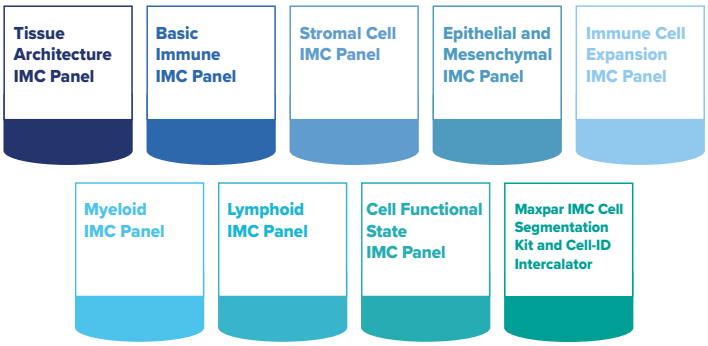
APPLICATION NOTE

Reveal Heterogeneity of Tumors with Whole Slide Imaging

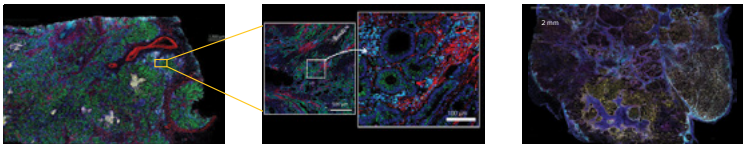
Quickly capture biological insights across the whole tumor at single-cell level

Interrogate the tumor microenvironment with the fastest, most comprehensive approach by utilizing:

READY-TO-GO IMC PANELS



IMC IMAGING MODES



Preview Mode | Cell Mode | Tissue Mode

Key takeaways

- Ready-to-go human immuno-oncology IMC panels provide swift high-parameter panel design with preselected, pathologist-verified antibodies
- Multiple imaging modes for Imaging Mass Cytometry™ (IMC™) systems enable simultaneous detection of protein targets, from whole slide tissue sections down to single-cell resolution
- Combining streamlined assembly of high-parameter panels with various imaging modes for IMC platforms means researchers now have access to the fastest, most comprehensive approach to interrogate the tumor microenvironment

Introduction

The tumor microenvironment (TME) represents a multifaceted terrain where a multitude of cancerous, normal and immune cells intricately coexist within diverse cellular neighborhoods and niches[1]. Understanding the complex spatial dynamics of cells and tissues and their interplay is crucial for comprehending and treating cancer, with the aim of enhancing patient outcomes[2]. Attaining such insights requires advanced imaging technologies capable of capturing multi-parametric spatial data at a throughput that meets the demands of modern research projects.

IMC technology is a spatial biology solution that has a significant advantage compared with cyclic immunofluorescence methods. By simultaneously detecting up to 40-plus targets, rather than using cycles, the IMC workflow enables the fastest time to high-parameter spatial biology insights. IMC technology achieves this through the utilization of variable resolution settings, enhancing the speed of data acquisition without compromising the quality of the results. Such efficiency in data collection positions IMC systems as the superior choice for comprehensive tissue imaging and analysis. Depending on the study objectives, IMC technology offers various imaging modes to explore TME heterogeneity, ranging from the entire tumor tissue section (Figure 1) down to single-cell resolution. This diverse spatial data collection range facilitates closer examination of various TME neighborhoods and cell types, resulting in a more extensive assessment of tumor biology.

