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Cytokine-Stimulated Phosphoflow of PBMC 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.

2. Materials and Equipment

2.1. PBMC, fresh or thawed frozen
2.2. Complete RPMI (RPMI with 10% FBS, P/S, glutamine)
2.3. Benzonase
2.4. Cytokine aliquots (IFNα, IFNγ, IL-6, IL-7, IL-10, IL-21, IL-2 etc)
2.5. 16% PFA
2.6. Methanol

2.7. CyFACS buffer (1x CyPBS with 0.1% BSA, and 0.05% Na Azide; made in MilliQ water). Do NOT use FBS!!
2.8. Phenotyping and phosphoprotein antibodies filtered with 0.1 um spin filters.
2.9. Ir-intercalator stock solution from DVS (Rh103-intercalator can be used).
2.10. 37°C water bath
2.11. Biosafety cabinet
2.12. Centrifuge
2.13. CO_2 incubator at 37°C
2.14. Calibrated pipettes
2.15. 8 or 12 pin aspirator

3. Procedure

Thaw PBMC

3.3. Warm media to 37°C in water bath. Each sample will require 20 ml of media with benzonase. Calculate the amount needed to thaw all samples, and prepare a separate aliquot of warm media with 1:10000 benzonase (25 U/ml). Remove samples from liquid nitrogen and transport to lab on dry ice.
3.4. Place 10ml of warmed benzonase media into a 15ml tube, making a separate tube for each sample.
3.5. Thaw frozen vials in 37°C water bath.
3.6. When cells are nearly completely thawed, carry to hood.
3.7. Add 1ml of warm benzonase media from appropriately labeled centrifuge tube slowly to the cells, then transfer the cells to the centrifuge tube. Rinse vial with more media from centrifuge tube to retrieve all cells.
3.8. Continue with the rest of the samples as quickly as possible.
3.9. Centrifuge cells at 1600 rpm (RCF=390) for 10 minutes at room temperature.
3.10. Remove supernatant from the cells and resuspend the pellet by tapping the tube.
3.11. Gently resuspend the pellet in 1ml warmed benzonase media. Centrifuge cells at 1600 rpm (RCF=390) for 10

minutes at room temperature. Remove supernatant from the cells and resuspend the pellet by tapping the tube.

3.12. Resuspend cells in 1ml warm media.

3.13. Count cells with Vicell (or hemocytometer if necessary). To count, take 20ul cells and dilute with 480ul PBS in vicell counting chamber. Load onto Vicell as PBMC with a 1:25 dilution factor.

3.14. Adjust the cell concentration to $5 * 10^6$ cells/ml with warm media (no more benzonase at this point.)

3.15.

3.16. Using a multichannel pipette, add 200 µl cells ($0.5 * 10^6$ cells) into each of eight wells of a 96-well deep well plate.

3.17. Rest for another 1 hour- 1.5 hours at 37°C in CO_2 incubator. During rest period, prepare the stimulation plate.

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		
IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa	IFNa		
IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg	IFNg		
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-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-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2		

Stimulate cells

3.18. Prepare cytokines at 5X concentrations, with enough volume to pipette 50µl into a well for each sample and control. See chart below for dilution for a full plate.

3.19. Add enough 5x cytokine (600µl for a full plate) into one row of a fresh deep well block to pipette.

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
Ionomycin	1000ng/ml final conc

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

3.20. Remove rested cells in the deep well block from incubator and stimulate by adding 50µl of 5x cytokines with multichannel plate to each row of patient samples. Change tips between each patient. Work as rapidly as possible.
3.21. Tap plate to mix, and incubate 15 minutes at 37°C in CO_2 incubator.
3.22. Remove cells from incubator at the 15 minutes and using a multichannel pipette, add 25µl 16% PFA to each row of patient samples in the deep well block. Pipette up and down to mix for each patient. Change tips between patients. Add PFA in the same order that you added the cytokine stimulation.
3.23. Incubate 10 minutes at room temperature.
3.24. Add 1.2 ml CyFACS buffer (1x CyPBS with 0.1% BSA, and 0.05% Na Azide; made in MilliQ water) to each well of the deep well block.
3.25. Centrifuge cells at 2000 rpm for 8 minutes at 4 °C.
3.26. Aspirate supernatant from the cells.

Surface Staining:

3.27. Make cocktail in CyFACS buffer 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 10 minutes at room temperature.

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

3.29. Wash cells with CyFACS buffer and centrifuge cells at 2000 rpm for 8 minutes at 4 °C. Aspirate.

3.30. 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.

3.31. Cells are stored overnight at this point at -80°C.
3.32. Remove samples from freezer.
3.33. Wash in CyFACS buffer.
3.34. Centrifuge cells at 2000rpm for 8 minutes at 4 °C. Aspirate

Intracellular Staining:

3.35. Make cocktail in CyFACS buffer 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 10 minutes at room temperature.
3.36. Add 20 ul of antibody cocktail to the cells in the deep well plate and let it incubate at RT for 30 minutes.

3.37. Wash in CyPBS.
3.38. Centrifuge cells at 2000rpm for 8 minutes at 4 °C. Aspirate
3.39. Make 1:200 dilution in CyPBS of Ir-intercalator. Add 20 uL of diluted Ir-intercalator solution to each sample, pipet to mix. Incubate on ice for 20 minutes.
3.40. Wash x 3 in MilliQ water .
3.41. Centrifuge cells at 2000rpm for 8 minutes at 4 °C. Aspirate
3.42. Acquire samples on the CyTOF, after standard instrument setup procedures.