MaxPar® Phospho-Protein Staining Protocol

Reagents and Materials:

- MaxPar® Metal Conjugated Antibodies
- MaxPar® Cell Staining Buffer (Cat# 201068)
- MaxPar® Fix and Perm Buffer (Cat# 201067)
- MaxPar® Water (Cat# 201069)
- MaxPar® Intercalator –Ir (Cat# 201192A [125 µM] or 201192B [500 µM])
- 16% Formaldehyde (Thermo Scientific Cat# 28908)
- Methanol (Fisher Scientific Cat# BP1105-4)
- Polystyrene or Polypropylene Round-bottom Tubes, 5 mL capacity, 12 x 75 mm
- Polystyrene or Polypropylene Round-bottom Tubes with Cell-Strainer Cap, 5 mL capacity, 12 x 75 mm
- 1.5 ml microfuge tubes
- Pipet tips with aerosol barrier
- Centrifuge capable of holding 5 ml tubes
- Vacuum aspirator
- Vortexer
- [Optional] Fc-receptor Blocking Solution (BD Cat# 553142 [mouse], BioLegend Cat# 422302 [human])
- [Optional] Cisplatin (Sigma Cat# 479306)
- [Optional] DMSO (ATCC Cat# 4-X)
- [Optional] Serum-Free and Complete Media

Important Notes Before Starting:

- This protocol should be followed for staining activation-induced phosphorylated antigens. For staining secreted proteins, including cytokines, and other intracellular antigens, please use the MaxPar Cytokine and Intracellular (cytoplasmic) Antigen Staining Protocol.

- For cell centrifugation steps, centrifugation should be performed for 5 minutes at 300g before cell fixation, and for 5 minutes at 800g after cell fixation. The increased centrifugation speed after cell fixation will result in greater cell recovery.

- DVS Sciences antibodies are pre-titrated and we recommend staining with 1 µl of each antibody for 3 million cells in a 100 µl staining volume; however antibodies can be titrated for individual experiments.

- An optional Fc-blocking step is recommended in the following protocol to prevent binding of MaxPar Metal Conjugated Antibodies to Fc receptors, which will result in high non-specific background signal. Fc receptors specific for IgG, including FcγR1 (CD64), FcγRII (CD32) and FcγRIII (CD16) are present on many cell types, with particularly high expression on myeloid, granulocyte and B cell lineages. Several antibody supply companies provide both human and mouse Fc-blocking reagents that can be used as indicated in the following protocol to minimize non-specific antibody binding.

Reagents and Solutions to Prepare in Advance:

Antibody Cocktails: prepare cocktails of MaxPar Metal Conjugated Antibodies, for both cell surface staining and phospho-proteins, in Cell Staining Buffer. It is recommended to prepare antibody cocktail in a total volume of 50 µL, so that when added to 50 µL of cells the total staining volume is 100 µL. *The antibody cocktail can be stored for up to 24 hours before staining.*

Intercalation Solution: prepare 1 mL of cell intercalation solution for each sample by adding MaxPar Intercalator-Ir into MaxPar Fix and Perm Buffer to a final concentration of 125 nM (a 1000X dilution of the 125 µM stock solution) and mix by vortexing. For example, for 10 samples, prepare intercalation solution by adding 10 µL of 125 µM Intercalator-Ir to 10 ml of Fix and Perm Buffer.

[Optional] Cisplatin: prepare a 25 mM (1000X) stock solution of cisplatin by dissolving 7.5 mg of cisplatin in 1 mL of DMSO. Aliquot and freeze at -20˚C for long-term storage.

[Optional] Serum-Free and Complete Media: If performing the optional cisplatin viability stain, pre-warm serum-free and complete media at 37˚C prior to beginning protocol. Use the same media that is normally used for cell culture.

Protocol:

[Optional] Cisplatin Viability Stain (if not performing cisplatin viability-stain, proceed to step #1 of the protocol below):

Note: *The following cisplatin-staining protocol has minimal impact on the phosphorylation of multiple targets when performed before PMA and Ionomycin cell activation . However, the impact of cisplatin-staining should be evaluated individually for all phospho-proteins and with different stimulation conditions.*

i. Wash cells to be stimulated with pre-warmed serum-free media and discard supernatant.
ii. Resuspend cells to 1 X 10^7/ml in pre-warmed serum-free media and add cisplatin to final concentration of 25 µM (1000X dilution of 25 mM stock solution).
iii. Mix well and incubate at 37˚C for 10 minutes.
iv. Quench cisplatin staining by washing with pre-warmed serum-containing complete media using 5-10X the volume of the stained cells, centrifuge and discard supernatant.
v. Place cells back in culture conditions for 15 minutes to allow cells to “rest”.
vi. Proceed with cell activation in step #1 below.

1. Prepare cells of interest from cell culture or primary tissue and activate desired signaling pathways by adding stimulus to cells for appropriate length of time.

2. At the end of stimulation, stop the signaling reaction by adding formaldehye to a final concentration of 1.6% (10X dilution of the 16% stock solution).

3. Mix gently and thoroughly, and incubate for 10 minutes at room temperature.

4. Transfer cells to an appropriate tube, and wash with MaxPar Cell Staining Buffer, using 5-10X the volume of the cell suspension; centrifuge and discard supernatant by aspiration.

5. Resuspend cells in MaxPar Cell Staining Buffer and aliquot 3 million cells into 5 mL tubes for each sample to be stained.

6. [*Optional*] Block Fc-mediated antibody binding: add Fc-Receptor Blocking Solution to each tube and incubate for 10 minutes at room temperature.

7. Without washing off Fc-Receptor Blocking Solution, add the surface antibody cocktail to each tube so the total staining volume is 100 µL.

8. Gently vortex samples and incubate for 30 minutes at room temperature.

9. Wash by adding 2 mL MaxPar Cell Staining Buffer to each tube, centrifuge and discard supernatant by aspiration.

10. Resuspend cells in residual volume by gently vortexing, and place cells on ice for 10 minutes to chill sample.

11. Add 1 mL of 4˚C methanol to each sample, mix gently, and incubate for 15 minutes on ice.

12. Wash cells with 2 mL MaxPar Cell Staining Buffer, centrifuge and discard supernatant by aspiration – repeat for a total of 2 washes.

13. Add phospho-protein antibody cocktail to each tube so the total staining volume is 100 µL.

14. Gently vortex and incubate for 30 minutes at room temperature.

15. Following the incubation, wash by adding 2 mL MaxPar Cell Staining Buffer to each tube, centrifuge and discard supernatant by aspiration.

16. Repeat for a total of two washes, and resuspend cells in residual volume by gently vortexing after final wash/aspiration.

17. Add 1 ml of the intercalation solution to each tube and gently vortex. Incubate for 1 hour at room temperature or leave overnight at 4^{o}C. *Note: Cells can be left at 4^{o}C in the intercalation solution up to 48 hours.*

18. Wash cells by adding 2 ml of MaxPar Cell Staining Buffer, centrifuge and discard supernatant by aspiration.

19. Repeat for a total of two washes with MaxPar Cell Staining Buffer.

20. Wash cells with 2 ml of MaxPar Water, centrifuge and discard supernatant by aspiration.

21. Leave cells pelleted until ready to run on CyTOF®. Immediately prior to CyTOF data acquisition, adjust cell concentration to 2.5-5 x 10^{5}/ml with MaxPar Water and filter cells into cell strainer cap tubes.

22. Acquire data on CyTOF.