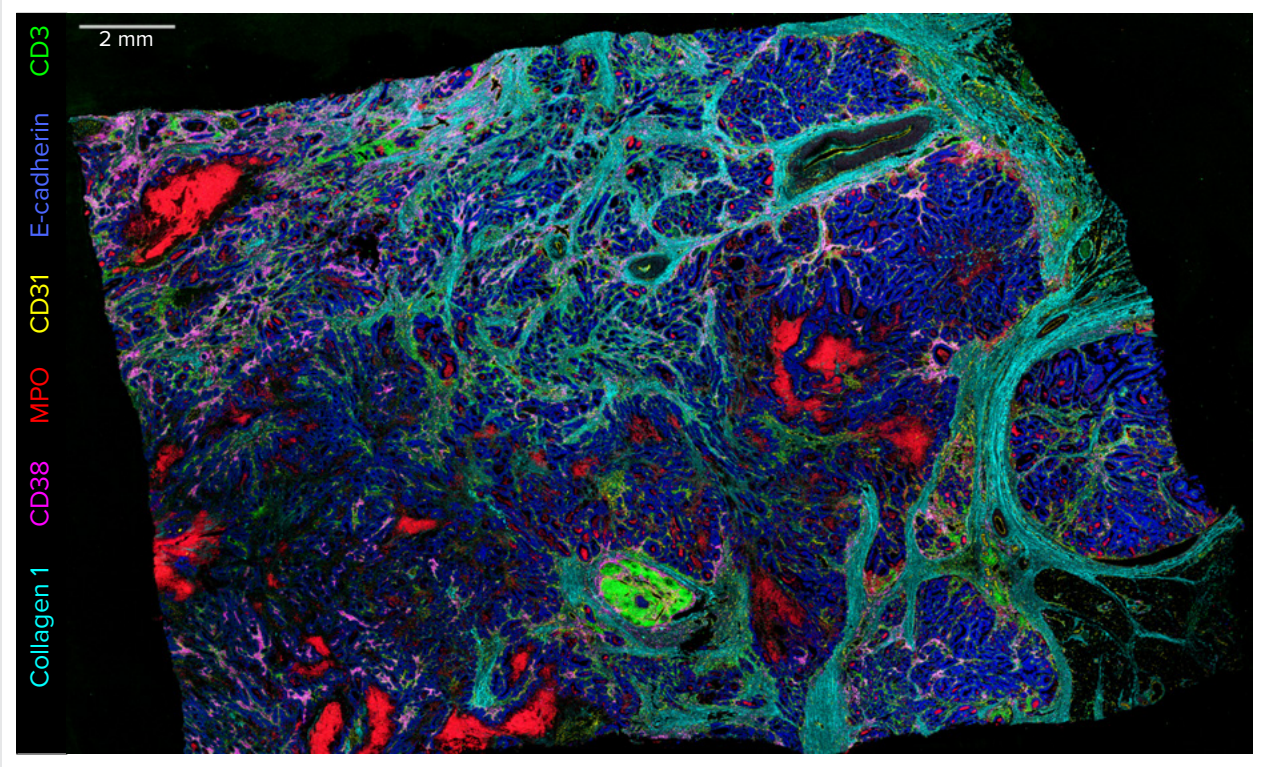


Figure 1. Whole slide Imaging Mass Cytometry technology provides a comprehensive investigation of the tumor tissue microenvironment. This multiplexed image demonstrates the application of Tissue Mode imaging using the Human Immuno-Oncology IMC Panel on human colon adenocarcinoma tissue. The novel WSI technique provides rapid and detailed assessment of various tissue structures including tumor-, immune- and stromal-specific features. Scale bar is 2 mm.

Here, we highlight the capability of IMC whole slide imaging (WSI) to provide streamlined and extensive evaluation of the human TME. We utilize the Human Immuno-Oncology IMC Panel, 31 Antibodies alongside the Human Immune Cell Expansion IMC Panel, 7 Antibodies. These panels were specifically chosen due to their inclusion of pathologist-verified antibodies that were preselected to provide the maximum immuno-oncological insights. Moreover, the modular assembly of these panels facilitated the swift, effortless design of our high-plex IMC panel. This streamlined approach to panel design not only expedited the experimental setup but also ensured that our panel was tailored precisely to our research objectives, enabling a comprehensive exploration of immuno-oncology dynamics within human breast cancer TME.

Study design

Panel design

A high-parameter 41-antibody panel, designed to highlight central features of human breast cancer TME (Figure 2, Table 1), is presented in this application note. The Human Immuno-Oncology IMC Panel was combined with the Human Immune Cell Expansion IMC Panel to further characterize the tumor immune landscape.

The Human Immuno-Oncology IMC Panel consists of 7 modular subpanels:

- The **Human Tissue Architecture IMC Panel, 4 Antibodies** detects the underlying structural composition of normal and tumorous tissue
- The **Human Stromal Cell IMC Panel, 4 Antibodies** detects the presence of stromal cell subtypes, including cancer-associated fibroblasts (CAFs)
- The **Human Basic Immune IMC Panel, 4 Antibodies** detects the presence of immune cell subtypes, including B cells, T cells, macrophages and a pan-leukocyte marker
- The **Human Lymphoid IMC Panel, 4 Antibodies** detects lymphoid cell subtypes of immune cell infiltrates, including T cell subtypes and NK cells
- The **Human Myeloid IMC Panel, 6 Antibodies** detects myeloid cell subtypes of immune cell infiltrates, including neutrophils and M2 macrophages
- The **Human Cell Functional State IMC Panel, 5 Antibodies** detects the functional state of the different cell subtypes, including key markers for proliferation, activation and exhaustion
- The **Human Epithelial and Mesenchymal IMC Panel, 4 Antibodies** detects epithelial and mesenchymal markers, including β-catenin

