Performance Lab:

Human Immune Monitoring Center
Holden T. Maecker, PhD, Director
1651 Page Mill Road, Palo Alto, CA 94304

Analyte (s)	CyTOF Proteomics (using Smart Tubes)
Technical Platform (s)	CyTOF Helios 3
Positive and negative controls, calibrators, and reference standards	See below SOP (Appendix 1)
Quality control parameters for specimens/analytes	See below SOP (Appendix 1)
Any critical pre-analytic variables	Sample quality, whole blood collection and storage at -80°C, antibody titration/validation (see Materials and Methods)

Table 1. Summary of analytical performance characteristics for CyTOF Proteomics

Accuracy	Equivalent to immunophenotypic PBMC phenotyping (see **Figures 2**)
Precision: Inter-assay	<15% C.V. for main cell populations [reference (1) and **Figure 3**] (n=121)
Precision: Intra-assay	<5% C.V. for most cell populations **[Figure 4]**
Analytical sensitivity	Cell frequency: ~1 in 50,000 cells depending on number of cells collected Signal intensity: 500 copies per cell to get 10 counts per cell (at LOD of 5000 reporter atoms per count and 100 reporter atoms per probe)
Analytical specificity including interfering substances	Antibody specificity determined by vendor
Reportable range	~0.0%-100%, based on collection of ~60,000 PBMC events
Reference interval (normal range)	Varies by population; see **Figure 5** for an example (14 batches run on 25 populations)
Standardization, harmonization, reproducibility, and ruggedness	N/A
Turn-around time	3 days

Failure rate of the assay <u>as it is to be performed in the trial</u>	<5%
Quality control and improvement procedures	1. 4-element normalization beads in each sample 2. Sample barcoding to improve reproducibly (2) 3. Inclusion of standard healthy control reference See section ”Sample acquisition, file processing and analysis” in the Appendix 1. SOP below
Actual number of samples of the reproducibility study	About 20-22 batches
How run-to-run variation (Coefficient of Variation; CV) was assessed and handled	Manual gating (Figure 1 gating strategy; Figure 3 CV)
How inter-laboratory variability in the measurements was assessed and how these sources of variation were minimized to maintain performance at all sites within acceptable limits and to prevent drift or bias in the assay.	N/A
Describe proficiency testing and results	Trained by Platform manager in side by side manner
Scoring procedures and type of data to be acquired: **• quantitative/continuously distributed** **• semi-quantitative/ordered categorical** **• qualitative/non-ordered categorical**	Quantitative/continuously distributed
Criteria and metrics for defining significant changes (e.g., between timepoints, between responders and non-responders)	Phospho: Anything above 1 for the median arcsinh ratios. Phenotype: Significance of changes should be determined at a group level, based on cutoff of $p<0.05$ using an appropriate statistical test.
Throughput (estimated number of samples in a given time period)	About 5 patients+1 (healthy control) donors for functional assays Depending on the assay
Any other performance data	Linearity: See **Figure 6**.

Materials and Methods. All validation was carried out on healthy donor whole blood collected in Smart Tubes (SMART TUBE Inc.). Smart tube is a validated system, commercially available that allows stimulation, fixation and storage of a whole blood sample in one tube while preserving surface markers expression and protein phosphorylation. It has been used for research and clinical studies for several years (3, 4) (cf Appendix 1). For comparison with the standardized PBMC analysis, PBMC were derived from either heparinized whole blood or leukapharesis products obtained from the Stanford Blood Center. Processing and cryopreservation were carried out as previously described (http://iti.stanford.edu/content/dam/sm/iti/documents/himc/protocols/SOP-WBStimSTBClusterTubesv1-0.pdf) within four hours of blood draw. On the day of staining, cells were thawed and washed as described (1).

Table 2. CyTOF Whole Blood phospho phenotyping panel. **(intracellular markers / surface markers)**

Metal Label	Specificity	Clone	Conjugated by
142 Nd	cCasp3	D3E9	Fluidigm
143 Nd	CD19	HIB19	Biolegend (in house)
144 Nd	pPLCg2	K86-689.37	Fluidigm
145 Nd	CD4	RPA-T4	Fluidigm
146 Nd	IgD	IA6-2	Fluidigm
147 Sm	CD20	2H7	Fluidigm
148 Nd	IgA	Polyclonal	Fluidigm
149 Sm	CD25	2A3	Fluidigm
150 Nd	pSTAT5	47	Fluidigm
151 Eu	CD123	6H6	Fluidigm
153 Eu	pSTAT1	4a	Fluidigm
154 Sm	CD45	H30	Fluidigm
155 Gd	CD27	L128	Fluidigm
156 Gd	pP38	D2F9	Fluidigm
157 Gd	CD24	ML-5	Biolegend (in house)
158 Gd	pSTAT3	4	Fluidigm
159 Tb	CD11c	Bu15	Fluidigm
160 Gd	CD14	M5E2	Fluidigm
161 Dy	CD141	BDCA-3	Milteni (in housee)
162 Dy	CD66b	80H3	Fluidigm
163 Dy	CD56	NCAM16.2	Fluidigm
164 Dy	IkBalpha	L35A5	Fluidigm
165 Ho	pCREB	87G3	Fluidigm
166 Er	CD16	B73.1	eBioscience (in house)
167 Er	CD38	HIT2	Fluidigm
168 Er	CD8	SK1	Fluidigm
169 Tm	CD45RA	HI100	Fluidigm
170 Er	CD3	UCHT1	Fluidigm
171 Yb	pERK1/2	D13.14.4E	Fluidigm
172 Yb	Ki67	B56	Fluidigm
174 Yb	HLA-DR	L243	Fluidigm
175	CD7	6B7	Biolegend (in house)
176 Yb	CD127	AO19D5	Fluidigm
209 Bi	CD11b	ICRF44	Fluidigm

Antibodies in the standard HIMC CyTOF phenotyping panel are shown in **Table 2**. Conjugations were carried out using MaxPar kits (Fluidigm), according to the manufacturer's directions. The conjugates were tested and titrated on PBMC from the same leukapharesis donor, with optimal titers chosen based on maximal separation of relevant positive and negative cell populations for each marker. Subsequent conjugations were compared back to the previous batch to ensure similar performance, with +/-20% median intensity of positive population as acceptability criteria.

For cross-site standardization, a simplified panel of 12 overlapping antibodies for PBMC and Whole Blood phospho-phenotyping were used, to which were added Ir-intercalator stock solution (#201192B) and EQ four-element beads (#201078). These were used at the manufacturer's recommended titer, with aliquots from the same reagent lot distributed to the different performance sites [see (1) for details].

