Poster #423

Exploring Healthy and Tumor Tissue Microenvironment with Immuno-Oncology Markers Using Multiplexed Hyperion Imaging System

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Abstract

To power immuno-oncology discovery, it is highly beneficial to explore healthy and tumor tissues with immuno-oncology markers using multiplexed analysis. The Hyperion™ Imaging System uses novel technology for tissue imaging that enables multiplexed analysis of protein expression in a single tissue sample. This methodology uses tissue sections stained with a cocktail of antigen-specific antibodies conjugated to different metal isotopes. In this study, we demonstrate how to generate high-parameter images with highly relevant immuno-oncology markers on the Hyperion Imaging System.

To detect multiple markers in one panel, we optimized the tissue staining protocol for signal detection and tissue preservation. For formalin-fixed, paraffin-embedded (FFPE) tissue staining, antigen retrieval conditions (temperature and incubation time) were optimized. We determined that antigen retrieval conditions of 96 °C for 30 minutes in basic retrieval solution enabled detection of nuclear markers such as FoxP3, along with other surface and cytoplasmic markers. To verify these methods, we also generated equivalent data to compare these results with immunofluorescence on FFPE tissue sections and examined co-localization and anti-localization of the antibodies with previously verified counter stains.

Using these optimized staining protocols, we generated images from various normal and tumor tissues (including diffuse large B cell lymphoma, colon adenocarcinoma, and bladder urothelial carcinoma) to show a combination of 5 structural, 1 cancer, 3 nuclear, and 18 immuno-oncology markers simultaneously. Together with other tissue architectural details, different immune cell types were identified in both normal and tumor tissues. This image resolution allowed for the visualization of proteins in the membranous, cytoplasmic, and nuclear cell compartments. Therefore, our data demonstrate that the Hyperion Imaging System provides a high-parameter imaging solution at subcellular resolution to characterize the immune repertoire in the tumor microenvironment.

Introduction

The Hyperion Imaging System is an innovative and high-throughput method of coupling laser ablation to mass cytometry (1). The benefit over traditional IHC techniques is the ability to multiplex over 40 markers in a single experiment, enabling high-content analysis of human tissue with fewer tissue samples required (2). Imaging Mass Cytometry™ (IMC™) workflow is diagrammed as following:

Metal assignment and antibody conjugation

Immuno-staining on FFPE/frozen tissue

Laser ablation and image data collection

Data analysis using MCD™ Viewer

Methods

IMC antibody verification process starts from staining SOP optimization by testing different antigen retrieval conditions so that both surface and intracellular markers can be detected in one panel. IMC staining images were compared with immunofluorescent staining images on sequential tissue sections (3). Verification specifications include positive tissue, negative tissue, co-localization marker, and counter-localization marker. The antibody verification workflow is indicated in Figure 1.

FFPE human sections were found to optimally stain according to our current Imaging Mass Cytometry Staining Protocol for FFPE Sections (Fluidigm PN 400322), stated as following:

1. FFPE human sections were pre-heated at 60 °C for 2 hr then dewaxed in xylene and finally rehydrated in descending grades of ethanol.
2. Antigen retrieval was performed at 96 °C for 30 min in Target Retrieval Solution (Agilent® S2367). Slides were left to cool down to 70 °C at room temperature (RT) then successively washed in dH2O and PBS for 10 min each.
3. Tissues were blocked in 3% BSA in PBS in a humid chamber for 45 min.
4. Tissues were stained with assay-dependent concentrations of metal-tagged antibodies in PBS, 0.5% BSA at 4 °C overnight in a humid chamber.
5. Slides were then rinsed twice with slow agitation, twice in PBS 0.2% Triton™ X, and twice in PBS for 8 min each.
6. Following these washes, tissues were stained with 0.3 µM Cell-ID™ Intercalator-Ir (Fluidigm PN 201192A) in PBS for 30 min at room temperature.
7. The samples were then rinsed for 5 min in dH2O and air-dried.

For IMC image acquisition dried, immunostained samples were inserted into the ablation chamber of the Hyperion Imaging System, where a pulsed 200 Hz UV laser is focused over a user-defined region of tissue, ablating adjacent 1 µm^2 spots, as the slide moves under the laser beam. The resulting plume travels to the plasma ion source, where the metal tags are ionized. Isotopes associated with individual 1 µm^2 spots are detected based on time-of-flight and then indexed against the source location. This indexed signal map is the image data acquired at the end of a scan. It is stored in MCD file format, and as an option, in TXT format.