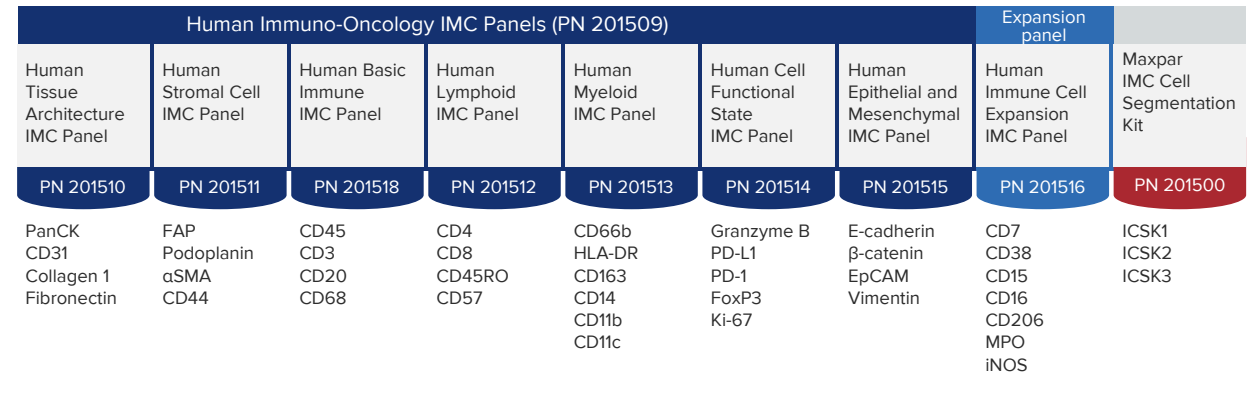


Figure 2. The Human Immuno-Oncology IMC Panel is a 31-antibody panel designed to detect relevant immuno-oncological processes in human tumors. The modular structure of the off-the-shelf IMC panel offers excellent flexibility to customize panels for application on any tumor sample. When combined with the Human Immune Cell Expansion IMC Panel and the Maxpar IMC Cell Segmentation Kit (ICSK), the fully loaded 41-marker panel permits the detection of lymphoid and myeloid immune cell subtypes and their activation states; the immunosuppressive, metastatic and growth state of tumors; tumor stemness and vascularization; tertiary lymphoid structures; the presence of CAFs; and extracellular matrix composition.

The Human Immune Cell Expansion IMC Panel consists of 7 antibodies, expanding the number of markers and detecting lymphoid and myeloid cell subtypes.

The high-parameter antibody panel was applied on tissue sections of invasive human breast carcinoma.

The tumor tissue slides were prepared and stained using optimized antibody dilutions. In addition, the Maxpar™ IMC Cell Segmentation Kit (ICSK) and Cell-ID™ Intercalator-Ir (identifies nuclei) were applied to facilitate single-cell image analysis.

Imaging modes

Tissue slides were ablated using the Hyperion™ XTi Imaging System, using the following imaging modes (Figure 3, Table 1):

- **Preview Mode** enables quick visualization for all 41 markers within minutes across the whole tissue. This fast scan provides guidance for selecting regions of interest (ROIs) to be acquired on the same slide in Cell Mode.
- **Cell Mode** facilitates acquisition of ROIs selected in Preview Mode at subcellular resolution for detailed characterization of individual cells and cell populations. Accelerate Cell Mode ROI selection with a Preview Mode image on the same slide.
- **Tissue Mode** is a complete WSI mode to visualize heterogeneity of the whole tissue at a macro level

Image analysis

Qualitative data analysis, multiplexed image rendering and single-channel image extractions were performed using MCD™ Viewer software. For tumor tissue data obtained using Preview Mode and Cell Mode, quantitative single-cell analysis was performed using a 2-step data analysis pipeline: Cellpose was used for cell segmentation, histoCAT was used for t-distributed stochastic neighbor embedding (t-SNE) for dimensionality reduction and PhenoGraph was used for clustering. The data obtained using Tissue Mode was analyzed with a pixel-clustering analysis approach using MCD SmartViewer software.

See Methods for additional experimental details regarding samples, staining, ablation and data analysis.