Helios (Fluidigm) instruments were set up using the manufacturer's recommended protocol, with detector voltages and Tb intensities from tuning solution tracked over time. Deviations from the previous run of more than 100 V detector

voltage or 10% Tb intensity, respectively, triggered cleaning of the nebulizer, spray chamber, and/or cones, as required, followed by re-tuning. . Samples were aquired in MilliQ-purified water using a narrow-bore (NB) injector.

Gating was done according to a standard FlowJo template (see **Figure 1** for an example). Gates were adjusted on a donor-specific basis, but only when there were clear shifts between negative and positive populations. No adjustments were made between replicates of the same donor in a given batch.

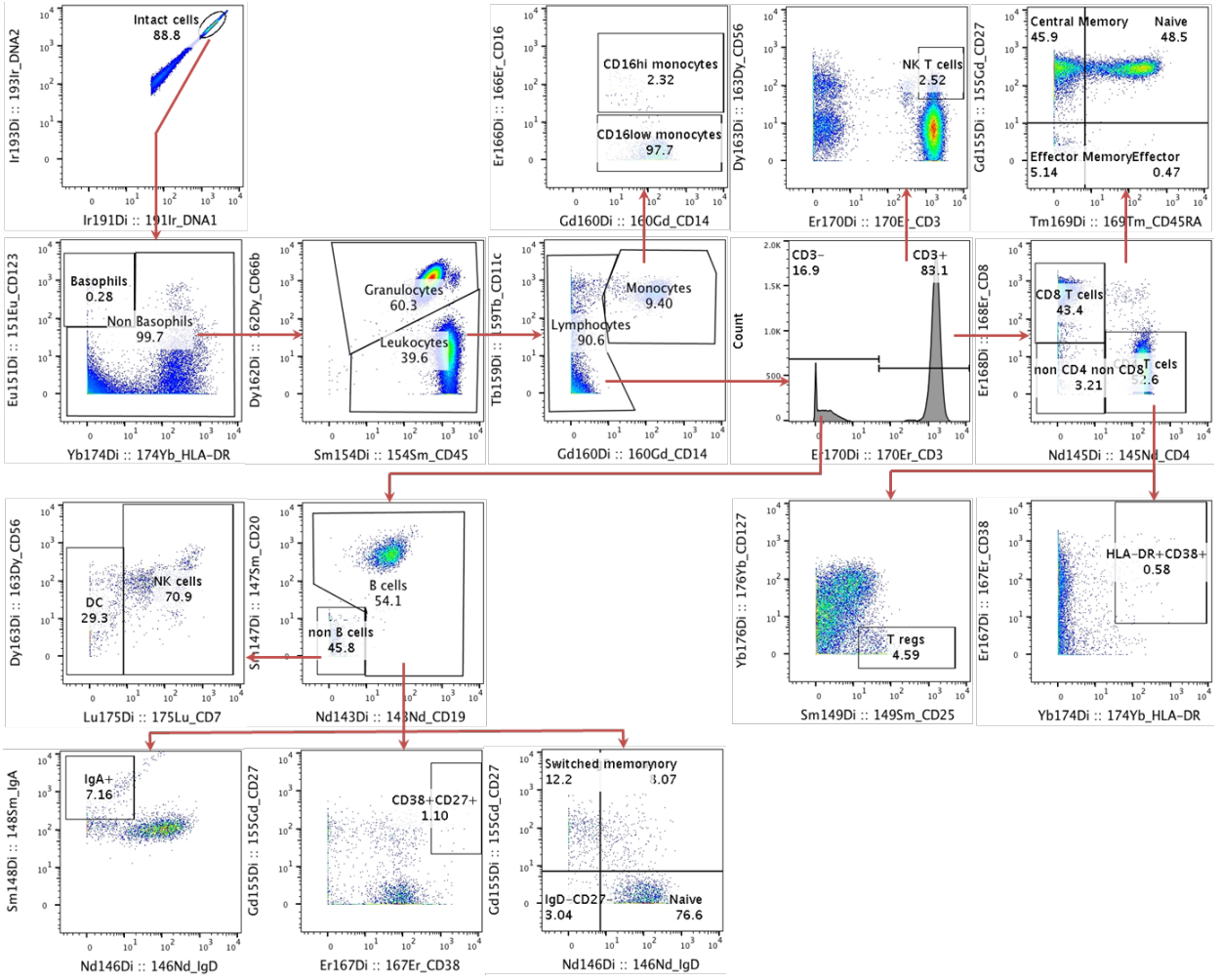


Figure 1. Manual gating template for WB Phospho-CyTOF phenotyping panel, shown on an example healthy subject WB.

Accuracy. Accuracy of CyTOF phospho-phenotyping of WB collected with the smart tube system was assessed by comparison to the standardized protocol of CyTOF surface phenotyping on PBMC. While the shape and positions of certain cell subsets differed somewhat between both staining protocols, the percentage of cells in each gated population was similar, as illustrated by **Figure 2**.