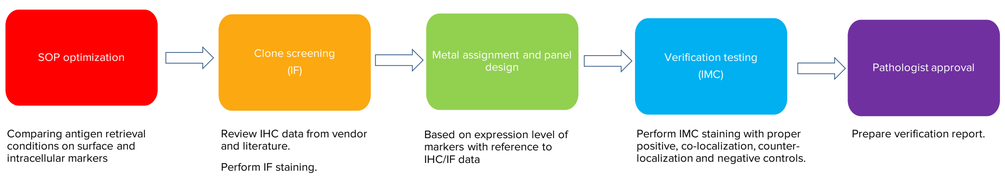

Figure 1. IMC antibody verification workflow.

Verifying IMC Staining SOP for FFPE Sections

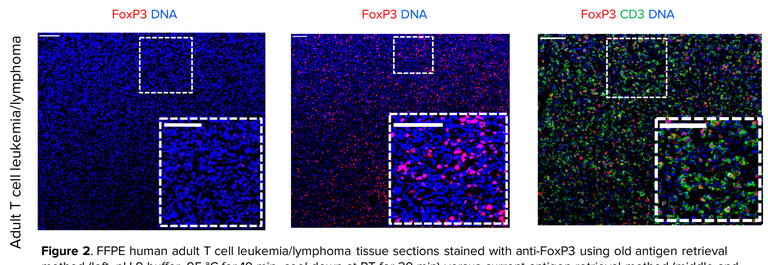

Figure 2. FFPE human adult T cell leukemia/lymphoma tissue sections stained with anti-FoxP3 using old antigen retrieval method (left, pH 9 buffer, 95 °C for 10 min, cool down at RT for 20 min) versus current antigen retrieval method (middle and right, pH 9 buffer, 96 °C for 30 min, cool down to 70 °C at RT for 10 min).

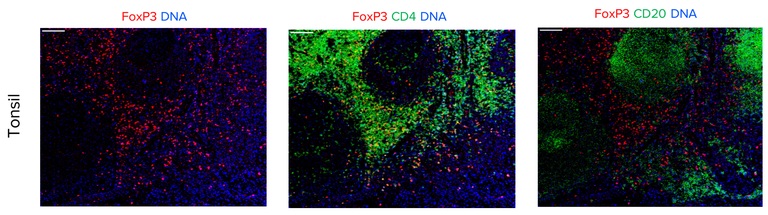

Figure 3. The transcription factor FoxP3 co-localizes with cell nuclei and CD4 staining, counter-localization with CD20 in FFPE sections of human tonsil.

Comparison of Different Applications IMC vs. IF

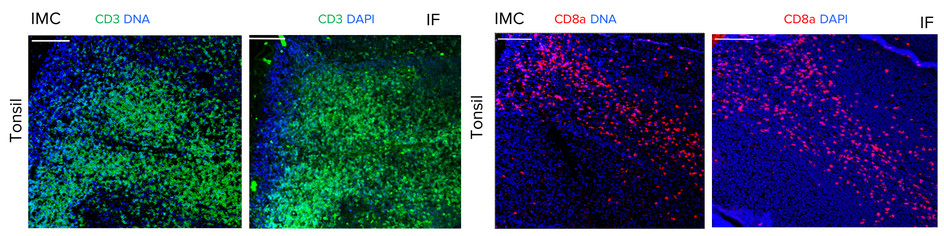

Figure 4. FFPE human tonsil sections stained with anti-CD3 antibody show equivalent staining patterns in both IMC (left) and IF (right).

Figure 5. FFPE human tonsil sections stained with anti-CD8a antibody show equivalent staining patterns in both IMC (left) and IF (right).

Summary

The Imaging Mass Cytometry staining method follows a workflow similar to traditional IF staining and generates comparable results while eliminating issues such as autofluorescence and spectral overlap.

The images generated from various normal and tumor tissues show a combination of structural, cancer, and immuno-oncology markers simultaneously. Different immune cell types can be identified in both normal and tumor tissues.

Delivering a comprehensive view from one scan, this technology can enable deep profiling of precious tissues at subcellular resolution to power immuno-oncology discovery.