Imaging workflows

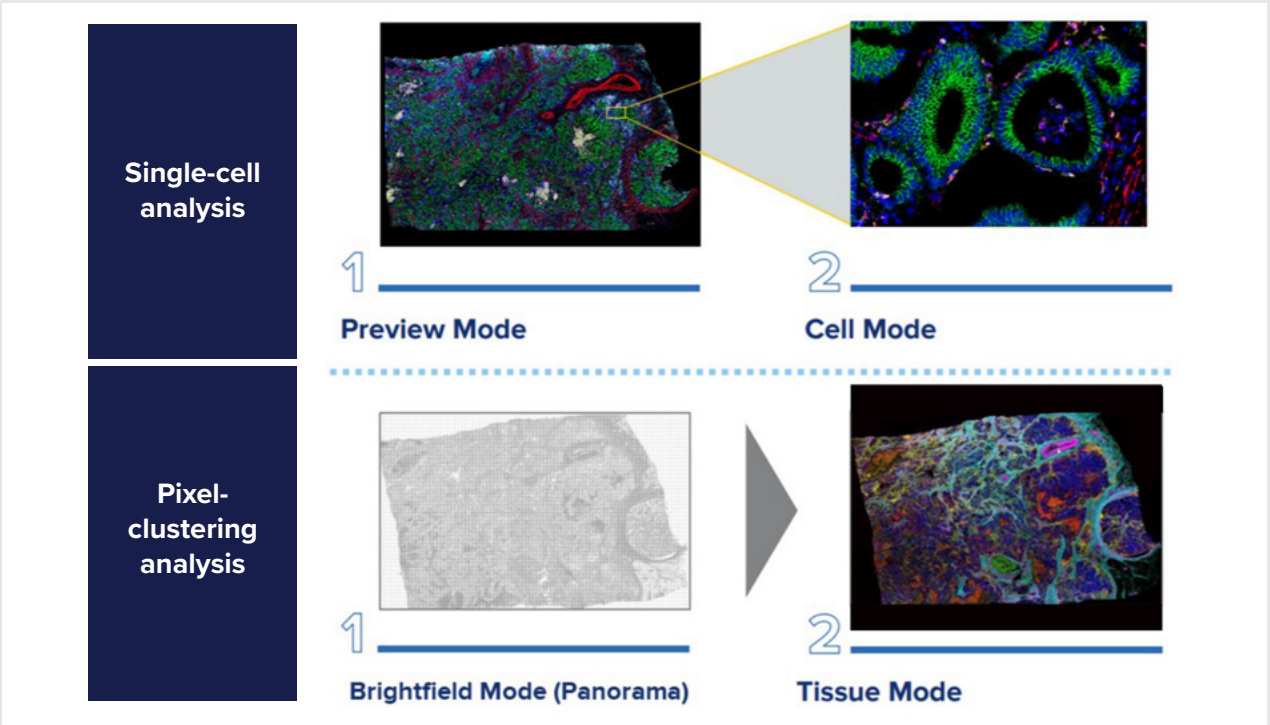


Figure 3. Versatile WSI modes for IMC systems accelerate quantitative spatial biology discoveries. Preview Mode rapidly scans the sample and generates useful data for guiding ROI placement when using Cell Mode. Cell Mode acquires data at single-cell resolution. Tissue Mode generates a high-quality scan of the entire tissue section in a matter of hours, with higher spot-size ablations enabling entire tissue analysis using pixel-clustering analysis. Combining these new imaging modes with the Hyperion XTi Slide Loader streamlines the IMC workflow and makes it a useful resource for high-throughput clinical and translational studies.

Results

Cell Mode in combination with Preview Mode reveals highly specialized tumor structures in breast cancer tissue

Based on Preview Mode data, 2 ROIs were selected for Cell Mode acquisition followed by single-cell analysis. The first ROI found at the tumor margin (Figure 4B, red inset) demonstrates a high abundance of T cells (denoted by CD3 expression). The marginal regions of the tumor contained significant enrichment of Ki-67+ proliferating tumor cells, accompanied by significant infiltration of immune cells, including T helper cells (CD3+, CD4+), cytotoxic T cells (CD3+, CD8+) and various myeloid cells. The second ROI (Figure 4B, cyan inset) showcases a tumor structure with a high abundance of stromal cells. We observed a highly organized and dense tumor structure harboring PD-L1+ and Ki-67+ tumor cells. Notably, this region also contained diverse CAF populations, which may play a role in enhancing the risk for tumor proliferation and growth[1, 2].

Subsequent cell clustering quantitatively resolved the cellular makeup of each tumor structure and isolated spatial localization patterns of specific cell populations.

Overall, the data presented so far highlights the power of Preview Mode to rapidly locate desirable areas of the tumor for subsequent subcellular resolution imaging with Cell Mode on the same slide. Furthermore, combining this imaging workflow with the Human Immuno-Oncology IMC Panel enhances the capabilities of IMC technology to effectively identify diverse TME organizations and immune responses to tumor development and progression for in-depth single-cell analysis.

WSI using Tissue Mode unveils tissue complexity and provides a deep dive into the TME

We applied Tissue Mode to capture the expression of all 41 markers in our high-parameter IMC panel across the entire breast cancer tissue under investigation. Tissue Mode imaging provides a global view of protein heterogeneity across the entire tissue section, enabled by the Human Immuno-Oncology IMC Panel to stain key targets.

Prominent heterogeneity was detected in the breast cancer tissue sample with specific areas of the tissue displaying a variety of specialized tissue structures (Figure 5). We also identified distinct tumor areas that were either devoid of, or enriched in, immune cells of lymphoid or myeloid origin. Additionally, we detected necrotic cores filled with components of extracellular matrix and various CAF subtypes.

Unlike Preview Mode, WSI with Tissue Mode data is quantifiable when analyzed with pixel-clustering analysis. Pixel-clustering analysis played a crucial role in deciphering the complex tissue landscape offered by high-parameter spatial biology. By analyzing the intensity and distribution of all 41 markers at the pixel level, the pixel-clustering algorithm was able to perform unsupervised clustering, automatically grouping pixels with similar characteristics. This unsupervised approach defined distinct tumor areas solely based on the combinatorial expression of markers in the Human Immuno-Oncology IMC Panel without prior assumptions about tissue composition. Identified tumor regions include immune-rich zones, highly replicating tumor regions, and potential communication hubs between immune cells and CAFs. This detailed tumor landscape encompasses complex interactions influencing tumor behavior.

These findings demonstrate the power of combining Tissue Mode imaging with pixel-clustering analysis to unveil the intricate heterogeneity of cancer tissues. This approach has the potential to rapidly provide valuable insights into tumor biology and guide the development of targeted therapies.