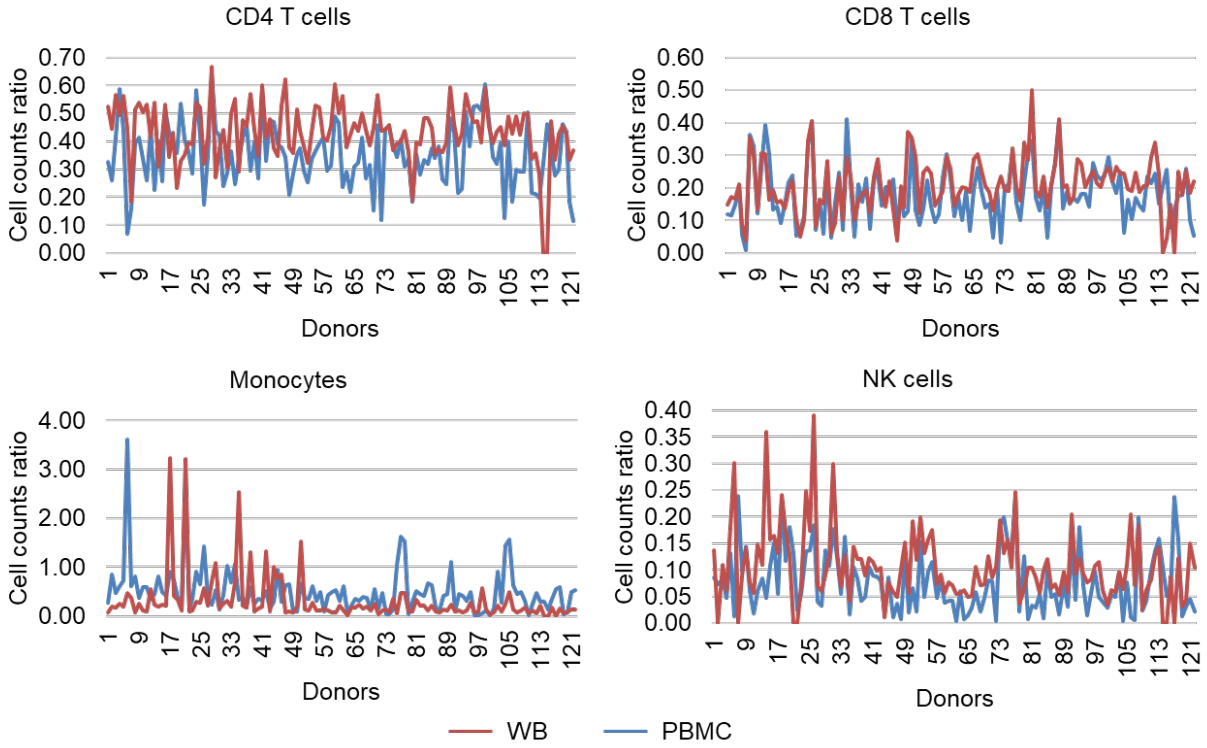


Figure 2. Example of relative population abundances (population cell count normalized on lymphocytes cell counts) determined by CyTOF in WB *vs* PBMCs, showing good numerical concordance.

Inter-Assay Precision. A single cryopreserved control is included with every batch of CyTOF phospho-phenotyping. Longitudinal analysis of selected cell subsets from this control over time is shown in **Figure 3**. Similar to data obtained from our cross-site standardization study (see below), the average CV of the main populations was about 10%. For smaller subsets as NK cells monocytes, the CVs were higher, yet always under 30%.

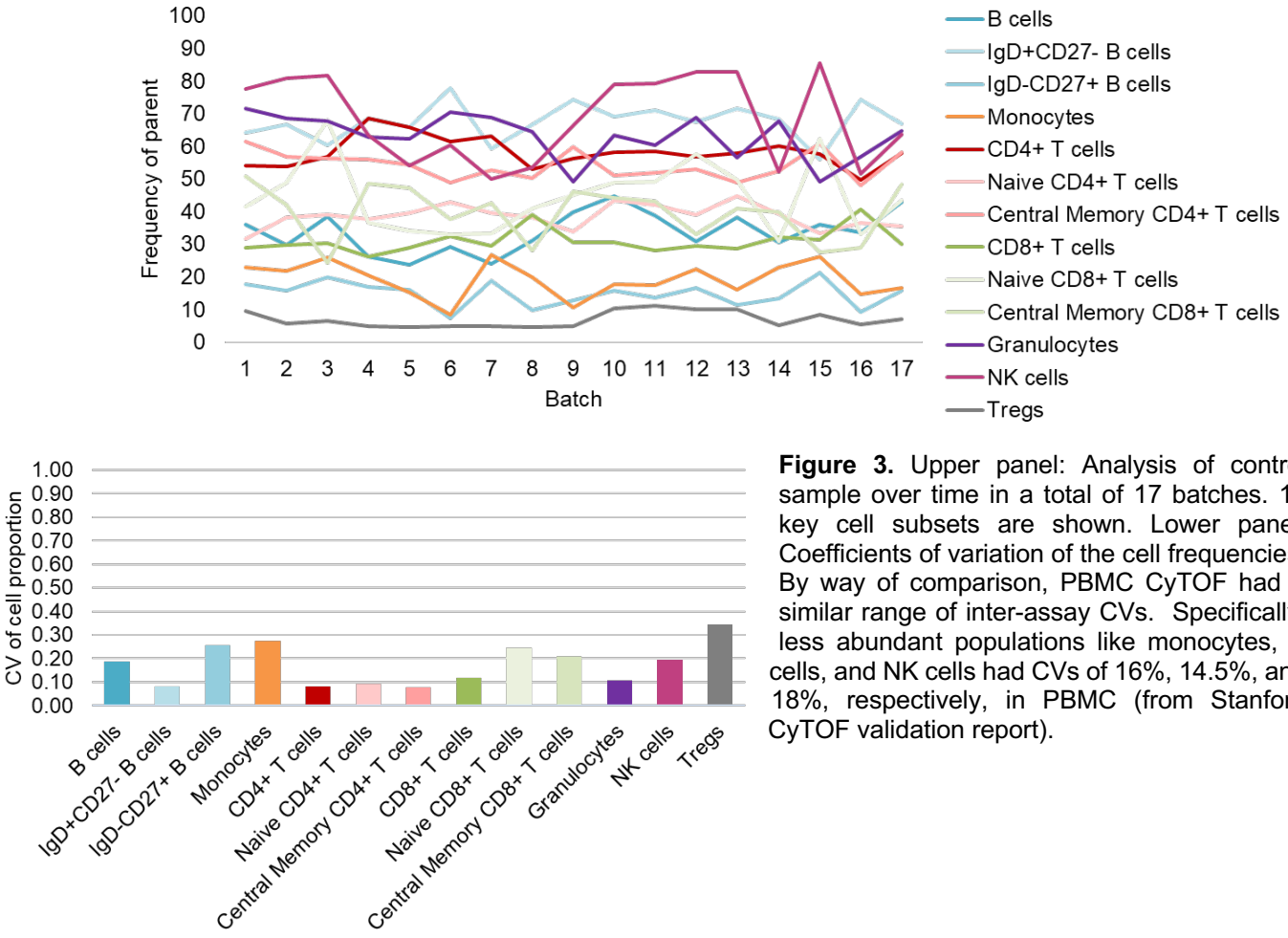


Figure 3. Upper panel: Analysis of control sample over time in a total of 17 batches. 13 key cell subsets are shown. Lower panel: Coefficients of variation of the cell frequencies. By way of comparison, PBMC CyTOF had a similar range of inter-assay CVs. Specifically, less abundant populations like monocytes, B cells, and NK cells had CVs of 16%, 14.5%, and 18%, respectively, in PBMC (from Stanford CyTOF validation report).

Intra-Assay Precision: To test the intra-assay precision of the phospho-CyTOF with Smart Tube assay, we ran three replicates of the same donor, spiked into three barcoded clinical samples and run on the same day and same CyTOF. After deconvolution, data from the control donor were collected and analyzed. The **Figure 4** illustrates the reproducibility of the data for the main cell populations and some phospho-proteins.