References

1. Giesen, C., Wang, H.A.O. Schapiro, D., Zivanovic, N., Jacobs, A., Hattendorf, B., Schüffler, P.J., Grolimund, D., Buhmann, J.M., Brandt, S., Varga, Z., Wild, P.J., Günther, D., Bodenmiller, B. "Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry." *Nature Methods* (2014).
2. Chang, Q., Ornatsky, O., Siddiqui, I., Loboda, A., Baranov, V., Hedley, D.W. "Imaging mass cytometry." *Cytometry Part A* (2017).
3. Mavropoulos, A., Lin, D., Lam, B., Chang, T.K.J., Bisgrove, D., Ornatsky, O. "Equivalence of Imaging Mass Cytometry and immunofluorescence on FFPE tissue sections." fluidigm.com

Antibody Verification Specifications

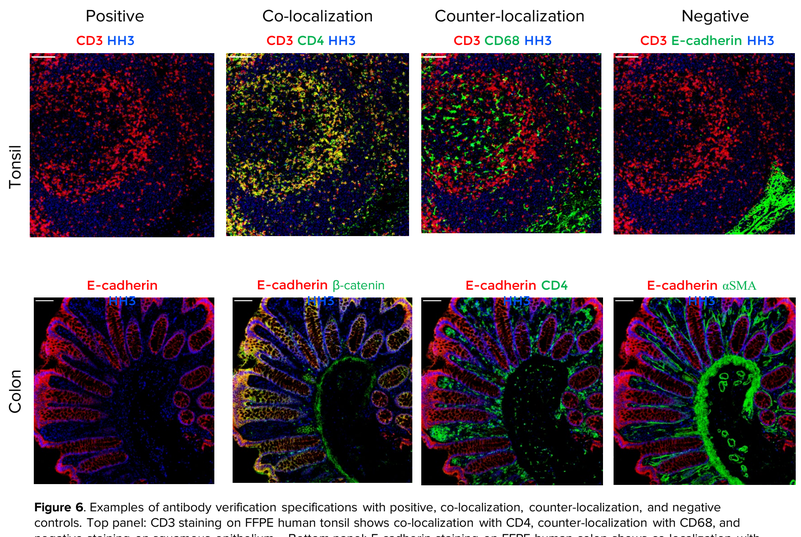

Figure 6. Examples of antibody verification specifications with positive, co-localization, counter-localization, and negative controls. Top panel: CD3 staining on FFPE human tonsil shows co-localization with CD4, counter-localization with CD68, and negative staining on squamous epithelium. Bottom panel: E-cadherin staining on FFPE human colon shows co-localization with β-catenin, counter-localization with CD4, and negative staining on smooth muscle tissue.

27-Marker Panel to Characterize Normal and Cancer Cells

	Fluidigm Cat. No.	Antibody and Clone	Tag	Concentration (ug/mL)	Expressing Cell Types
Structural	3141017D	aSMA (1A4)	141Pr	1.25	Smooth muscle actin
	3143029D	Vimentin (RV202)	143Nd	5	Intermediate filament for mesenchymal tissue
	3148020D	Pan-keratin (C11)	148Nd	5	Intermediate filaments, structural proteins of epithelial and hair forming cells
	3158029D	E-cadherin (24E10)	158Gd	2.5	Epithelia cells
	3169023D	Collagen I (poly)	169Tm	5	Connecting tissue
Nuclear	3168022D	Ki-67 (B56)	168Er	10	Proliferating cells
	3176023D	HH3 (D1H2)	176Yb	5	All nucleated cells
	201192A	DNA1	191Ir	0.3125 µM	All nucleated cells
	201192A	DNA2	193Ir	0.3125 µM	All nucleated cells

