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Protocol status: Working
We use this protocol and it's working

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Staining protocol for Imaging Mass Cytometry

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ABSTRACT

In Imaging Mass Cytometry (IMC), a high-resolution laser is combined with a mass cytometer that permits mass spectrometry-based, spatially reserved high-dimensional analysis of intact formalin-fixed paraffin-embedded (FFPE) tissues. The protocol summarizes the staining procedure for IMC using a cocktail of heavy metal conjugated primary antibodies identifying specific antigens.

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GUIDELINES

Protocol Quality Control metrics

- Antibody validation: All primary antibodies included in the panel are validated either by the vendor or internal validation. Strategies considered for antibody validation include: 1) knockout/knockdown of gene of interest, 2) independent antibody verification, 3) morphological validation by performing IF using single common protocol on human kidney and positive control tissue (lymph node, tumors etc), 4) morphological validation by a renal pathologist blinded to the antibody used and 5) counterstaining for additional markers of the same cell type.
- Aperio scanned image of PAS-stained section of the entire biopsy is evaluated for tissue morphology and integrity.
- Use 5 µm thick sections for staining. For better results, stain and ablate the tissue within 2 weeks of sectioning.
- Entire biopsy is ablated with increments in approximately 3 mm2 area to complete ablation of the cortical and medullary regions. Regions with artifactual section damage are excluded.
- Each IMC analysis includes a section from a reference kidney, both cortex and medulla (quality control), on the same slide as an internal standard.
- IMC is performed on the Hyperion imaging system. Prior to ablation of tissue, the machine undergoes routine tuning and calibration for mass spectrometric detection of all the heavy metals used in the antibody panel each time.
- For each IMC ablation, the steps of antigen retrieval and cocktail hybridization will be confirmed by visual inspection of the resident cell type markers using mcd viewer to identify the nuclei, proximal tubules, glomeruli, thick ascending limb, distal convoluted tubules and collecting duct to confirm that the correct region was ablated and analyzed.

MATERIALS

Xylenes - JT Baker (9490-05)
Ethanol – Decon Labs (2805M)
10X Phosphate buffered saline (PBS) - Gibco (70011044)
10X Tris buffered saline (TBS) - BioRad (1706435)
Triton X-100 - Sigma (X100RS-5G)
Epitope Retrieval Reagent pH 9 (10x) - Leica Biosystems (RE7113-CE)
Bovine serum albumin (BSA) - American Bio (AB0048)
Double distilled water
PAP pen - Abcam (ab2160)
Maxpar IMC Cell Segmentation - Standard Biotools (201500)
Intercalator-Ir - Standard Biotools (201192A)
Antibodies (as listed on Table 1)

BEFORE START INSTRUCTIONS

	Type	Target	Cell Type	Species	Vendor (Cat No.)	Dilution	Metal Conjugate
	Resident Cell Panel	Beta catenin	Tubular epithelium	Mouse	Standard Biotools (3147005A)	1:500	147Sm
		Aqp1	Proximal tubule	Rabbit	Abcam (ab178352-1001)	1:2500	173Yb
		Megalin	Proximal tubule	Mouse	Millipore (MABS489)	1:250	174Yb
		Uromodulin	Thick ascending limb	Rat	R&D Systems (MAB5175)	1:1600	151Eu
		Calbindin	Distal convoluted tubule	Mouse	ThermoFischer (MA524135)	1:400	142Nd
		CK7	Collecting duct	Mouse	Standard Biotools (3164028D)	1:150	164Dy
		Nestin	Podocytes	Mouse	Abcam (ab6320-1001)	1:200	146Nd
		Vimentin	Fibroblasts, pericytes, podocytes	Mouse	Abcam (ab8978-1001)	1:400	150Nd
		CD31	Endothelium	Mouse	Abcam (ab212712-1001)	1:100	149Sm
		ERG	Endothelium	Rabbit	Abcam (ab214796-1001)	1:500	166Er
		alpha-SMA	Smooth muscle, mesengial	Mouse	Standard Biotools (3141017D)	1:1000	141Pr
		WT1	Podocytes	Mouse	ThermoFischer (MA146028)	1:100	176Yb
		Aqp2	Collecting duct	Rabbit	Abcam (ab230170)	1:200	154Sm
	Immune Cell Panel	CD68	Macrophages	Mouse	Fluidigm (3159035D)	1:800	159Tb
		CD14	Pro-Inflammatory Macrophages (M1)	Rabbit	Fluidigm (3144025D)	1:100	144Nd
		CD163	Alternative Activated Macrophages (M2)	Mouse	Bio-Rad (MCA1853)	1:100	148Nd
		CD206	Alternative Activated Macrophages (M2)	Rabbit	Abcam (AB64693)	1:400	163Dy
		CD11c	Dendritic cell	Rabbit	Abcam (AB216655)	1:200	167Er
		CD3	T cell	Mouse	Novus Biologicals (NBP2-54392-100)	1:250	170Er
		CD4	Helper T cells	Rabbit	Fluidigm (3156033D)	1:100	156Gd