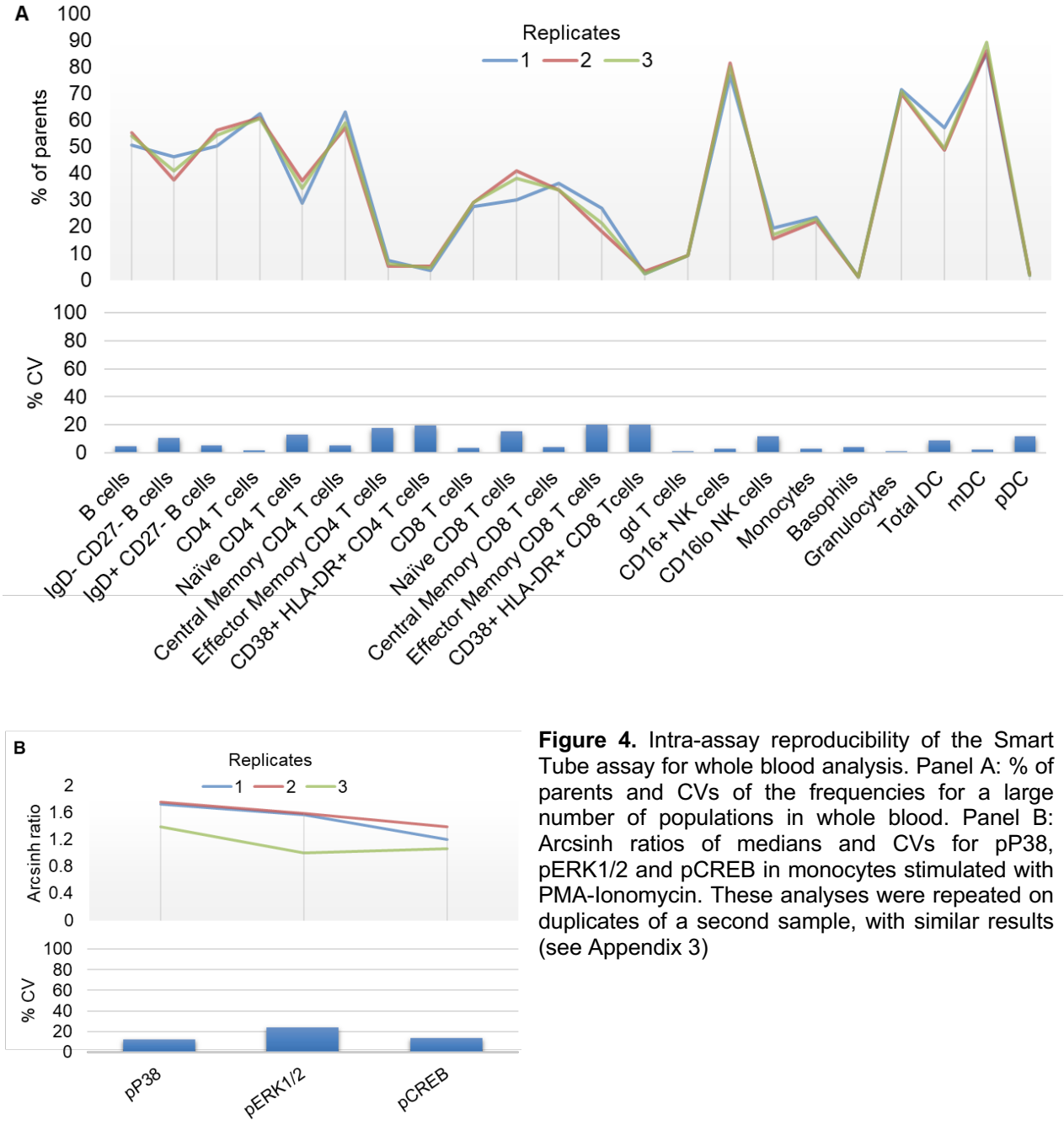


Figure 4. Intra-assay reproducibility of the Smart Tube assay for whole blood analysis. Panel A: % of parents and CVs of the frequencies for a large number of populations in whole blood. Panel B: Arcsinh ratios of medians and CVs for pP38, pERK1/2 and pCREB in monocytes stimulated with PMA-Ionomycin. These analyses were repeated on duplicates of a second sample, with similar results (see Appendix 3)

Normal Ranges. With hundreds of healthy subjects that we have analyzed by CyTOF phenotyping, we can determine normal ranges for 200+ cell phenotypes. An example is shown in **Figure 5**. These ranges tend to be wide, and are influenced by age and sex, as

exemplified in the Figure. Complete data for all gated populations in this study are available on request.

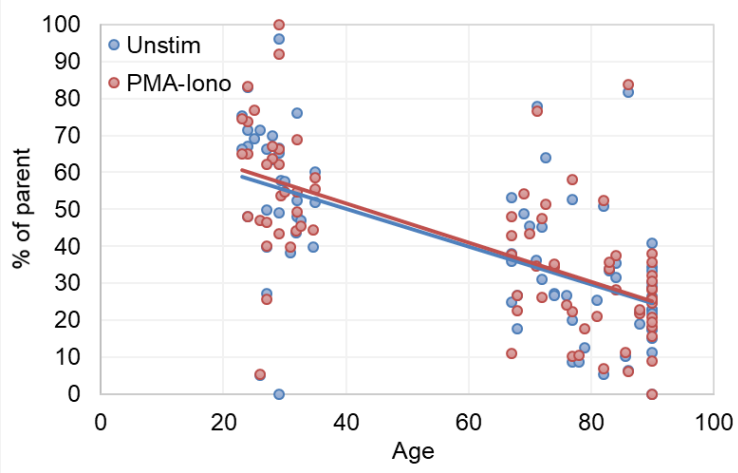


Figure 5. Example of normal range for a cell subset (CD27+ CD45RA+ CD8+ T cells) in CyTOF analysis on WB, accounting for age and stimulation status. This subset has a significant correlation with the age of the subjects when the stimulation itself does not affect the cell discrimination. Data on many more subsets is available upon request.

Linearity. Signal linear dynamic range was tested using either (a) cell-based analysis, with serial dilution of Tb-labeled antibody, or (b) solution-based analysis, using serial dilutions of tuning solution (Fluidigm) and recording Tb and Tm mean and median intensities (**Figure 6**). Both methods show relatively linear dynamic range over approximately 4 logs.

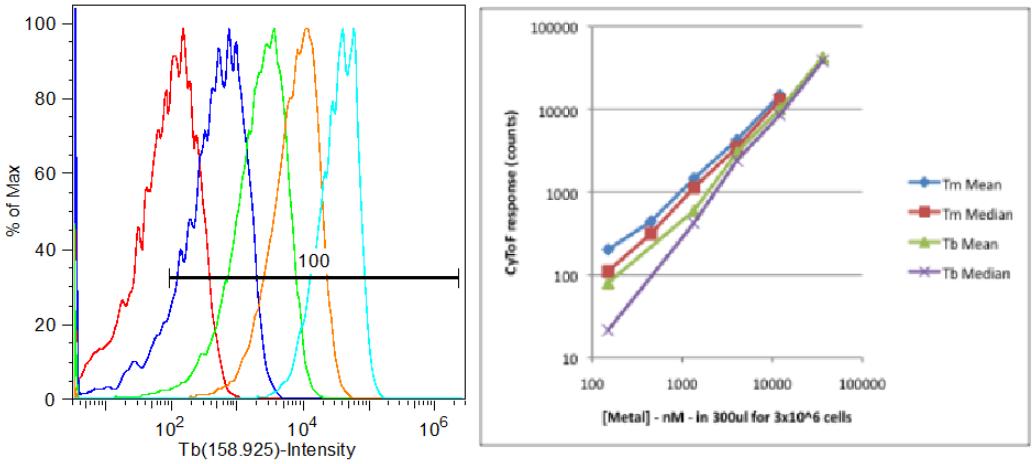


Figure 6. Cell-based (left) or solution-based (right) analyses of linearity both show approximately 4-log linear dynamic range of CyTOF.

