Maxpar Phosphoprotein Staining with Fresh Fix

WARNING 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

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

*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
- Serum-Free and Complete Media
- Methanol (Cat. No. BP1105-4, Fisher Scientific™)
- 1.5 mL microfuge tubes
- Pipet tips with aerosol barrier

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 Cytoplasmic/Secreted Antigen Staining with Fresh Fix (PN 400279).

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.

Formaldehyde solution: It is critical to prepare fresh formaldehyde (FA) solution to effectively fix cells stained with the Maxpar antibodies. Be sure to open the single-use formaldehyde ampule and prepare the FA solution immediately before use in the fixation process.

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. The increased centrifugation speed after cell fixation will result in greater cell recovery.

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. Cell surface and phosphoprotein antibodies are diluted in Maxpar Cell Staining Buffer. However, antibodies should be titrated for individual experiments.

Cell viability staining: Prior to Step 1 of the protocol, cells can be stained with Cell-ID Cisplatin to identify viable cells.

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 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 monocytes, granulocytes, and B cells.

Reagents and Solutions to Prepare in Advance

Antibody Cocktail

Prepare cocktails of Maxpar metal-conjugated antibodies, for both cell surface staining and phosphoproteins, in Cell Staining Buffer. It is recommended to prepare antibody cocktails in a total volume of 50 µL so that when it is 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 Maxpar Fix and Perm Buffer.

(Optional) Serum-Free and Complete Media

If performing the optional cisplatin viability stain, 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

Prepare Cells

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 5X Fix I Buffer to a final concentration of 1X.
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 for 5 minutes at 800 *x g* and discard supernatant by aspiration.
5. Resuspend cells in Maxpar Cell Staining Buffer and aliquot 1–3 million cells, in a volume of 50 µL, into 5 mL 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 with Surface Antibodies

1. (Optional) Fc 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 antibody cocktail to each tube so the total staining volume is 100 µL (50 µL 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, and remove supernatant by aspiration.

Stain Cells with Phosphoprotein Antibodies

1. Gently vortex to resuspend cells in residual volume. Place cells on ice for 10 minutes to chill sample.
2. Add 1 mL of 4° C methanol to each sample, mix gently, and incubate for 15 minutes on ice.
3. Wash cells with 2 mL Maxpar Cell Staining Buffer, centrifuge, and discard supernatant by aspiration.
4. Repeat step 3 for a total of two washes.
5. Resuspend pellet in residual volume with gentle vortexing.
6. Add phosphoprotein antibody cocktail to each tube so the total staining volume is 100 µL.
7. Gently pipet to mix each tube and incubate the tubes at room temperature for 30 minutes.
8. Following the incubation, wash by adding 2 mL Maxpar Cell Staining Buffer to each tube, centrifuge at 800 x g for 5 mintues and remove supernatant by aspiration.
9. Repeat Step 8 for a total of 2 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.

   NOTE 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.

2. 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.
3. Incubate tubes for 10 minutes at room temperature.
4. Centrifuge cells at 800 x *g* for 5 minutes.
5. Increased centrifuge speed after cell fixation results in greater cell recovery.
6. 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 Carefully aspirate and discard supernatant. Gently vortex to resuspend cells in residual volume.

4 Wash cells by adding 2 mL of Maxpar Cell Acquisition Solution to each tube and gently vortex. Centrifuge tubes at 800 x g for 5 min.

5 Carefully aspirate and discard supernatant. Gently vortex to resuspend cells in residual volume.

6 (Optional) Repeat wash by adding another 2 mL of Maxpar Cell Acquisition Solution to each tube and gently vortex. Reserve a small volume (approximately 10 µL) from each tube to count cells.

NOTE The additional wash with Maxpar Cell Acquisition Solution helps with removing additional debris from samples.

7 Centrifuge tubes at 800 x *g* for 5 minutes. While tubes are in the centrifuge, go to Step 8.

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

9 Carefully remove all of the supernatant from centrifuged samples (Step 7) by aspiration.

10 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.

11 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.

12 Acquire data on Helios™ (WB Injector).

Appendix: 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.

