Cytokine-Stimulated Phosphoflow of Whole Blood using CyTOF Mass Cytometry

1. Principle

Phosphorylation of tyrosine, serine, and threonine residues is critical for the control of protein activity involved in various cellular events. An assortment of kinases and phosphatases regulate intracellular protein phosphorylation in many different cell signaling pathways, such as T and B cell signaling, those regulating apoptosis, growth and cell cycle control, plus those involved with cytokine, chemokine, and stress responses. Phosphoflow assays combine phospho-specific antibodies with the power of flow cytometry to enhance phospho protein study. In our assay, PBMCs are stimulated by cytokines, fixed, surface-stained with a cocktail of antibodies labeled with MAXPAR metal-chelating polymers and permeabilized with methanol. They are then stained with intracellular phospho-specific antibodies.
We use a CyTOF™ mass cytometer to acquire the ICP-MS data. The current mass window selected is approximately AW 103-203, which includes the lanthanides used for most antibody labeling, as well as iridium and rhodium for DNA intercalators. Subsequent analysis of the dual count signal data using FlowJo software allows for cell types to be analyzed based on the dual count signal in each mass channel. The percentage of each cell type is determined and reported as a percent of the parent cell type. Median values are reported to quantitate the level of phosphorylation of each protein in response to stimulation. Comparing the level of phosphorylation between samples can offer insight to the status of the immune system. Whole blood stimulation is the closest to in vivo condition and it allows for assessment of granulocyte population as well as lymphocytes and monocytes.

2. Materials and Equipment

2.1. Whole blood

2.2. Complete RPMI (RPMI with 10% FBS, P/S, glutamine)

2.3. Nunc Coded Cryobank Vials (Cluster tubes)

2.4. Cytokine aliquots (IFNα, IL-6, IL-7, IL-10, IL-21, LPS, PMA/Ionomycin etc)
2.5. Methanol
2.6. DPBS (-Ca, -Mg)
2.7. Phenotyping and phosphoprotein antibodies filtered with 0.1 um spin filters.
2.8. Ir-intercalator stock solution from DVS (Rh103-intercalator can be used).
2.9. 37°C water bath
2.10. Biosafety cabinet
2.11. Centrifuge
2.12. CO_2 incubator at 37°C
2.13. Calibrated pipettes
2.14. 8 or 12 pin aspirator
2.15. Smart Tube Proteomic Stabilizer
2.16. Smart Tube 1X Thaw-Lyse buffer

3. Procedure

Prepare Stims in Cluster Tubes.

3.3. Prepare cytokines at 5X concentrations in Complete RPMI (RPMI with 10% FBS, P/S, glutamine), with enough volume to pipette 50µl into a well for each sample and control. See chart below for dilution for a full plate.

3.4. The Cluster tubes with the aliquoted stims can be frozen away at -80C until further use.

4. Example of a full plate:

1	2	3	4	5	6	7	8	9	10	11	12
unstim	unstim	unstim	unstim	unstim	unstim	unstim	unstim	unstim	unstim	unstim	unstim
IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa
IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6
IL-7	IL-7	IL-7	IL-7	IL-7	IL-7	IL-7	IL-7	IL-7	IL-7	IL-7	IL-7
IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10	IL-10
IL-21	IL-21	IL-21	IL-21	IL-21	IL-21	IL-21	IL-21	IL-21	IL-21	IL-21	IL-21
LPS	LPS	LPS	LPS	LPS	LPS	LPS	LPS	LPS	LPS	LPS	LPS
PMA/Io	PMA/Io	PMA/Io	PMA/Io	PMA/Io	PMA/Io	PMA/Io	PMA/Io	PMA/Io	PMA/Io	PMA/Io	PMA/Io

5. **Example of cytokine stims**:

IFNa : PBL Interferon source Catalog # 11105-1
Final concentration of stim used= 10000 units/ ml

IFNg2 : BD Pharmingin Catalog # 554617
Final concentration of stim used= 50 ng/ ml

IL6: BD Pharmingin Catalog # 550071
Final concentration of stim used= 50 ng/ ml

IL7: BD Pharmingin Catalog # 554608
Final concentration of stim used= 50 ng/ ml

IL10: BD Pharmingin Catalog # 554611
Final concentration of stim used= 50 ng/ ml

IL21: GIBCO Catalog # PHC0214
Final concentration of stim used= 50 ng/ ml

IL2: BD Pharmingin Catalog # 554603
Final concentration of stim used= 50 ng/ ml

CD3 = 2.5 ul in 990ul (Final conc 500ng/ml) BD Pharmingin Catalog # 555329
CD28 = 10 ul in above media Final (conc 2000ng / ml)
BD Pharmingin Catalog #555725