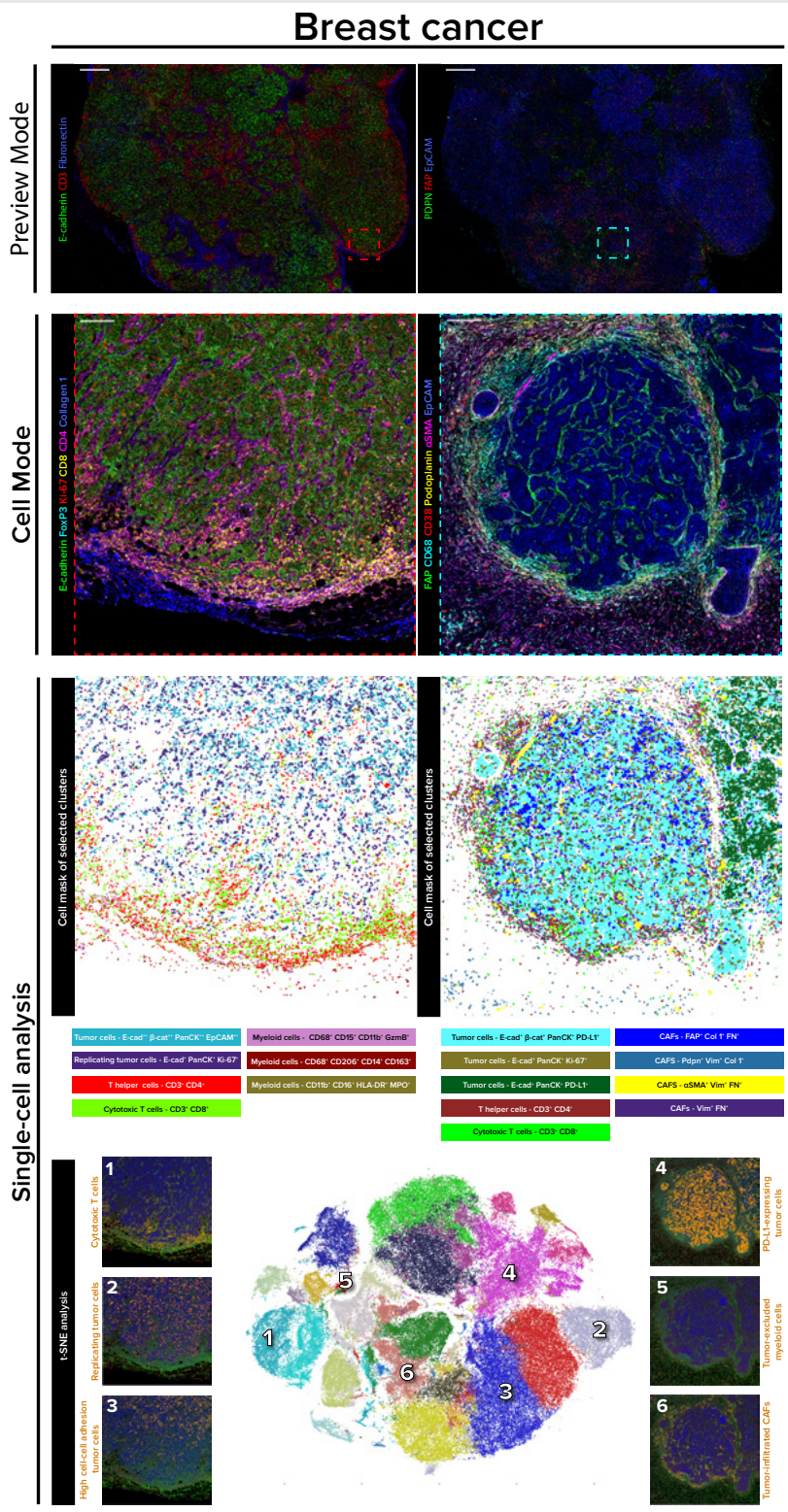


Figure 4. Streamlined single-cell analysis of breast cancer spatial biology using Preview Mode, Cell Mode and the Human Immuno-Oncology IMC Panel. A) Preview Mode: Rapid scanning using Preview Mode demonstrated the heterogeneous landscape of the entire breast cancer (invasive ductal carcinoma) tissue, which assisted in guiding placement of several ROIs at relevant positions for (B) Cell Mode acquisition (red inset). Multiple types of lymphoid and myeloid immune cells were detected at the tumor margin (cyan inset). A tumor structure with low immune penetration was detected near necrotic tissue. (C) Single-cell analysis: Subtypes of CAFs and immune cells were identified and observed by single-cell analysis to localize at the margin of the PD-L1-expressing tumor structure. Single-cell analysis quantitatively delineated cell phenotypes of tumor and immune cell populations in all acquired ROIs. t-SNE and PhenoGraph clustering analysis successfully segregated and spatially resolved subsets of activated and dormant tumor cells and immune cell populations, which could then be mapped back to the segmented cell mask for each tissue (insets 1–6). Scale bar is 200 µm.