Appendix 1: SOP

Cytokine-Stimulated Phosphoflow of Whole Blood Flu samples using CyTOF Mass Cytometry

Adapted from: https://bio-protocol.org/e1495

MATERIALS and REAGENTS

Whole blood phosphoflow Flu samples and a standard healthy control donor reference were stimulated, fixed and stored at HIMC biobanking according to HIMC "WB Stim & Smart Tube Stabilizer – Cluster Tubes Cryopreservation" protocol.

- Smart Tube 1X Thaw-Lyse buffer Fisher Cat# 1028711
- 16% PFA (Alfa Aesar, catalog number: 4368)
- Methanol (Thermo Fisher Scientific, catalog number: A452SK-1)
- Phenotyping and phosphoprotein antibodies filtered with 0.1 um spin filters (EMD Millipore, model: UFC30VV00)
- Ir-intercalator stock solution (Fluidigm Cat#: 201192B)
- Fluidigm Cell-ID™ 20-Plex Pd Barcoding Kit (Cat#201060)
- Fluidigm MaxPar PBS (Cat # 201058)
- Fluidigm Cell Staining Buffer (Cat# 201068)
- Deep Well plate (Costar Cat# 3960)

Each donor has 8 conditions (always in the following order):

1. Unstimulated
2. IFN alpha
3. IL6
4. IL7
5. IL10
6. IL21
7. LPS
8. PMA/Ionomycin

Sample Experiment Workflow

- Thaw 12 donor samples at the time (x 8 stims) *(Please note that this protocol can be scaled down according to the number of donors)*
- Lyse, wash, barcode all 96 samples in two 48-well plate format;
- Combine all 8 barcoded stims (per donor) in one well (end up with 6 barcoded samples per 48 well plate);
- Surface stain all 12 pooled barcoded samples;
- Methanol-freeze and store at -80C until the CyTOF run;
- Before the run, perform intracellular staining on 6 pooled barcoded samples (one plate or batch);
- Ir interchalaltor-stain and run on CyTOF

Lysis:

1. Thaw samples in ice cold water for about 15 min.
2. Transfer 500 uL of sample into each well of 48-deep well plate with 2 mL of 1x Thaw-Lyse buffer, resuspend well and let it sit for 10 minutes at RT.
3. Centrifuge cells at 1800 (650 g) rpm for 10 minutes at RT, aspirate supernatant from the cells.
4. Repeat lysis: add 2 ml of 1x Thaw-Lyse buffer to each well, resuspend and let it sit for 10 minutes at RT.
5. Centrifuge cells at 650 g for 10 minutes at RT, aspirate supernatant from the cells.

Barcoding:

1. Barcode Perm Buffer: Prepare 4 mL for each sample to barcode by mixing 1 part Maxpar 10X Barcode Perm with 9 parts Maxpar PBS; store at 4℃ for up to one week. Prepare 500 ml for 12 samples (use entire bottle of 10X MaxPar Perm Buffer).
2. Wash each sample with 1 mL of Barcode Perm Buffer (Centrifuge cells at 650 g for 10 minutes at RT, aspirate supernatant from the cells). Repeat for total of 3 washes.
3. Resuspend each sample to be barcoded completely in 800 µL Barcode Perm Buffer.
4. Resuspend barcodes completely in 100 µL Barcode Perm Buffer and transfer 110 µl to the appropriate samples. Mix the sample immediately and completely.

5. Incubate for 30 minutes at RT.
6. Wash with 2 mL of Maxpar Cell Staining Buffer (Centrifuge cells at 650 g for 10 minutes at RT, aspirate supernatant from the cells). Repeat for total of two washes.
7. Resuspend each sample in 200 µL Maxpar Cell Staining Buffer. Combine all eight barcoded samples from a given donor into one well using same plate, add 1 mL of Cell Staining Buffer to each well of combined samples and centrifuge at 650 g for 10 min at RT.

Now we have 12 barcoded pooled samples (12 donors) in two plates.

Surface staining:

1. Prepare surface antibody cocktail in Cell Staining Buffer according to previously determined titration. Filter in 0.1 um spin filter (centrifuge in a tabletop microcentrifuge (RCF=14,000) for 5 minutes at 4C).
2. Add 100 uL surface cocktail to each sample, resuspend and incubate at RT for 30 min. L SEP
3. Wash cells in 1 mL/well with Cell Staining buffer and centrifuge cells at 800g for 10 min at 4C. Discard supernatant by aspiration. L SEP
4. Repeat step 3 for total of two washes

Additional fix and permeabilization:

1. Add 200 µL of 4% PFA in PBS to each sample, incubate for 10 min at RT.
2. Wash with 1mL PBS, spin at 800g, aspirate
3. Permeabilize cells by adding 600 µL ice cold MeOH to each sample and resuspend by Pipetting up and down. Store overnight or longer at -80°C.

Intracellular staining

1. Remove one 48-well plate with 6 samples from -80C.
2. Wash each sample with 1 mL PBS buffer. Centrifuge cells at 730 g for 10 min at 4C. Discard supernatant by aspiration.
3. Repeat step 1 for total of two washes.
4. Prepare intracellular antibody cocktail in PBS according to previously determined titration. Filter in 0.1 um spin filter (centrifuge in a tabletop microcentrifuge (RCF=14,000) for 5 minutes at 4C.
5. Add 100 µL of antibody cocktail to each sample and incubate at RT for 30 min.
6. Wash in 1 mL PBS. Centrifuge cells 730 g for 10 min at 4C, aspirate.

7. Repeat wash step above.
8. Make 1:2000 dilution in 2% PFA/PBS of Ir-intercalator. Add 200 µl of diluted Ir-intercalator to each sample, pipet to mix. Incubate at RT for 20 min or overnight at 4C.
9. Wash with 1ml PBS per sample. Centrifuge cells 730 g for 10 min at 4C, aspirate.
10. Wash with 1 MilliQ water per sample. Centrifuge cells 2000 RPM for 10 min at 4C, aspirate. Repeat wash with 1mL MilliQ water for total of two washes.

