

Appendix 1: SOP**Cytokine-Stimulated Phosphoflow of Whole Blood Flu samples using CyTOF Mass Cytometry**

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

Note that both channels Ta and Nb have to be on for the acquisition.

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) ^[L]_[SEP]
- Methanol (Thermo Fisher Scientific, catalog number: A452SK-1) ^[L]_[SEP]
- Phenotyping and phosphoprotein antibodies filtered with 0.1 um spin filters (EMD Millipore, model: UFC30VV00) ^[L]_[SEP]
- Ir-intercalator stock solution (Fluidigm Cat#: 201192B) ^[L]_[SEP]
- 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) ^[L]_[SEP]

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 intercalator-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 °C 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 μ L 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 μ m spin filter (centrifuge in a tabletop microcentrifuge (RCF=14,000) for 5 minutes at 4C).
2. Add 100 μ L surface cocktail to each sample, resuspend and incubate at RT for 30 min. 
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. 
4. Repeat step 3 for total of two washes

Additional fix and permeabilization:

1. Add 200 μ L 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 μ L 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 μ m spin filter (centrifuge in a tabletop microcentrifuge (RCF=14,000) for 5 minutes at 4C).
5. Add 100 μ L 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 IFN α 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 (IFN α , 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 IFN α 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, I κ B and pp38 stimulation by LPS and PMA/Ionomycin in monocytes.

Appendix 2: Stims

HUMAN IMMUNE MONITORING CENTER

Preparation of IFN α for Stimulation of 200 μ l 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 10×10^6 units/ml stock concentration

For example, if **Activity** was 2.09×10^6 units/ml, and 109 μ l of PBS to original vial to make $2.09 \times 10^5 / 209 \mu$ l.

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

Methods

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

Math:

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

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

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

Preparation of IL6 for Stimulation of 200 μ l whole blood sample

Materials

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

To make stock: add 50 μ l PBS to make 100 μ g/ml stock concentration

Aliquot to 5 μ l 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 1×10^6 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 1×10^6 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 1×10^6 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 1×10^6 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 1×10^6 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 1×10^6 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.