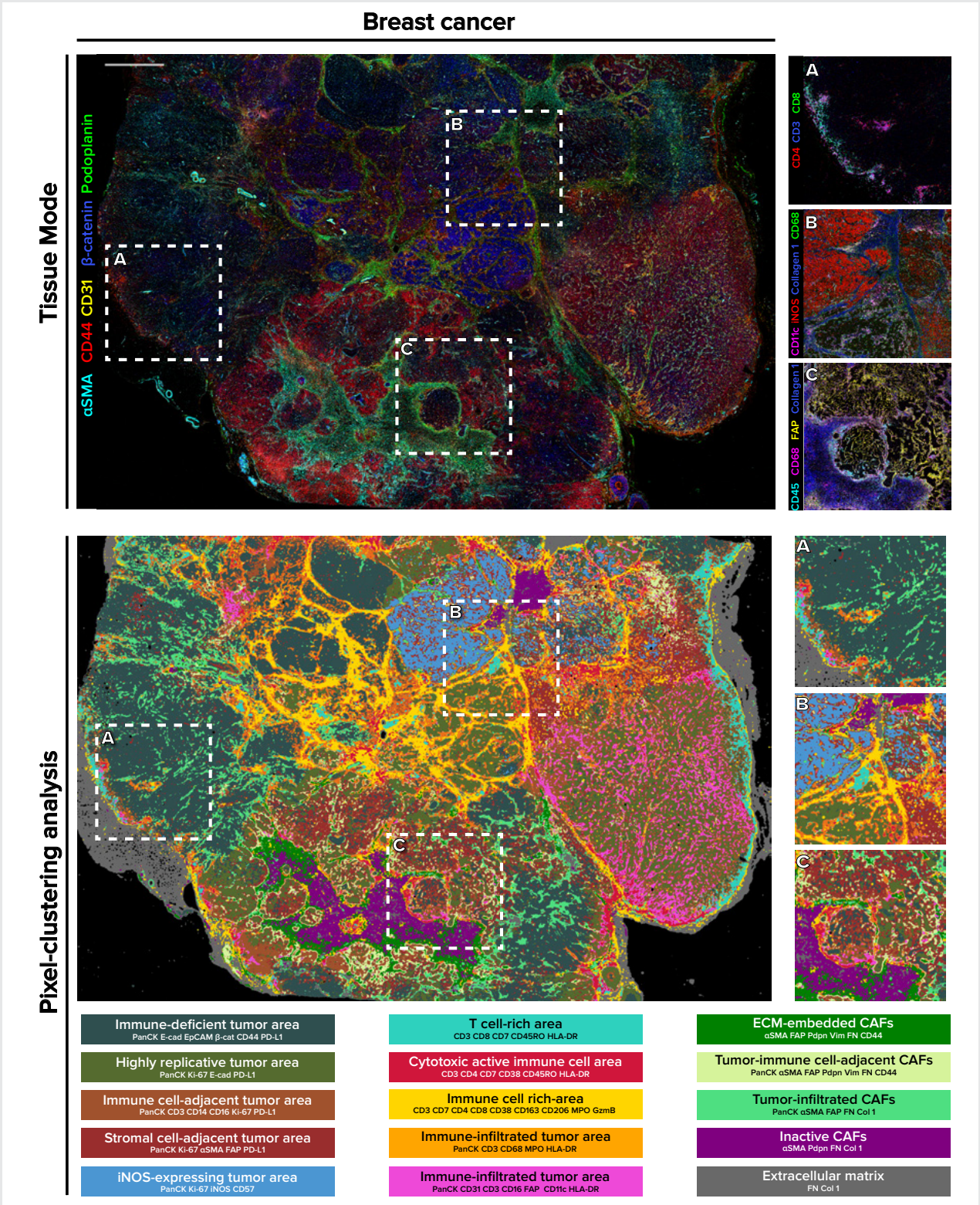


Figure 5. Rapid whole tissue spatial biology analysis of breast cancer using Tissue Mode and the Human Immuno-Oncology IMC Panel. Tissue Mode imaging successfully captured the expression pattern of Human Immuno-Oncology IMC Panel markers in breast cancer tissue. Prominent heterogeneity across the tissue was detected, with multiple specialized tumor structures identified: Lymphocyte localization was observed at the tumor margins (A, inset); the presence of specialized tumor cells expressing iNOS surrounded by collagen 1-rich extracellular matrix was detected (B, inset); and tissue compartments containing interactive niches of CAFs and immune cells were identified (C, inset). Unsupervised pixel-clustering analysis along with hierarchical clustering highlighted several tumor areas such as immune-deficient, highly replicative, immune cell-enriched, stromal cell-enriched and high iNOS expression. Areas with high immune cell infiltrations containing cytotoxic T cells, T helper cells and myeloid cells were detected. Interactive niches of several CAF subtypes were visualized and their spatial localization patterns were observed. Scale bar is 2 mm.

Conclusions

In this application note, we demonstrated the enhanced capabilities of IMC technology to capture dozens of biologically relevant parameters through utilization of Hyperion XTi Imaging System WSI workflows in combination with the ready-to-go, comprehensive Human Immuno-Oncology IMC Panel. This panel combined with the new WSI workflows detected clinically relevant components of the TME, including various tumor subtypes, immune cells, stromal cells and cellular compartments. Altogether, these findings provide insight into the state of the tumor tissue and empower researchers to evaluate parameters related to patient survival and treatment.

Our results indicate that each imaging mode offers unique yet complementary advantages for tumor imaging and provides critical information about the tumor at different scales.

With Preview Mode, within minutes we confidently identified ROIs containing elevated levels of clinically relevant tissue characteristics based on the expression pattern of the 41 protein targets in the panel.

These ROIs were then further investigated using Cell Mode single-cell-resolution imaging on the same slide.

Single-cell analysis of tissues in Cell Mode revealed the cellular interplay of various cell subtypes, including immune infiltrates, proliferating PD-L1+ tumor cells and stromal cells.