	Fluidigm Cat. No.	Antibody and Clone	Tag	Concentration (ug/mL)	Expressing Cell Types
Immuno-oncology	3144025D	CD14 (EPR3653)	144Nd	5	Monocytes, macrophages, Langerhans cells and granulocytes
	3145017D	CD33 (poly)	145Nd	10	Monocytes, activated T cells, granulocytes, myeloid progenitors, and mast cells
	3146020D	CD16 (EPR16784)	146Nd	5	NK, T cells, DC, macrophages and monocytes
	3147021D	CD163 (EDHu-1)	147Sm	5	Macrophages and monocytes
	3149028D	CD11b (EPR1344)	149Sm	2.5	DC, NK, granulocytes, macrophages and monocytes
	3152016D	CD45 (CD45-2B11)	152Sm	5	Hematopoietic cells (not erythrocytes and platelets)
	3153028D	CD223 (D2G40)	153Eu	5	Activated T cells, NK cells, Tregs
	3155016D	FoxP3 (236A/E7)	155Gd	5	Tregs
	3156033D	CD4 (EPR6855)	156Gd	1.25	Helper T cells, macrophages and monocytes
	3159035D	CD68 (KP1)	159Tb	0.625	DC, macrophages and monocytes, granulocytes
	3161029D	CD20 (H1)	161Dy	1	T subset, B cells
	3162034D	CD8a (C8/144B)	162Dy	2.5	Cytotoxic T, NK cells, lymphoid dendritic cells
	3165039D	PD-1 (EPR4877(2))	165Ho	5	Activated T and B cells
	3166031D	CD45RA (HI100)	166Er	5	B cell and naive T cell subsets, monocytes and medullary thymocytes.
	3167021D	Granzyme B (EPR20129-217)	167Er	5	NK, T cells
	3170019D	CD3 (poly)	170Er	2.5	T cells
	3174025D	HLA-DR (LN3)	174Yb	5	B cells, activated T cells, monocytes and macrophages, dendritic cells, other APCs
	3175036D	CD25 (EPR6452)	175Lu	10	Activated T and B cells, some thymocytes, pre B cells, Tregs, and oligodendrocytes.

Immune Markers Expressed in Normal Immune Tissues

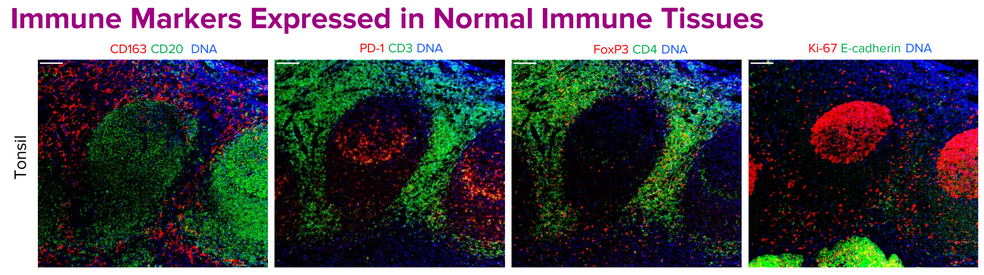

Figure 7. Expression of CD163, CD20, PD-1, CD3, FoxP3, CD4, Ki-67, and E-cadherin in FFPE human tonsil tissue.

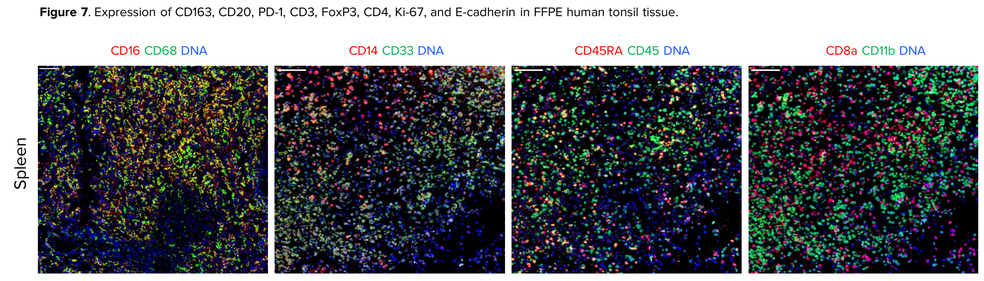

Figure 8. Expression of CD16, CD68, CD14, CD33, CD45RA, CD45, CD8a, and CD11b in FFPE human spleen tissue.

