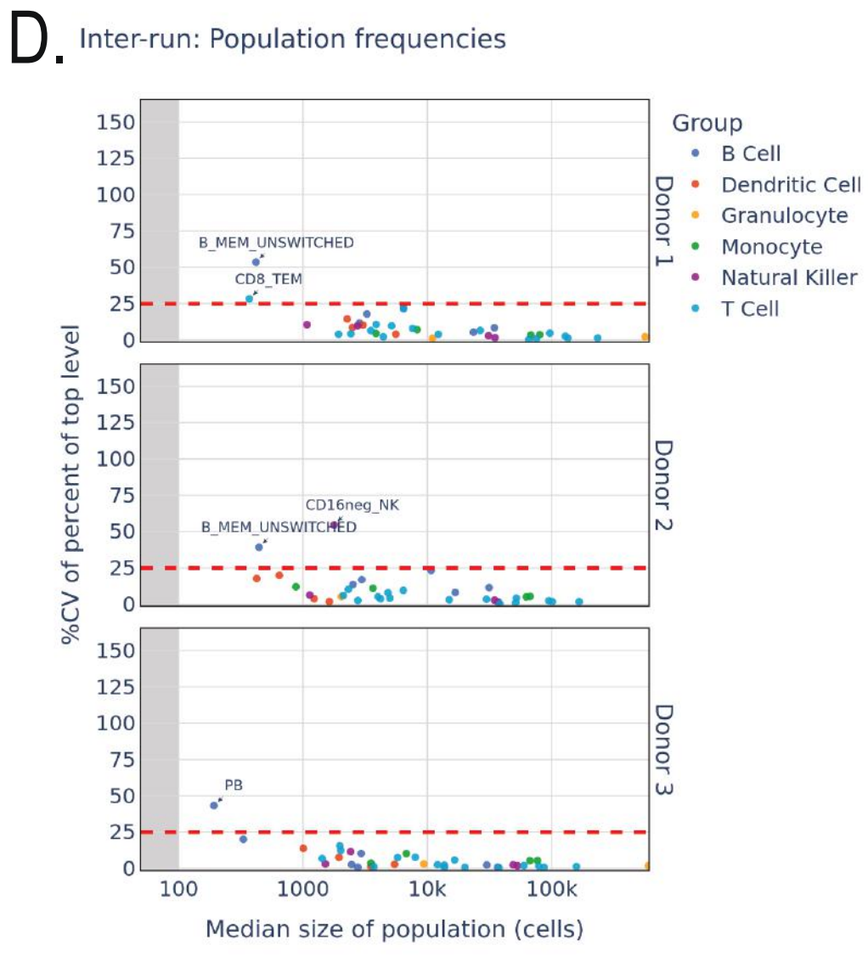


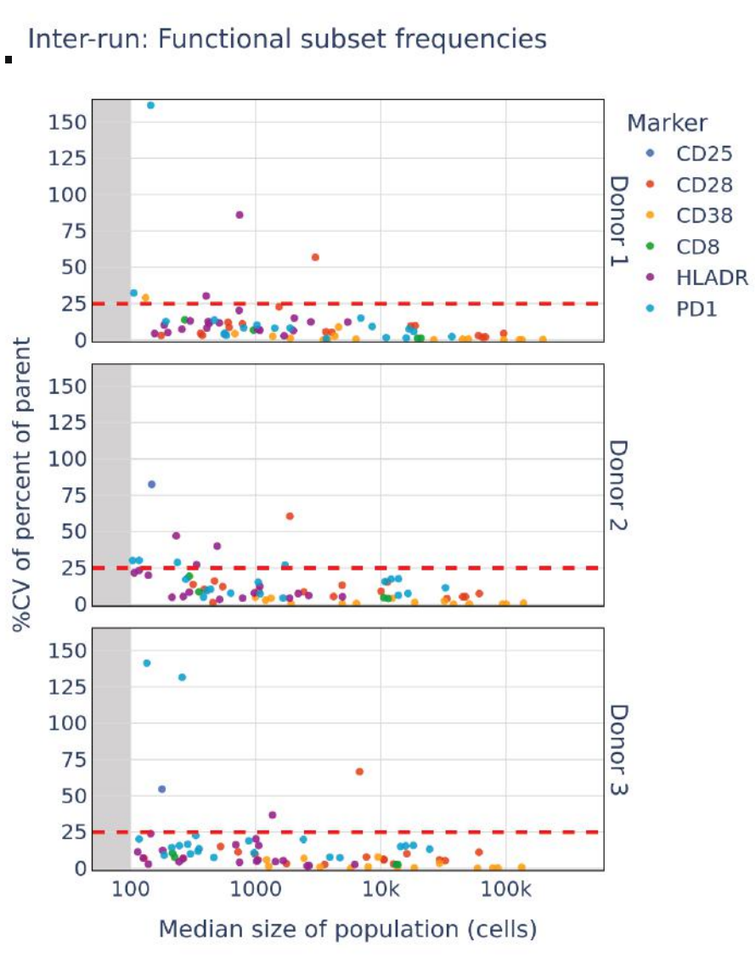
Figure 7

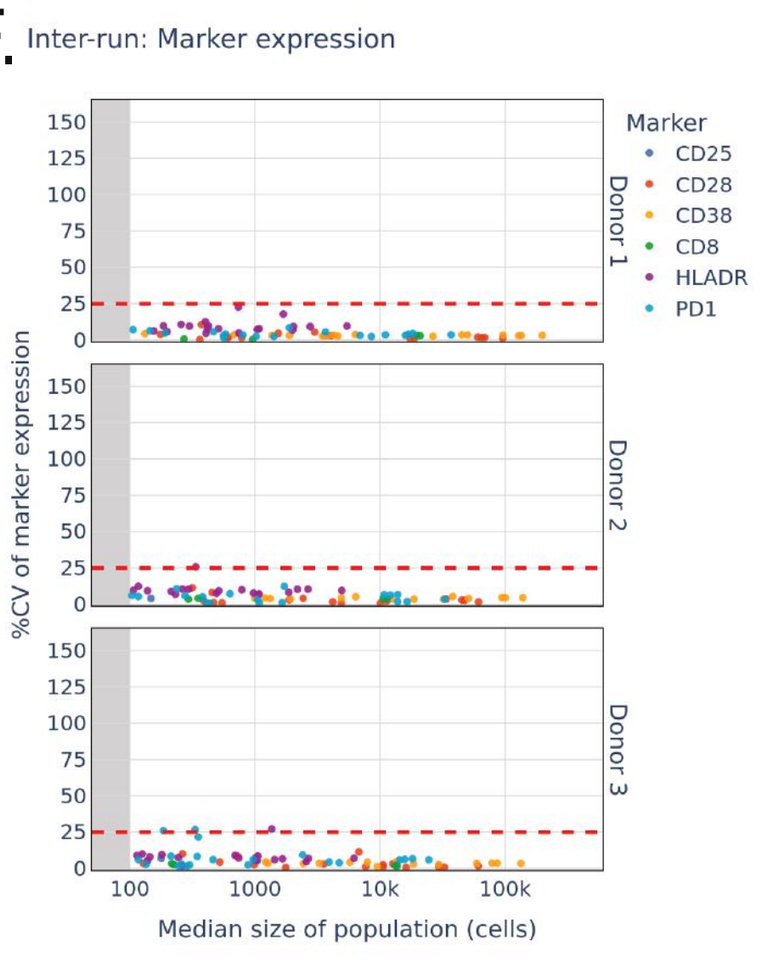

A.
Intra-run: Population frequencies
%CV of percent of top level
Median size of population (cells)
Group
B Cell
Dendritic Cell
Granulocyte
Monocyte
Natural Killer
T Cell
Donor 1
Donor 2
Donor 3
CD16neg_NK
CD16neg_NK
100
1000
10k
100k


B.
Intra-run: Functional subset frequencies
%CV of percent of parent
Median size of population (cells)
Marker
CD25
CD28
CD38
CD8
HLADR
PD1
Donor 1
Donor 2
Donor 3
100
1000
10k
100k


C.
Intra-run: Marker expression
%CV of marker expression
Median size of population (cells)
Marker
CD25
CD28
CD38
CD8
HLADR
PD1
Donor 1
Donor 2
Donor 3
100
1000
10k
100k


D.
Inter-run: Population frequencies
%CV of percent of top level
Median size of population (cells)
Group
B Cell
Dendritic Cell
Granulocyte
Monocyte
Natural Killer
T Cell
Donor 1
Donor 2
Donor 3
B_MEM_UNSWITCHED
CD8_TEM
CD16neg_NK
B_MEM_UNSWITCHED
PB
100
1000
10k
100k


E.
Inter-run: Functional subset frequencies
%CV of percent of parent
Median size of population (cells)
Marker
CD25
CD28
CD38
CD8
HLADR
PD1
Donor 1
Donor 2
Donor 3
100
1000
10k
100k


F.
Inter-run: Marker expression
%CV of marker expression
Median size of population (cells)
Marker
CD25
CD28
CD38
CD8
HLADR
PD1
Donor 1
Donor 2
Donor 3
100
1000
10k
100k