By using Tissue Mode, we were able to partition the highly complex immune and tumor compartments of the entire breast cancer tissue in a matter of hours and quantitatively measure the relative proportions of each compartment using MCD SmartViewer software powered by pixel-clustering algorithms. These novel imaging modes collectively permit more focused and deeper evaluation of intratumoral heterogeneity of cancer tissues and spatial interactions between cell populations.

Overall, Hyperion XTi imaging workflows together with the Human Immuno-Oncology IMC Panel offer a powerful tool for investigating and understanding the intricacies of the TME, establishing the new standard for evaluating spatial biology.

Tips for success

- For best results, use freshly cut FFPE tissue samples when possible
- Perform a 3-point titration and include positive control tissue for all antibodies when optimizing their working concentrations on tumor tissue. Recommended dilution ranges for each antibody can be found in the technical datasheet (TDS).
- Antibody titrations should be separately optimized for Cell Mode and Tissue Mode
- After staining, samples should be stored at room temperature in slide holders inside a sealed bag in a non-humid environment
- Customers should reach out to their local Field Applications Scientist (FAS) for ordering and product support. To be connected to an FAS, contact **technical support**.

Methods

Panel design

Antibodies were selected based on the best fit for the immuno-oncology application on tissues and full compatibility with other IMC panels from Standard BioTools (for example, the Maxpar Neuro Phenotyping IMC Panel Kit; see the TDS for the Human Immuno-Oncology IMC Panel for more information on compatible subpanels). For ordering information on the panel used in this application note, refer to Table 1.

Tissue

A commercially available human multicancer tissue microarray (TMA) was obtained from **tissuearray.com**. The TMA was composed of 12 multiple-organ tumors with matched or unmatched normal-adjacent or cancer-adjacent tissue (core size 1.5 mm). The colon adenocarcinoma and invasive breast carcinoma single tissue sections were obtained from BioChain and stored according to the manufacturer's recommendation before use. Several regions of the tumor tissues were ablated. Varying ROIs were selected, including tumor cores and margins.

Staining

Slide preparation and staining were conducted according to the Imaging Mass Cytometry Staining Protocol for FFPE Sections (400322). Briefly, slides were baked at 60 °C for 2 hr followed by dewaxing in xylene and stepwise rehydration in descending grades of ethanol. Slides were washed in Maxpar Water and inserted into preheated Dako Target Retrieval Solution (pH 9) for 30 min at 96 °C. Subsequently, slides were washed in 1X Maxpar PBS and blocked with 3% bovine serum albumin in 1X PBS for 45 min at room temperature (RT). Antibody cocktails were prepared at recommended dilutions (Table 1), added to slides and incubated overnight at 4 °C in a humified chamber. The following day, slides were washed and stained with Cell-ID Intercalator-Ir in 1X PBS at RT for 30 min for DNA labeling. The slides were washed, air-dried and prepared for acquisition as described below.

Imaging

Imaging was performed using the Hyperion XTi Imaging System with CyTOF™ Software v9.0. Before ablation, instrument tuning was performed using a tuning slide.

Preview Mode

In Preview Mode, the tumor sections were ablated at 800 Hz with 1 µm resolution at 25 µm spacing to rapidly capture a subsampled image of all expressed markers in the antibody panel. This does not impact the ability to perform additional imaging and was used to guide ROI placement for Cell Mode. The time taken to acquire the 22.7 mm x 15.4 mm image was approximately 40 min.

Cell Mode

For Cell Mode imaging, each 2 mm x 2 mm ROI was selected and ablated at 800 Hz with optimized laser power and 1 µm resolution. The time taken to acquire each ROI was approximately 1.5 hr.

Tissue Mode

In Tissue Mode, the full single tumor sections were ablated at 800 Hz with optimized laser power at 5 µm resolution. The time taken to acquire the 28.7 mm x 16 mm image was approximately 8 hr.

QC and preprocessing

MCD Viewer software v1.0.560.6 was used to render multiplexed and single-channel 16-bit TIFF images. For qualitative verification of staining, images for each channel were rendered and verified to ensure absence of nonspecific and background staining. Raw single-channel OME-TIFF files were exported for further analysis.

Cell segmentation

The Maxpar IMC Cell Segmentation Kit and Cell-ID Intercalator-Ir were used to label the cell membrane and nuclei of all cells present in the TME, respectively. Cellpose v2.2.3 was used for performing cell segmentation. Cellpose[3] is a deep learning-based segmentation method. The pre-trained Cellpose tissue-net model was optimized on the data acquired to identify the nuclei and membranes of the cells represented in the samples. Images containing individual cell masks were generated and extracted for downstream single-cell analysis.

Single-cell analysis

Single-channel OME-TIFF and cell masks for the tumor ROIs were loaded into histoCAT v1.76. t-SNE dimensionality reduction and PhenoGraph clustering were performed. Masks representing specific clusters were plotted onto ROIs rendered with ICSK channels and cell quantities for each cluster were extracted and documented. All clusters were plotted on the t-SNE graphs and heat maps.

