Maxpar Cytoplasmic/Secreted Antigen Staining with Fresh Fix

Before handling any chemicals, refer to the safety data sheet (SDS) provided by the manufacturer, and observe all relevant precautions.

Reagents and Materials

Required Materials

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Catalog Number</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-Staining Reagents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell-ID™ Cisplatin—100 µL</td>
<td>201064</td>
<td>−20 °C in single-use aliquots</td>
</tr>
<tr>
<td>Cell-ID Intercalator-Ir—125 µM, 25 µL</td>
<td>201192A</td>
<td>−20 °C in single-use aliquots</td>
</tr>
<tr>
<td>Maxpar® Cell Staining Buffer—500 mL</td>
<td>201068</td>
<td>4 °C. Do not freeze.</td>
</tr>
<tr>
<td>Maxpar Fix and Perm Buffer</td>
<td>201067</td>
<td>4 °C. Do not freeze.</td>
</tr>
<tr>
<td>Maxpar Fix I Buffer (5X)</td>
<td>201065</td>
<td>4 °C. Do not freeze.</td>
</tr>
<tr>
<td>Maxpar Perm-S Buffer</td>
<td>201066</td>
<td>4 °C. Do not freeze.</td>
</tr>
<tr>
<td>Maxpar metal-conjugated antibodies</td>
<td>Various</td>
<td>4 °C. Do not freeze.</td>
</tr>
<tr>
<td>Maxpar PBS—500 mL</td>
<td>201058</td>
<td>4 °C. Do not freeze.</td>
</tr>
<tr>
<td>Maxpar Cell Acquisition Solution—200 mL/6-pack (6<em>200 mL)</em></td>
<td>201240, 201241</td>
<td>4 °C. Do not freeze.</td>
</tr>
<tr>
<td>Maxpar Water</td>
<td>201069</td>
<td>4 °C. Do not freeze.</td>
</tr>
<tr>
<td>EQ™ Four Element Calibration Beads—100 mL</td>
<td>201078</td>
<td>4 °C. Do not freeze.</td>
</tr>
<tr>
<td>Tuning Solution—250 mL</td>
<td>201072</td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

*For Helios with WB Injector only

Other Required Materials and Equipment

- Pierce™ 16% Formaldehyde (w/v), Methanol-free [Cat. No. 28906 (10 x 1 mL)/Cat. No. 28908 (10 x 10 mL), Thermo Scientific™]
- Polypropylene round-bottom tubes, 5 mL capacity, 12 x 75 mm
• Polypropylene round-bottom tubes with cell-strainer cap, 5 mL capacity, 12 x 75 mm
• Centrifuge capable of holding 5 mL tubes
• Vacuum aspirator
• Vortex
• (Optional) Fc receptor-blocking reagent

Important Notes Before Starting

This protocol should be followed for staining cytokines and other secreted proteins in addition to intracellular antigens that are localized outside of the nucleus (for example, IkBα, cleaved caspase-3). For phosphoprotein staining, use the Maxpar Phosphoprotein Staining with Fresh Fix Protocol (PN 400278).

If staining antigens that are secreted, including cytokines, it is necessary to block their secretion from the cell in order to enable their detection by the Helios™ system. Treatment of cells with monensin or brefeldin A during cell activation inhibits the intracellular protein/secretory transport pathway, resulting in accumulation of secreted proteins in the lumen of the endoplasmic reticulum and Golgi apparatus. Optimal blocking time should be evaluated for specific targets, activations, and cell types.

Reagent handling: Retrieve, mix, and centrifuge reagents as directed. Frozen aliquots of Cell-ID Intercalator-Ir and Cell-ID Cisplatin should be used only once, and only immediately after thawing. Avoid multiple freeze/thaw cycles.

Centrifuge speeds: For cell centrifugation steps, centrifugation should be performed for 5 minutes at 300 x g before cell fixation, and for 5 minutes at 800 x g after cell fixation. Increased centrifugation speed after cell fixation results in greater cell recovery.

Cell viability staining: Prior to step 3 of the protocol, cells can be stained with Cell-ID Cisplatin to identify viable cells.

Antibodies: Fluidigm antibodies are pre-titrated, and we recommend staining with 1 μL of each antibody for 1–3 million cells in a 100 μL staining volume. However, antibodies should be titrated for individual experiments. Cell surface antibodies are diluted in Cell Staining Buffer and cytoplasmic antibodies are diluted in Perm-S Buffer.

FcR blocking: An optional Fc receptor-blocking step is recommended in the following protocol to prevent binding of Maxpar metal-conjugated antibodies to Fc receptors. Binding results in high nonspecific background signal. Fc receptors specific for IgG, including FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), are present on many cell types, with particularly high expression on monocytes, granulocytes, and B cells.

The protocol recommends staining of surface markers prior to cell fixation to achieve optimal signal-to-noise ratio in surface marker detection. Surface staining may also be performed either following fixation, or concurrently with intracellular staining, following both fixation and permeabilization. However, staining surface antigens post-fixation and/or permeabilization may result in decreased signal-to-noise ratio, and should be evaluated for individual antigens/clones.
Shorter fixation times may result in improved detection of surface markers. This should be evaluated for individual antigens and antibody clones.