Sample acquisition, file processing and analysis:

1. Acquire samples on the Helios at 300 events/sec rate (final dilution of sample should be 750,000 cells/ ml); collect one million events per sample.
2. Normalize FCS file using Fluidigm normalization software.
3. Concatenate if needed.
4. Debarcode each sample with Fluidigm Debarcoder and generate 8 FCS files per donor (plus one unassigned event fcs file). Each fcs file corresponds to one stim condition.
5. Upload ALL RESULTING FILES (“BATCH”) into single FlowJo workspace. For example, if six barcoded samples were run on a given day, resulting “batch” workspace should contain 48 individual fcs files.
6. Debarcoded fcs files gated using WB phospho template.
7. Upload “Batch” FlowJo workspace (naming the file “date_batch”) into Sherpa.
8. Zip Debarcoded individual donor files and Flowjo workspace and upload into Sherpa.
9. QC of each batch is performed in cytobank. Debarcoded fcs files are uploaded into community.cytobank.org, major cell population are gated using cytobank gating template: intact cells, granulocytes, monocytes, lymphocytes, CD3, B cells, NK cells and workspace with signaling markers histogram overlay is generated for each donor for all stimulation conditions. This step will ensure that no mistake was made during collection/smart tube labeling process. If the mistake is detected, re-debarcode and rename the files giving them proper _Stim extensions.
10. For further QC of each stim condition, medians of phospho markers that should be positive for specific stim condition in certain cell populations are extracted into Excel spreadsheet. Medians are averaged per batch (average of 6 donors) per phospho marker per stim and plotted in Excel, with corresponding unstimulated condition sample readout plotted on the same chart, resulting in at least one phosphoflow readout being positive for one stim.

Following phosphoflow markers and cell populations are analyzed:

- Stat1 stimulation by IFNa in monocytes;
- Stat3 stimulation by IL6 in monocytes, by IL10 in monocytes and lymphocytes, and by IL21 in lymphocytes;
- Stat5 stimulation by IL7 in lymphocytes
- p38 stimulation by LPS in monocytes and by PMA/Ionomycin in monocytes and lymphocytes;
- CREB stimulation by LPS in monocytes;
- Erk stimulation by LPS in monocytes and by PMA/Ionomycin in monocytes and lymphocytes.

11. For alternative QC analysis, pCyTOF (whole blood) v1+v2 phospho readouts of [archsin (stim) - archsin(unstim)] ratios were downloaded from Stanford Data Miner to Excel spreadsheet for every batch run in the years 2013-2016. The resulting table was further reduced to contain only the best readouts corresponding to seven stimulation conditions (IFNa, IL6, IL7, IL10, IL21, LPS and PMA/Ionomycin). The bar diagrams were constructed for each stimulation condition (one or more readout per condition). The examples of graphed readouts include:

- Stat1, 3 and 5 stimulation by IFNa in monocytes;
- Stat3 stimulation by IL10 and IL6 in monocytes;
- Stat3 stimulation by IL21 in B cells;
- Stat5 stimulation by IL7 in CD4+ T cells;
- Creb, IkB and pp38 stimulation by LPS and PMA/Ionomycin in monocytes.

Appendix 2: Stims

HUMAN IMMUNE MONITORING CENTER

Preparation of IFNa for Stimulation of 200 ul whole blood sample

Materials

IFN Alpha 2: pbl assay science cat. # 11105-1, volume 0.1 ml in PBS

Pay attention to Activity, as it varies from lot to lot

To make stock: add enough volume to make 10X10^6 units/ml stock concentration

For example, if **Activity** was 2.09X10^6 units/ml, and 109 ul of PBS to original vial to make 2.09X10^5/209ul.

Aliquot to 5 ul per tube, store at -80C.

Methods

1. Make **intermediate dilution**, working on ice: Thaw a stock aliquot, add 3 ul of stock IFNa to 600 ul of RPMI-1640. Mix thoroughly. This volume will be enough to stim 12 samples. Scale accordingly if required.
2. Add 50 ul of intermediate IFNa dilution to 200 ul of whole blood or 200 ul PBMC containing 1x10^6 cells, and follow stim protocol.
3. **Final concentration is 10,000 units/ml IFNa**

Math:

Stock concentration is 10X10^6 units/ml (add 109ul PBS to 100 ul of 2.09X10^6 units/ml making 2.09X10^6 units/209ul or 1X10^6 units/100ul or 10X10^6 units/1ml)

Intermediate concentration is 50,000 units/ml (3 ul of stock to 600 ml RPMI or 1/200 dilution)

Final concentration is 10,000 units/ml (50 ul of intermediate stock to 200 ul of whole blood or 200 ul PBMC or 1/5 dilution)

Preparation of IL6 for Stimulation of 200 ul whole blood sample

Materials

IL6: BD Biosciences cat. # **550071**, concentration 200 ug/ml, size 10 ug (in 50 ul volume)

To make stock: add 50 ul PBS to make 100ug/ml stock concentration

Aliquot to 5 ul per tube, store at -80C.

Methods

1. Make **intermediate dilution**, working on ice: Thaw a stock aliquot, add 2.5 ul of stock IL6 to 1000 ul of RPMI-1640. Mix thoroughly. This volume will be enough to stim 20 samples. Scale accordingly if required.
2. Add 50 ul of intermediate IL6 dilution to 200 ul of whole blood or 200 ul PBMC containing 1x10^6 cells, and follow stim protocol.
3. **Final concentration is 50 ng/ml**

Math:

Stock concentration is 100ug/ml

Intermediate concentration is 250 ng/ml (2.5 ul of stock to 1000 ml RPMI or 1/400 dilution)

Final concentration is 50 ng/ml (50 ul of intermediate stock to 200 ul of whole blood or 200 ul PBMC or 1/5 dilution)

Preparation of IL7 for Stimulation of 200 ul whole blood sample

Materials

IL7: BD Biosciences cat. # **554608**, 5 micrograms lyophilized powder

To make stock: add 50 ul PBS to make 100ug/ml stock concentration

Aliquot to 5 ul per tube, store at -80C.

Methods

1. Make **intermediate dilution**, working on ice: Thaw a stock aliquot, add 2.5 ul of stock IL7 to 1000 ul of RPMI-1640. Mix thoroughly. This volume will be enough to stim 20 samples. Scale accordingly if required.
2. Add 50 ul of intermediate IL7 dilution to 200 ul of whole blood or 200 ul PBMC containing 1x10^6 cells, and follow stim protocol.
3. **Final concentration is 50 ng/ml**

Math:

Stock concentration is 100ug/ml

Intermediate concentration is 250 ng/ml (2.5 ul of stock to 1000 ml RPMI or 1/400 dilution)

Final concentration is 50 ng/ml (50 ul of intermediate stock to 200 ul of whole blood or 200 ul PBMC or 1/5 dilution)

Preparation of IL10 for Stimulation of 200 ul whole blood sample

Materials

IL10: BD Biosciences cat. # **554611**, concentration 100 ug/ml, size 50 ug (in 50 ul volume)

Aliquot to 5 ul per tube, store at -80C.