Pixel-clustering analysis

Tissue Mode pixels were fed directly into the modified FlowSOM clustering workflow. Preprocessing involved application of a Gaussian blur filter followed by an arcsinh transformation and rescaling. For each sample, all pixels were grouped into 100 clusters using a self-organizing map. The 100 clusters were then grouped into 20 meta-clusters using consensus agglomerative clustering. Phenotypically similar meta-clusters were manually consolidated to arrive at the final number of meta-clusters for each sample.

IMC Panel	Marker	Clone	Metal	Concentration (µg/mL)		Part Number
				Tissue Mode	Cell Mode	
Human Tissue Architecture IMC Panel, 4 Antibodies (201510)	Pan-cytokeratin	AE-1/AE-3	141Pr	0.2	1.0	3141021D
	CD31	EPR3094	151Eu	1.0	5.0	3151025D
	Collagen 1	Polyclonal	89Y	1.0	5.0	3089006D
	Fibronectin	EPR23110-46	171Yb	0.2	1.0	3171027D
Human Stromal Cell IMC Panel, 4 Antibodies (201511)	FAP	E1V9V	161Dy	0.5	2.5	3161035D
	Podoplanin	D2-40	164Dy	0.5	2.5	3164032D
	αSMA	1A4	209Bi	0.125	0.5	3209017D
	CD44	IM7	153Eu	1.0	5.0	3153029D
Human Basic Immune IMC Panel, 4 Antibodies (201518)	CD45	D9M8I	152Sm	0.25	2.5	3152018D
	CD3ε	D7A6E	170Er	1.0	5.0	3170023D
	CD20	H1	115In	1.0	5.0	3115001D
	CD68	KP1	159Tb	0.25	1.0	3159035D
Human Lymphoid IMC Panel, 4 Antibodies (201512)	CD4	EPR6855	156Gd	1.0	5.0	3156033D
	CD8	C8/144B	162Dy	0.5	2.5	3162034D
	CD45RO	UCHL1	173Yb	0.2	1.0	3173016D
	CD57	NK/804	163Dy	0.5	2.5	3163033D
Human Myeloid IMC Panel, 6 Antibodies (201513)	CD66b	BLR111H	160Gd	0.25	1.25	3160030D
	HLA-DR	LN3	174Yb	0.2	1.0	3174025D
	CD163	EDHu-1	147Sm	1.0	5.0	3147021D
	CD14	EPR3653	175Lu	0.5	2.5	3175041D
	CD11b	EPR1344	144Nd	0.25	1.25	3144030D
	CD11c	D3V1E	154Sm	0.5	2.5	3154027D
Human Cell Functional State IMC Panel, 5 Antibodies (201514)	Granzyme B	EPR20129-217	176Yb	0.125	1.0	3176028D
	PD-L1	73-10	166Er	5.0	10.0	3166032D
	PD-1	D4W2J	165Ho	5.0	10.0	3165045D
	FoxP3	PCH101	155Gd	5.0	10.0	3155018D
	Ki-67	B56	150Nd	0.5	2.5	3150035D
Human Epithelial and Mesenchymal IMC Panel, 4 Antibodies (201515)	E-cadherin	24E10	158Gd	0.5	2.5	3158029D
	β-catenin	5H10	169Tm	0.25	2.5	3169031D
	EpCAM	EPR20532-222	172Yb	0.5	2.5	3172034D
	Vimentin	D21H3	149Sm	0.125	1.25	3149032D
Human Immune Cell Expansion IMC Panel, 7 Antibodies (201516)	CD7	EPR4242	143Nd	0.5	2.5	3143032D
	CD15	W6D3	145Nd	0.5	2.5	3145021D
	CD16	EPR16784	146Nd	0.5	2.5	3146020D
	iNOS	SP126	168Er	1.0	5.0	3168029D
	CD38	EPR4106	142Nd	0.5	2.5	3142022D
	CD206	5C11	167Er	0.5	2.5	3167026D
	MPO	EPR20257	148Nd	0.4	2.0	3148024D

Table 1. A high-parameter 41-antibody panel designed to highlight central features of human breast cancer TME

Required reagents

Standard BioTools Products		Part Number
Cell-ID™ Intercalator-Ir (125 µM)		201192A
Human Immune Cell Expansion IMC Panel, 7 Antibodies		201516
Human Immuno-Oncology IMC Panel, 31 Antibodies		201509
Maxpar IMC Cell Segmentation Kit		201500
Maxpar PBS		201058
Maxpar Water		201069
Third-Party Reagents		**Part Number**
Commercial Alcohols	Ethyl Alcohol Anhydrous, USP	P006EAAN
Agilent	Dako Target Retrieval Solution, pH 9 (x10)	S236784-2
Sigma-Aldrich	Bovine Serum Albumin solution, 10% in DPBS	A1595
	m-Xylene ReagentPlus, 99%	185566-1L
Thermo Fisher Scientific	Triton X-100 Surfact-Amps Detergent Solution	85111

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2. Hinshaw, D.C. and Shevde, L.A. “The tumor microenvironment innately modulates cancer progression.” *Cancer Research* 79 (2019): 4,557–4,566.
3. Stringer, C. et al. “Cellpose: a generalist algorithm for cellular segmentation.” *Nature Methods* 18 (2021): 100–106.

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