Reagents and Solutions to Prepare in Advance

**Antibody Cocktail**

Prepare antibody cocktails for surface markers in Cell Staining Buffer, and for cytoplasmic/secerted antigens in Perm-S Buffer. It is recommended to prepare antibody cocktails, such that the volume of each test is 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 Cell-ID Intercalator-Ir into Maxpar Fix and Perm Buffer to a final concentration of 125 nM (a 1,000X 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.

**Fix I Buffer**

Prepare 1 mL of 1X Fix I Buffer for each sample to be stained. For example, for 10 samples, prepare 10 mL of Fix I Buffer by combining 2 mL of 5X Fix I Buffer and 8 mL of PBS. Alternatively, if staining post-fixation, 5X Fix I Buffer can be added directly to culture medium to a final concentration of 1X.

Protocol

**Prepare Cells**

1. Prepare cells of interest from cell culture or primary tissue and activate cells by adding stimulus to cells for appropriate length of time. If staining cytokines or other secreted proteins, add protein-transport inhibitor to the culture medium during the final hours of the stimulation to block protein secretion.

2. Following stimulation, transfer cells to a tube of appropriate size, centrifuge cells, and discard supernatant by aspiration.

3. Resuspend cells in Maxpar Cell Staining Buffer and aliquot 1–3 million cells in a volume of 50 μL into 5 mL polypropylene tubes for each sample to be stained.

**NOTE** Adjust the volume in which cells are re-suspended to account for volume of Fc Receptor Blocking solution if used.
Stain Cells with Surface Antibodies

1. (Optional) FcR blocking: Add Fc receptor-blocking solution to each tube and incubate for 10 minutes at room temperature. Without washing off Fc receptor-blocking solution, continue with protocol.

2. Add 50 µL of the surface marker antibody cocktail to each tube so the total staining volume is 100 µL (50 µL of cell suspension + 50 µL antibody cocktail). (See Antibody Cocktail Table for mixing volumes).

3. Gently pipet to mix each tube and incubate the tubes at room temperature for 15 minutes.

4. Gently vortex samples and incubate for an additional 15 minutes at room temperature.

5. Following the incubation, wash by adding 2 mL Maxpar Cell Staining Buffer to each tube, centrifuge at 300 x g for 5 minutes and remove supernatant by aspiration and gently vortex to resuspend cells in residual volume.

6. Repeat Step 5 for a total of two washes. Resuspend cells thoroughly in residual volume by gently vortexing after final wash/aspiration.

Stain Cells with Cytoplasmic/Secreted Protein Antibodies

1. Fix cells by adding 1 mL of 1X Maxpar Fix I Buffer to each tube, gently vortex, and incubate at room temperature for 10-30 minutes.

2. Wash cells with 2 mL of Maxpar Perm-S Buffer, centrifuge for 5 min at 800 x g, and discard supernatant.

3. Repeat Step 2 for a total of two washes with Maxpar Perm-S Buffer.

4. Add 50 µL of the cytoplasmic/secerted antibody cocktail to each tube so the total staining volume is 100 µL (50 µL of cell suspension + 50 µL antibody cocktail).

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

6. Following the incubation, wash by adding 2 mL Maxpar Cell Staining Buffer to each tube, centrifuge for 5 min at 800 x g and discard supernatant by aspiration.

7. Repeat for a total of two washes, and resuspend cells in residual volume by gently vortexing after final wash/aspiration.
Fix Cells (Fresh Fix)

**IMPORTANT** It is essential to thoroughly disrupt the pellet by vortexing before adding the fresh fixative at this step.

1. Prepare a fresh 1.6% FA solution from the 16% formaldehyde stock ampule. Dilute 1 part of the stock formaldehyde with 9 parts Maxpar PBS.

2. For example, to prepare the 1.6% FA solution for one sample, add 100 μL of 16% stock formaldehyde to 900 μL of Maxpar PBS. Include 10% volume overage for multiple samples.

3. Add 1 mL of the 1.6% FA solution to each tube (containing 1–3 million cells in suspension) and gently vortex to mix well.

4. Incubate tubes for 10 minutes at room temperature.

5. Centrifuge cells at 800 x g for 5 minutes.

6. Increased centrifuge speed after cell fixation results in greater cell recovery.

7. Carefully aspirate and remove supernatant. Gently vortex to resuspend cells in residual volume.

Stain Cells with Cell-ID Intercalator-Ir

1. Prepare 1 mL of intercalation solution for each sample by adding Cell-ID Intercalator-Ir into Maxpar Fix and Perm Buffer to a final concentration of 125 nM (a 1,000X dilution of the 125 μM stock solution) and vortex to mix.

NOTE For example, to prepare intercalation solution for one sample, add 1 μL of 125 μM Intercalator-Ir to 1 mL of Maxpar Fix and Perm Buffer. Include 10% in excess volume for multiple samples.