Methods

1. Make **intermediate dilution**, working on ice: Thaw a stock aliquot, add 2.5 ul of stock IL10 to 1000 ul of RPMI-1640. Mix thoroughly. This volume will be enough to stim 20 samples. Scale accordingly if required.
2. Add 50 ul of intermediate IL10 dilution to 200 ul of whole blood or 200 ul PBMC containing 1x10^6 cells, and follow stim protocol.
3. **Final concentration is 50 ng/ml**

Math:

Stock concentration is 100ug/ml

Intermediate concentration is 250 ng/ml (2.5 ul of stock to 1000 ml RPMI or 1/400 dilution)

Final concentration is 50 ng/ml (50 ul of intermediate stock to 200 ul of whole blood or 200 ul PBMC or 1/5 dilution)

Preparation of IL21 for Stimulation of 200 ul whole blood sample

Materials

IL21 Life Technologies Cat. No. PHC0214, 10ug lyophilized powder

To make stock: add 100 ul PBS to make 100ug/ml stock concentration

Aliquot to 5 ul per tube, store at -80C.

Methods

1. Make **intermediate dilution**, working on ice: Thaw a stock aliquot, add 2.5 ul of stock IL21 to 1000 ul of RPMI-1640. Mix thoroughly. This volume will be enough to stim 20 samples. Scale accordingly if required.
2. Add 50 ul of intermediate IL21 dilution to 200 ul of whole blood or 200 ul PBMC containing 1x10^6 cells, and follow stim protocol.
3. **Final concentration is 50 ng/ml**

Math:

Stock concentration is 100ug/ml

Intermediate concentration is 250 ng/ml (2.5 ul of stock to 1000 ml RPMI or 1/400 dilution)

Final concentration is 50 ng/ml (50 ul of intermediate stock to 200 ul of whole blood or 200 ul PBMC or 1/5 dilution)

Preparation of LPS for Stimulation of 200 ul whole blood sample

Materials

LPS: **Sigma -Aldrich,** Cat. No. L7770, 1 mg lyophilized powder

To make stock: add 1000 ul milliQ water to make 1mg/ml stock concentration

Aliquot to 5 ul per tube, store at -20C.

Methods

1. Make **intermediate dilution**, working on ice: Thaw a stock aliquot, add 2.5 ul of stock LPS to 500 ul of RPMI-1640. Mix thoroughly. This volume will be enough to stim 10 samples. Scale accordingly if required.
2. Add 50 ul of intermediate LPS dilution to 200 ul of whole blood or 200 ul PBMC containing 1x10^6 cells, and follow stim protocol.
3. **Final concentration is 1 ug/ml**

Math:

Stock concentration is 1 mg/ml

Intermediate concentration is 5 ug/ml (2.5 ul of stock to 500 ml RPMI or 1/200 dilution)

Final concentration is 1 ug/ml (50 ul of intermediate stock to 200 ul of whole blood or 200 ul PBMC or 1/5 dilution)

Preparation of PMA/Ionomycin for Stimulation of 200 ul whole blood sample

Materials

PMA: Sigma Catalog # P8139, 1mg powder

Ionomycin Calbiochem Catalog # 407952, 1mg powder

To make PMA stock: add 1000 ul DMSO to 1mg vial to make 1 mg/ml stock concentration

Aliquot to 10 ul per tube, store at -20C.

To make Ionomycin stock: add 100 ul DMSO to 1mg vial to make 10 mg/ml stock concentration

Aliquot to 10 ul per tube, store at -20C.

Methods

1. Make first **intermediate dilution of PMA**: thaw a stock aliquot at RT, add 1 ul of stock PMA to 100 ul of RPMI-1640. Mix thoroughly.
2. Make first **intermediate dilution of Ionomycin**: thaw a stock aliquot at RT, add 1 ul of stock PMA to 9 ul of RPMI-1640. Mix thoroughly.
3. Make **second intermediate dilution** of PMA/Ionomycin: add 2.5 ul of first intermediate dilutions of PMA and 2.5 ul of first intermediate dilutions of Ionomycin to 500 ul RPMI-1640. Mix thoroughly. This volume will be enough to stim 10 samples. Scale accordingly if required.
4. Add 50 ul of second intermediate PMA/Ionomycin dilution to 200 ul of whole blood or 200 ul PBMC containing 1x10^6 cells, and follow stim protocol.
5. **Final concentration is 10 ng/ml PMA and 1 ug/ml Ionomycin**

Math:

Stock concentration of PMA is 1mg/ml, stock concentration of ionomycin is 10mg/ml

First intermediate concentration of PMA is 10 ug/ml (1 ul of stock to 100 ml RPMI or 1/100 dilution),

First intermediate concentration of ionomycin is 1 mg/ml (1 ul of stock to 9 ml RPMI or 1/10 dilution)

Second intermediate concentration of PMA is 50 ng/ml (2.5 ul of first intermediate stock to 500 ml RPMI or 1/200 dilution),

Second intermediate concentration of ionomycin is 5 ug/ml (2.5 ul of first intermediate stock to 500 ml RPMI or 1/200 dilution),

Final concentration of PMA is 10 ng/ml (50 ul of second intermediate stock to 200 ul of whole blood or 1/5 dilution)

Final concentration of ionomycin is 1 ug/ml (50 ul of second intermediate stock to 200 ul of whole blood or 1/5 dilution)

Appendix 3: Supplementary figures (Intra-assay reproducibility of the Smart Tube assay for whole blood analysis on a additional donor)

Image A: % of parents and CVs of the frequencies for a large number of populations in whole blood.