		CD8a	Cytotoxic T cells	Mouse	Fluidigm (3162034D)	1:300	162Dy
		CD20	B cells	Mouse	Fluidigm (3161029D)	1:150	161Dy
		MBP	Eosinophils	Mouse	Novus Biologicals (NBP1-42140-MTO)	1:20	143Nd
		MPO	Neutrophils	Rabbit	Abcam (AB236022)	1:500	172Yb
		Chymase	Mast cells	Rabbit	Abcam (AB233729)	1:400	165Ho
		CD56	NK cells	Mouse	Cell Signaling (97174SF)	1:200	175Lu
	Injury Cell Panel	Kim1	Epithelial injury/repair	Mouse	R&D Systems (MAB1750-100)	1:300	160Gd
		Ki67	Proliferation	Mouse	Fluidigm (3168022D)	1:100	168Er
		IL9	Cytokine	Rabbit	Abcam (ab181397)	1:250	153Eu
		FACL4	Ferroptosis	Rabbit	Abcam (ab240135)	1:400	155Gd
		MCP-1	Cytokine	Mouse	Novus Biologicals (NBP2-22115)	1:300	169Tm
		TNFa	Cytokine	Rabbit	Abcam (ab271989)	1:300	145Nd
		LC3b	Autophagy	Rabbit	Abcam (ab221794-1001)	1:200	158Gd
		VCAM-1	Epithelial injury/repait	Rabbit	Abcam (ab271899-1001)	1:200	152Sm
	Segmentation Kit	ICSK1	Membrane	-	Standard Biotools (201500)	1:400	195Pt
		ICSK2	Membrane	-	Standard Biotools (201500)	1:400	196Pt
		Intercalator	DNA	-	Standard Biotools (201192A)	1:1000	191Ir/193Ir

Table 1. Antibody Panel

Staining Tissue Sections

4h 20m

1 Bake the slides Overnight at 60°C. Ensure that all visible wax has been removed

2 Dewax the slides in xylenes in the fume hood for 00:10:00 10m

3 Repeat step 2 and dewax the slides in fresh xylene in the fume hood for 00:10:00 10m

4 Hydrate the slides in descending grades of ethanol (100%, 95%, 80%, 70%) for 00:05:00 each. 5m

5 Wash the slides in double distilled water for 00:05:00 in a Coplin jar placed on an orbital shaker plate with gentile agitation. 5m

6 Perform antigen retrieval bu immersing slides in 1X epitope retrieval bugger (pH 9.0) for 00:20:00 in steamer 20m

6.1 Prepare 1X epitope retrieval buffer from its 10X stock solution.

7 Cool slides for 00:40:00 40m

8 Wash the slides in double distilled water for 00:05:00 in a Coplin jar with gentle agitation on an orbita shaker. Perform this step twice. 5m

9 Wash the slides with 1X TBS for 00:10:00 with gentle agitation on an orbital shaker. 10m

10 Use a PAP pen to draw an outline encircling the sample.

11 Block with 3% BSA in 1X TBS for 01:00:00 at room temperature in a hydration chamber. 1h

11.1 You can use an empty pipette tip box where the slides rest on the tip shelf and the bottom is filled halfway with water.

11.2 Blocking solution should be diluted from 10% BSA freshly made from powder. The remaining 10% BSA should be aliquoted and stored at -20°C and diluted at time of use.

11.3 Use enough blocking solution to cover the section.

12 Prepare the antibody cocktail calculating the total volume of antibodies at the concentrations specific for the assay and bring the volume up to a final volume using 0.5% BSA. in 1X TBS.

12.1 Dilutions can be found on Table 1.

13 Place the slides in a hydration chamber and pipette the antibody mix on to the section.

13.1 Store the antibody cocktail mix on ice and add it on to the samples within 1-2 hours of preparation for best results.

14 Incubate overnight with the antibody cocktail at 4°C in a hydration chamber.

15 Wash the slides in 0.1% Triton X-100 in 1X TBS for 00:05:00 in Coplin jars with slow agitation on an orbital shaker. 5m

16 Repeat step 15 two more times.

17 Wash the slides in 1X TBS for 00:05:00 with gentle agitation on an orbital shaker. 5m

18 Repeat step 17.

19 Stain the tissue with Intercalator-Ir in 1X PBS (1:1000) for 01:00:00 at room temperature in a hydration chamber. 1h

20 Wash the slides in double distilled water for 00:05:00 with gentle agitation on an orbital shaker. 5m

21 Air dry the slides for at least 00:20:00 at room temperature. 20m

Imaging Mass Cytometry

22 Load the stained slide into the pre-tuned and pre-calibrated Hyperion IMC System.

23 Create a new project on the CyTOF software.

24 Draw a panorama that covers the tissue that will be ablated.

25 Determine regions of interest (ROIs) to ablate with increments less than $3mm^2$ on the tissue, ensuring that cortical and medullary regions are separate within the panorama created. Regions with artificial section damage are excluded.

26 Create or select an antibody/metal template.

27 Make sure to select "generate txt file".

28 Save the project file and start ablation of tissue.

Image Verification

29 Once ablation is complete, load the file on MCD viewer to check the quality of each image and channel.