Comparing Immune Profiles of Normal and Cancerous FFPE Tissues

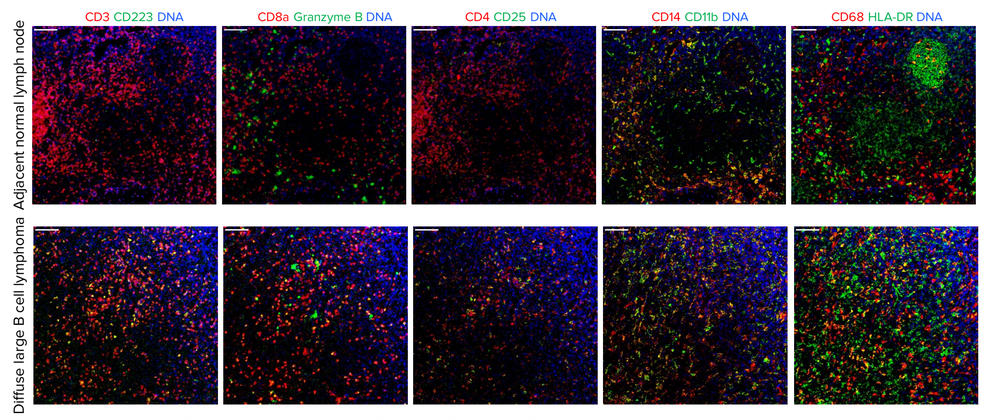

Figure 9. Expression of CD3, CD223, CD8a, granzyme B, CD4, CD25, CD14, CD11b, CD68, and HLA-DR in FFPE normal human lymph node (top panel) and diffuse large B cell lymphoma (DLBL, bottom panel) tissues.

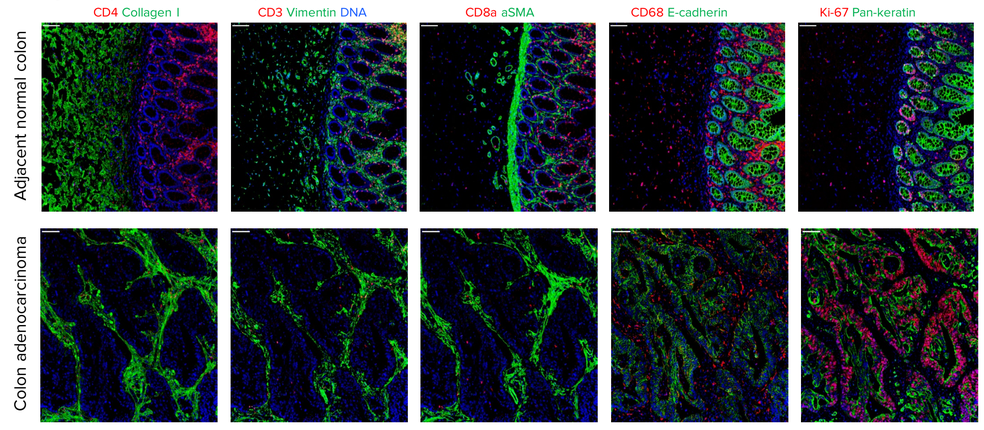

Figure 10. Expression of CD4, collagen I, CD3, vimentin, CD8a, aSMA, CD68, E-cadherin, Ki-67, and pan-keratin in FFPE normal human colon (top panel) and colon adenocarcinoma (bottom panel) tissues. Detection of tumor-infiltrating T cells are shown in the carcinoma tissues.

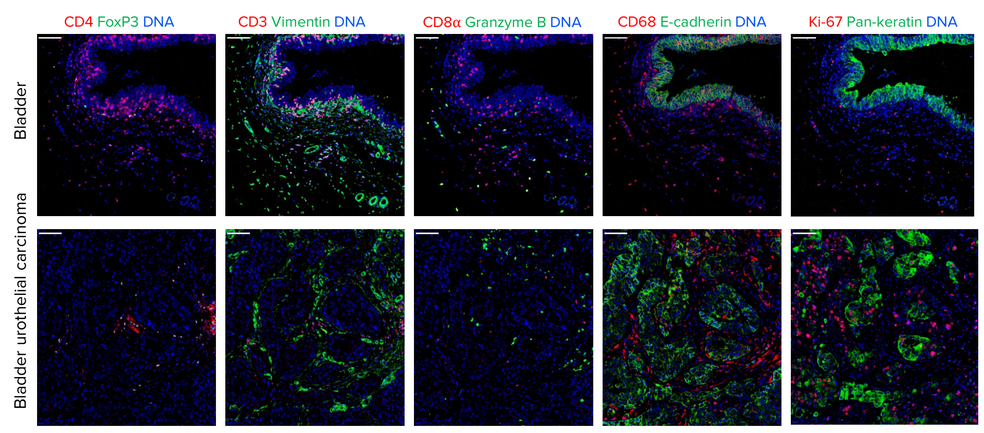

Figure 11. Expression of CD4, FoxP3, CD3, vimentin, CD8a, granzyme B, CD68, E-cadherin, Ki-67, and pan-keratin in FFPE normal human bladder (top panel) and bladder urothelial carcinoma (bottom panel) tissues. Detection of Treg cell population is shown in carcinoma tissue.

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