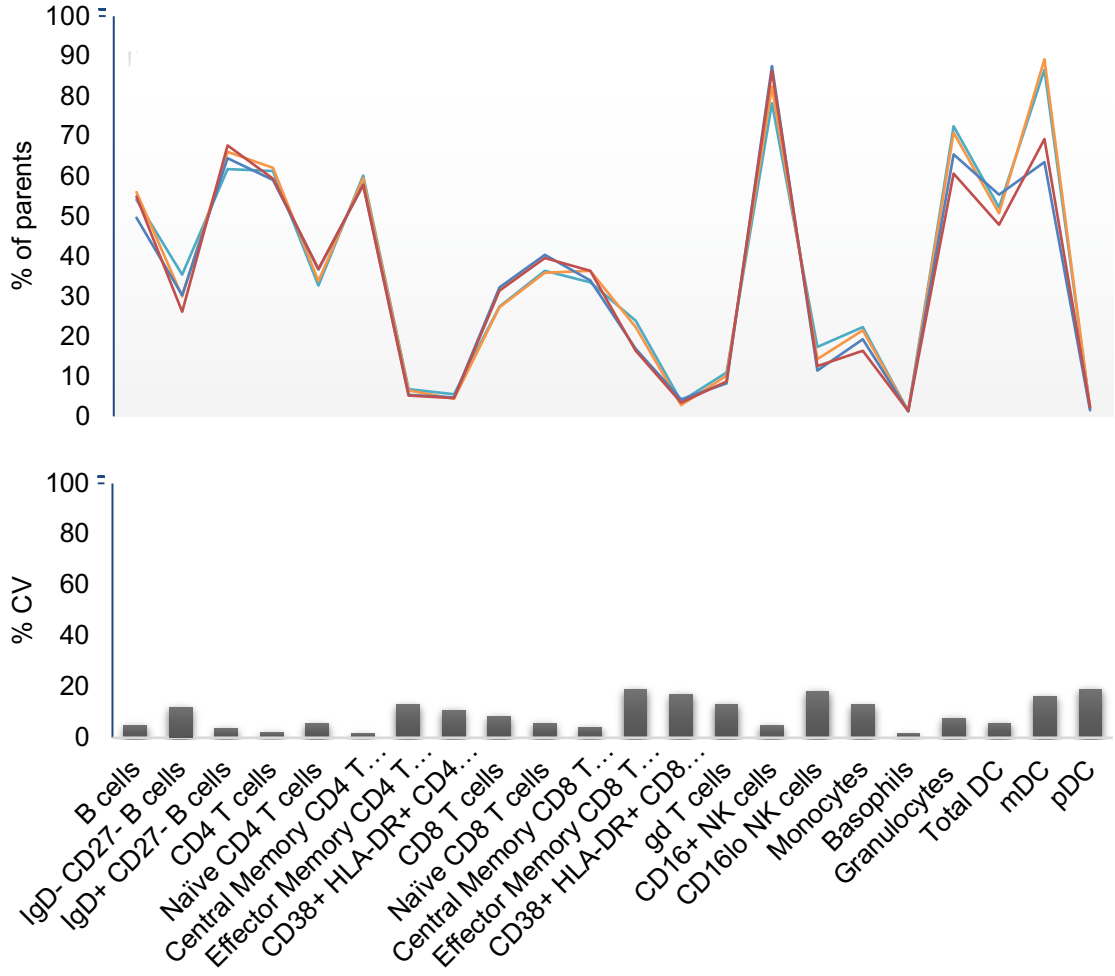
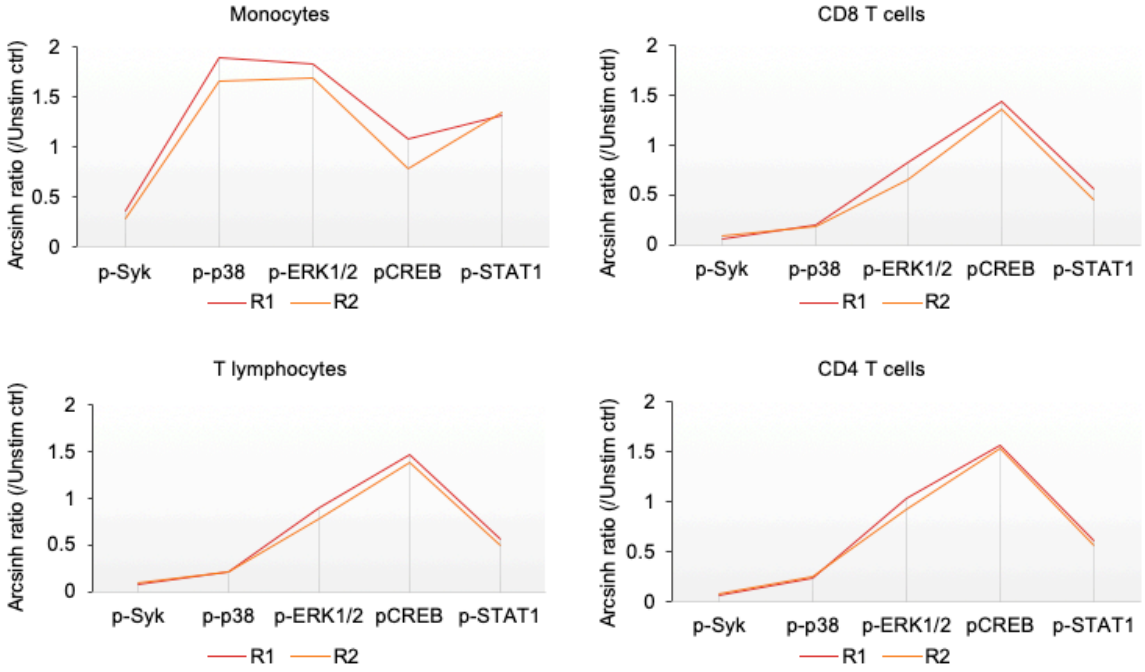


Image B: Arcsinh ratios of medians and CVs for pP38, pERK1/2 and pCREB in monocytes stimulated with PMA-Ionomycin.



References

1. Leipold, M. D., G. Obermoser, C. Fenwick, K. Kleinstuber, N. Rashidi, J. P. McNevin, A. N. Nau, L. E. Wagar, V. Rozot, M. M. Davis, S. DeRosa, G. Pantaleo, T. J. Scriba, B. D. Walker, L. R. Olsen, and H. T. Maecker. 2018. Comparison of CyTOF assays across sites: Results of a six-center pilot study. *J Immunol Methods* 453: 37-43.
2. Mei, H. E., M. D. Leipold, A. R. Schulz, C. Chester, and H. T. Maecker. 2015. Barcoding of live human peripheral blood mononuclear cells for multiplexed mass cytometry. *J Immunol* 194: 2022-2031.
3. Gaudilliere, B., G. K. Fragiadakis, R. V. Bruggner, M. Nicolau, R. Finck, M. Tingle, J. Silva, E. A. Ganio, C. G. Yeh, W. J. Maloney, J. I. Huddleston, S. B. Goodman, M. M. Davis, S. C. Bendall, W. J. Fantl, M. S. Angst, and G. P. Nolan. 2014. Clinical recovery from surgery correlates with single-cell immune signatures. *Sci Transl Med* 6: 255ra131.
4. Smets, T., F. Stevenaert, H. Adams, 3rd, and G. Vanhoof. 2018. Deep Profiling of the Immune System of Multiple Myeloma Patients Using Cytometry by Time-of-Flight (CyTOF). *Methods Mol Biol* 1792: 47-54.