2. Add 1 mL of the cell intercalation solution to each tube and gently vortex. Incubate for 1 hour at room temperature or leave overnight at 4 °C.

NOTE Cells can be left at 4 °C in the cell intercalation solution up to 48 hours before data acquisition.

Prepare Cells for Acquisition

For CyTOF, CyTOF 2 and Helios (HT Injector)

1. Centrifuge tubes containing cells in intercalation solution at 800 x g for 5 minutes.

2. Wash cells by adding 2 mL of Maxpar Cell Staining Buffer, centrifuge at 800 x g for 5 minutes, and remove supernatant by aspiration.

3. Resuspend cells in 1 mL of Maxpar Water. Reserve a small volume (approximately 10 μL) from each tube to count cells.
4 Centrifuge tubes at 800 x g for 5 minutes. While tubes are in the centrifuge, go to Step 5.

5 Count cells in the reserved volume from each tube. Make sure to note the cell concentration for each tube.

6 Carefully remove all of the supernatant from centrifuged samples (Step 4) by aspiration.

7 Prepare sufficient volume of 0.1X EQ beads to re-suspend all samples in the experiment by diluting 1 part beads to 9 parts Maxpar Water.

8 Leave cells pelleted until ready to run. Immediately prior to data acquisition, adjust cell concentration to 5.0 x 10^5 (CyTOF and CyTOF 2) or 1.0 x 10^6 cells/mL (Helios™ HT Injector) or concentration appropriate for the sample type in the diluted EQ bead solution. Filter cells into cell strainer cap tubes.

9 Acquire data on CyTOF, CyTOF 2 or Helios (HT Injector).

**For Helios (WB Injector)**

**IMPORTANT** Check with your Helios operator to confirm whether the HT or WB Injector is in use.

1 Centrifuge tubes containing cells in intercalation solution at 800 x g for 5 minutes.

2 Wash cells by adding 2 mL of Maxpar Cell Staining Buffer, centrifuge at 800 x g for 5 minutes, and remove supernatant by aspiration.

3 Resuspend cells in 1 mL of Maxpar Cell Acquisition Solution. Reserve a small volume (approximately 10 μL) from each tube to count cells.

4 Centrifuge tubes at 800 x g for 5 minutes. While tubes are in the centrifuge, go to Step 5.

5 Count cells in the reserved volume from each tube. Make sure to note the cell concentration for each tube.

6 Carefully remove all of the supernatant from centrifuged samples (Step 4) by aspiration.

7 Prepare sufficient volume of 0.1X EQ beads to re-suspend all samples in the experiment by diluting 1 part beads to 9 parts Maxpar Cell Acquisition Solution.

8 Leave cells pelleted until ready to run on Helios™. Immediately prior to data acquisition, adjust cell concentration to 1.0 x 10^6 cells/mL or concentration appropriate for the sample type in the diluted EQ bead solution. Filter cells into cell strainer cap tubes.

9 Acquire data on Helios™ (WB Injector).
## Appendix A: Antibody Cocktail Preparation Guide

The following guide can be used to prepare the Maxpar metal-conjugated antibody cocktail in Maxpar Cell Staining Buffer. Prepare the antibody cocktail in a 1.5 mL tube by first adding Cell Staining Buffer and then adding each of the antibodies. Combine 50 µL of the complete antibody cocktail with each sample to be stained.

### Antibody Cocktail Preparation Guide

<table>
<thead>
<tr>
<th>(a) Number of Samples</th>
<th>(d) Vol of Antibody (µL)</th>
<th>(b) Number of Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.3</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>10.6</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>15.8</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>21.1</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>26.4</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>31.7</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>37.0</td>
<td>14</td>
</tr>
<tr>
<td>8</td>
<td>42.3</td>
<td>16</td>
</tr>
<tr>
<td>9</td>
<td>47.6</td>
<td>18</td>
</tr>
<tr>
<td>10</td>
<td>52.9</td>
<td>20</td>
</tr>
<tr>
<td>11</td>
<td>58.2</td>
<td>22</td>
</tr>
<tr>
<td>12</td>
<td>63.5</td>
<td>24</td>
</tr>
<tr>
<td>13</td>
<td>68.8</td>
<td>26</td>
</tr>
<tr>
<td>14</td>
<td>74.1</td>
<td>28</td>
</tr>
<tr>
<td>15</td>
<td>79.4</td>
<td>30</td>
</tr>
<tr>
<td>16</td>
<td>84.7</td>
<td>32</td>
</tr>
<tr>
<td>17</td>
<td>90.0</td>
<td>34</td>
</tr>
</tbody>
</table>

**TO USE THE TABLE:** Locate the row matching the number of samples to be processed (a) and the column for the number of antibodies to stain the sample (b). Use the table to determine the Total Volume of Cell Staining Buffer needed (c). Add this volume of Cell Staining Buffer to your mastermix tube. Again locate the row of the numbers to be processed (a) and in the adjacent column determine the volume per antibody (d). Add the indicated volume of each antibody solution to the mastermix tube.