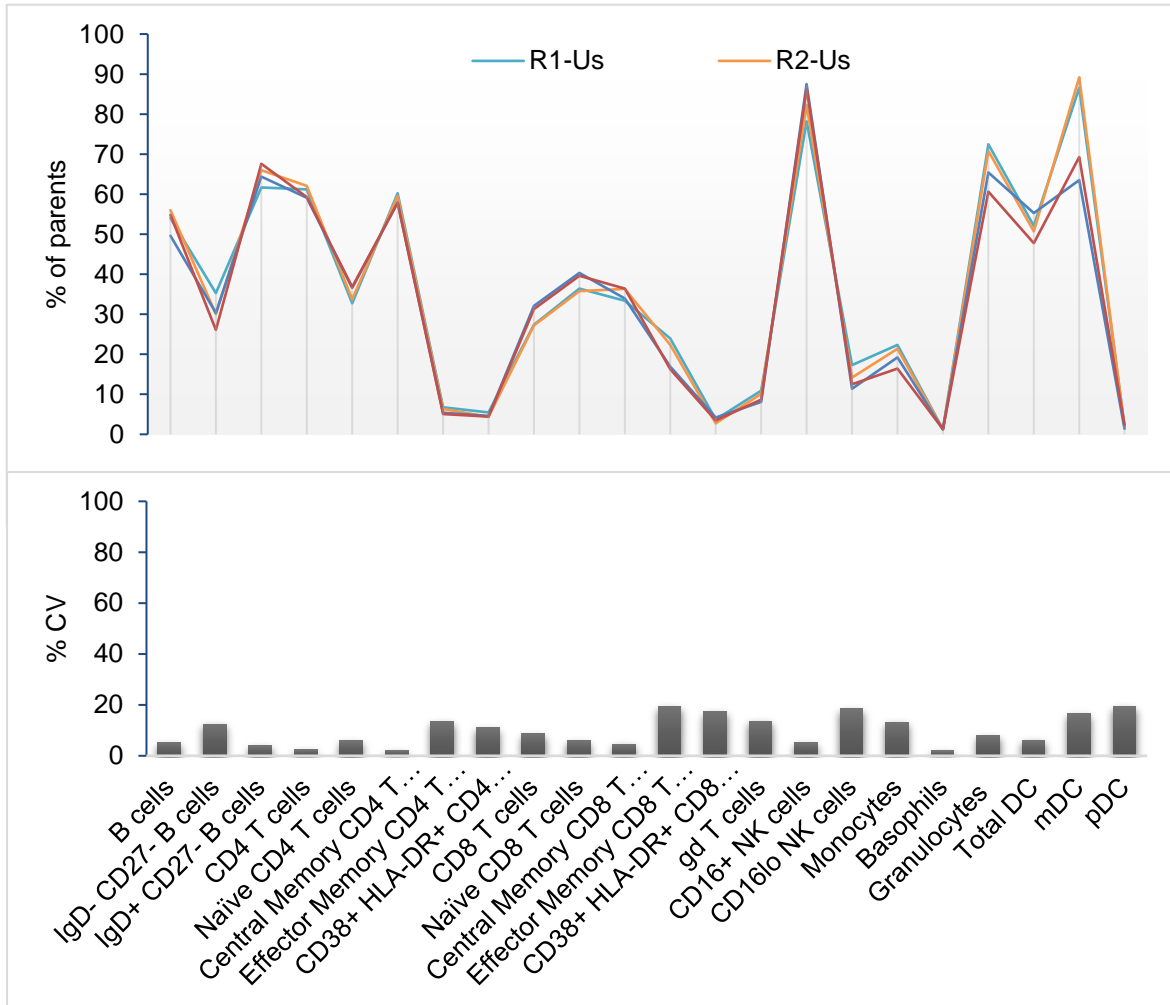
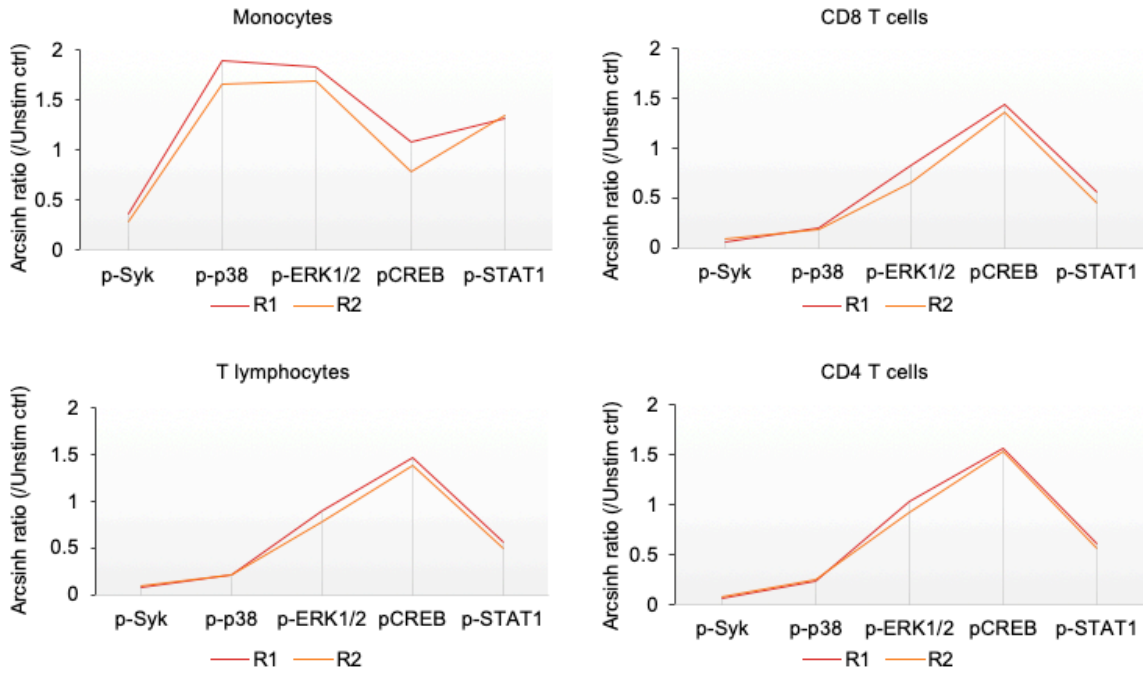


Image B: Arcsinh ratios of medians and CVs for pP38, pERK1/2 and pCREB in monocytes stimulated with PMA-Ionomycin.



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.