LPS: Sigma catalog# L7770
Final concentration of stim used= 1 ug/ ml

PMA	10ng/ml final conc./ ml
Ionomycin	1000ng/ml final conc/ml

IL5: Peprotech Catalog# 200-05
Final concentration of stim used= 10 ng/ ml

IL17A: Peprotech Catalog# 200-17
Final concentration of stim used= 50 ng/ ml

IL17E: Peprotech Catalog# 200-24
Final concentration of stim used= 50 ng/ ml

6. **Stimulation :**

6.1 Rest the blood collected from donors in incubator at 37 C in CO_2 incubator for 1 hour. Just before use, take out the required number of cluster tubes with the stims from the -80 freezer and let warm at 37 C water bath for 5-10 minutes.

6.2 Aliquot 200 ul of whole blood into column 1 of the cluster tube plate. Change tips between each patient.
Repeat with all the columns of tubes depending on the number of donors. Work as rapidly as possible.
Tap plate to mix, and incubate 15 minutes at 37°C in CO_2 incubator.

6.3 Remove cells from incubator at 15 minutes and using a multichannel pipette, add 250 µl of 1x Proteomic Stabilizer (Smart Tube, Inc) to each column of patient samples in the Cluster tube block. Pipette up and down to mix for each patient. Change tips between patients. Add the stabilizer in the same order that you added the cytokine stimulation.

6.4 Incubate for 10 minutes at room temperature. At this point it can be frozen away at -80 °C until it is ready to be surface stained.

7. Surface Staining:

7.1 Take out the required number of frozen stimmed cluster tubes with whole blood as can be comfortably run on the cyTOF in 1 day. Thaw in cold water for about 15 min.

7.2 Using a pipette, transfer the fixed sample into 15ml tube with 1x Thaw-Lyse and let it sit for 10 minutes at RT.

7.3 Centrifuge cells at 1650 rpm for 8 minutes at RT. Aspirate supernatant from the cells.

7.4 Add 15 ml, 1x Thaw-Lyse and let it sit for 5 minutes at RT. Centrifuge cells at 1650 rpm for 8 minutes at RT. Aspirate supernatant from the cells.

7.5 Now add ~ 200- 500 ul of PBS to the tube and transfer the pellet to a labeled deep well plate. Transfer all samples this way.

7.6 Centrifuge cells at 2000 rpm for 10 minutes at 4°C. Aspirate supernatant from the cells so that about 50 ul remains at the bottom of each well.

7.7 Make cocktail in PBS of metal-chelating polymer-labeled surface antibodies according to previously determined titration. Make sufficient volume for each sample to have 20 uL of cocktail. Pipet into 0.1 um spin filter and centrifuge in a tabletop microcentrifuge (RCF=14,000) for 2 minutes at room temperature.

7.8 Add 20 ul of antibody cocktail to the cells in the deep well plate, vortex to mix and let it incubate at RT for 30 minutes.

7.9 Wash cells with PBS and centrifuge cells at 2000 rpm for 10 minutes at 4 °C. Aspirate.

7.10. Permeabilize the cells by adding 600µl cold MeOH to each well of the deep well block using a multichannel pipette. Pipette up and down to mix for each patient. Change tips between patients. Cells are stored overnight at this point at -80°C.

7.11 Remove samples from freezer. Add 1.2 ml of PBS. Centrifuge cells at 2000 rpm for 10 minutes at 4 °C. Aspirate

8. Intracellular Staining:

8.1 Make cocktail in PBS of metal-chelating polymer-labeled intracellular antibodies according to previously determined titration. Make sufficient volume for each sample to have 20 uL of cocktail. Pipet into 0.1 um spin filter and centrifuge in a tabletop microcentrifuge (RCF=14,000) for 2 minutes at room temperature.

8.2 Add 20 ul of antibody cocktail to the cells in the deep well plate, vortex to mix and let it incubate at RT for 30 minutes.

8.3 Add 1.6 ml of PBS. Centrifuge cells at 2000 rpm for 10 minutes at 4 °C. Aspirate

8.4 Make 1:200 dilution in PBS of Ir-intercalator. Add 20 uL of diluted Ir-intercalator solution to each sample, pipet to mix. Incubate on ice for 20 minutes.

8.5 Wash x 3 in MilliQ water .Centrifuge cells at 2000 rpm for 10 minutes at 4 °C. Aspirate

8.6 Bring up the samples in 1 ml MilliQ water and filter samples through a cell strainer. Acquire samples on the CyTOF, after standard instrument setup procedures.