(a) Number of Samples	**(d)** Vol of Antibody (µl)	**(c)** Vol. Cell Staining Buffer (µl)	**(b)** Number of Antibodies																																				
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**
1	1.1		53.9	52.8	51.7	50.6	49.5	48.4	47.3	46.2	45.1	44	42.9	41.8	40.7	39.6	38.5	37.4	36.3	35.2	34.1	33	31.9	30.8	29.7	28.6	27.5	26.4	25.3	24.2	23.1	22	20.9	19.8	18.7	17.6
2	2.2		108	106	103	101	99	96.8	94.6	92.4	90.2	88	85.8	83.6	81.4	79.2	77	74.8	72.6	70.4	68.2	66	63.8	61.6	59.4	57.2	55	52.8	50.6	48.4	46.2	44	41.8	39.6	37.4	35.2
3	3.3		162	158	155	152	149	145	142	139	135	132	129	125	122	119	116	112	109	106	102	99	95.7	92.4	89.1	85.8	82.5	79.2	75.9	72.6	69.3	66	62.7	59.4	56.1	52.8
4	4.4		216	211	207	202	198	194	189	185	180	176	172	167	163	158	154	150	145	141	136	132	128	123	119	114	110	106	101	96.8	92.4	88	83.6	79.2	74.8	70.4
5	5.5		270	264	259	253	248	242	237	231	226	220	215	209	204	198	193	187	182	176	171	165	160	154	149	143	138	132	127	121	116	110	105	99	93.5	88
6	6.6		323	317	310	304	297	290	284	277	271	264	257	251	244	238	231	224	218	211	205	198	191	185	178	172	165	158	152	145	139	132	125	119	112	106
7	7.7		377	370	362	354	347	339	331	323	316	308	300	293	285	277	270	262	254	246	239	231	223	216	208	200	193	185	177	169	162	154	146	139	131	123
8	8.8		431	422	414	405	396	387	378	370	361	352	343	334	326	317	308	299	290	282	273	264	255	246	238	229	220	211	202	194	185	176	167	158	150	141
9	9.9		485	475	465	455	446	436	426	416	406	396	386	376	366	356	347	337	327	317	307	297	287	277	267	257	248	238	228	218	208	198	188	178	168	158
10	11		539	528	517	506	495	484	473	462	451	440	429	418	407	396	385	374	363	352	341	330	319	308	297	286	275	264	253	242	231	220	209	198	187	176
11	12.1		593	581	569	557	545	532	520	508	496	484	472	460	448	436	424	411	399	387	375	363	351	339	327	315	303	290	278	266	254	242	230	218	206	194
12	13.2		647	634	620	607	594	581	568	554	541	528	515	502	488	475	462	449	436	422	409	396	383	370	356	343	330	317	304	290	277	264	251	238	224	211
13	14.3		701	686	672	658	644	629	615	601	586	572	558	543	529	515	501	486	472	458	443	429	415	400	386	372	358	343	329	315	300	286	272	257	243	229
14	15.4		755	739	724	708	693	678	662	647	631	616	601	585	570	554	539	524	508	493	477	462	447	431	416	400	385	370	354	339	323	308	293	277	262	246
15	16.5		809	792	776	759	743	726	710	693	677	660	644	627	611	594	578	561	545	528	512	495	479	462	446	429	413	396	380	363	347	330	314	297	281	264
16	17.6		862	845	827	810	792	774	757	739	722	704	686	669	651	634	616	598	581	563	546	528	510	493	475	458	440	422	405	387	370	352	334	317	299	282
17	18.7		916	898	879	860	842	823	804	785	767	748	729	711	692	673	655	636	617	598	580	561	542	524	505	486	468	449	430	411	393	374	355	337	318	299
18	19.8		970	950	931	911	891	871	851	832	812	792	772	752	733	713	693	673	653	634	614	594	574	554	535	515	495	475	455	436	416	396	376	356	337	317
19	20.9		1024	1003	982	961	941	920	899	878	857	836	815	794	773	752	732	711	690	669	648	627	606	585	564	543	523	502	481	460	439	418	397	376	355	334
20	22		1078	1056	1034	1012	990	968	946	924	902	880	858	836	814	792	770	748	726	704	682	660	638	616	594	572	550	528	506	484	462	440	418	396	374	352
21	23.1		1132	1109	1086	1063	1040	1016	993	970	947	924	901	878	855	832	809	785	762	739	716	693	670	647	624	601	578	554	531	508	485	462	439	416	393	370
22	24.2		1186	1162	1137	1113	1089	1065	1041	1016	992	968	944	920	895	871	847	823	799	774	750	726	702	678	653	629	605	581	557	532	508	484	460	436	411	387
23	25.3		1240	1214	1189	1164	1139	1113	1088	1063	1037	1012	987	961	936	911	886	860	835	810	784	759	734	708	683	658	633	607	582	557	531	506	481	455	430	405
24	26.4		1294	1267	1241	1214	1188	1162	1135	1109	1082	1056	1030	1003	977	950	924	898	871	845	818	792	766	739	713	686	660	634	607	581	554	528	502	475	449	422
25	27.5		1348	1320	1293	1265	1238	1210	1183	1155	1128	1100	1073	1045	1018	990	963	935	908	880	853	825	798	770	743	715	688	660	633	605	578	550	523	495	468	440

TO USE THE TABLE: Locate the row matching the number of samples to be processed (a) and the column for the number of antibodies used 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 matching the number of samples 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.

For technical support visit fluidigm.com/support.

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