A Deep-Learning Approach for Tissue Mapping and Cell Phenotyping in Imaging Mass Cytometry Data Analysis

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Introduction

Imaging Mass Cytometry™ (IMC™) technology is a multiplexed imaging technique that generates high-dimensional spatial data at subcellular resolution without the complications of autofluorescence and cyclic imaging. IMC technology has two distinct whole slide imaging (WSI) modes: Preview Mode (PM) and Tissue Mode (TM). PM rapidly scans stained tissue to provide a comprehensive overview within minutes, while TM provides fast acquisition of the entire tissue at 5-micron resolution, mapping out the distribution of over 40 markers and revealing tissue heterogeneity. Both WSI modes enable researchers to make informed decisions about selecting tissue areas that warrant closer examination at single-cell resolution. Following PM, regions of interest (ROIs) are selected on the same slide for high-resolution imaging using Cell Mode (CM). This facilitates single-cell analysis of the ROIs identified during PM. These imaging modes together with an automated slide loader function support nonstop acquisition of tissue samples.

Methods and materials

Tissue sections of immunotherapy-treated lung cancer were stained with a 34-marker IMC panel by combining the Human Immuno-Oncology IMC Panel, 31 Antibodies with the Maxpar™ IMC Cell Segmentation Kit to study spatial organization and cellular interactions in the tissue. Images were acquired on the Hyperion™ XTi Imaging System (Standard BioTools), first in PM and then in CM with automatic selection of ROIs using Phenoplex™ software (Visiopharm®). ROIs were automatically selected based on three criteria: 1) tertiary lymphoid structures (TLSs) expressing CD20 and CD3; 2) granzyme B-rich areas; and 3) areas with a high number of CD68 and vimentin double-positive cell clusters. An adjacent serial section was acquired in TM for a whole slide morphologic segmentation comparison.

Tissue segmentation for all modes was performed via training a deep-learning AI algorithm embedded within Phenoplex software to recognize morphological features such as vessels and TLSs. Single-cell analysis of the images generated in CM was performed with cell segmentation based on iridium DNA channels. Cellular populations and phenotyping were performed using the Phenoplex guided workflow. This data was used to compare the immune contexture through a series of t-SNE plots partitioned by spatial region and clinical variables.

31-marker pathologist-verified antibody panel

Human Immuno-Oncology IMC Panel, 31 Antibodies (PN 201509)							ICSK
Human Tissue Architecture IMC Panel, 4 Antibodies	Human Stromal Cell IMC Panel, 4 Antibodies	Human Lymphoid IMC Panel, 4 Antibodies	Human Myeloid IMC Panel, 6 Antibodies	Human Cell Functional State IMC Panel, 5 Antibodies	Human Epithelial and Mesenchymal IMC Panel, 4 Antibodies	Human Basic Immune IMC Panel, 4 Antibodies	Maxpar IMC Cell Segmentation Kit and Cell-ID™ Intercalator-Ir
PN 201510	PN 201511	PN 201512	PN 201513	PN 201514	PN 201515	PN 201516	PN 201500 PN 201192B
Pan-cytokeratin CD31 Collagen 1 Fibronectin	FAP Podoplanin αSMA CD44	CD4 CD8 CD45RO CD57	CD66b HLA-DR CD163 CD14 CD11b CD11c	Granzyme B PD-L1 PD-1 FoxP3 Ki-67	E-cadherin β-catenin EpCAM Vimentin	CD45 CD20 CD45	ICSK1 ICSK2 ICSK3 DNA1 DNA2

Figure 1. Pathologist-verified 31-marker antibody panel. The Human Immuno-Oncology IMC Panel is designed to explore immuno-oncological processes in human tumors. It includes 31 pathologist-verified antibodies in the base panel and is optimized for FFPE tissues. The panel's modular structure allows for customization, making it suitable for various translational and clinical samples. When combined with the Maxpar IMC Cell Segmentation Kit, it enables the detection of immune cell subtypes, tumor characteristics and microenvironment components, and the presence of cancer-associated fibroblasts (CAFs). This comprehensive approach enhances understanding of the tumor microenvironment (TME) in immuno-oncology research.

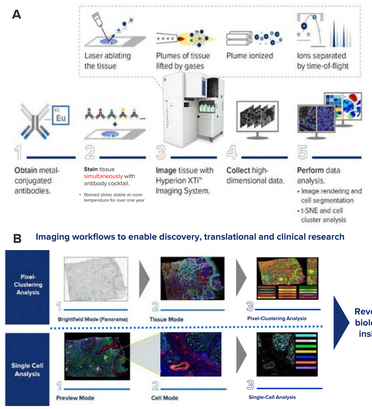


Figure 2. Imaging Mass Cytometry workflows. (A) IMC technology offers a streamlined workflow that simplifies translational and clinical application of multiplexed tissue analysis. The five-step process, which consists of obtaining metal-conjugated antibodies, staining tissues with antibody cocktails, imaging tissues with the Hyperion XTi Imaging System, and the collection and analysis of high-dimensional data, can be accomplished in as little as 72 hours (two slides with two 4 mm² ROI each). **(B)** The novel WSI modes for IMC technology offer a customized workflow for specific imaging applications. Here we highlight two simple ways for a user to get started. For single-cell analysis, start with PM, which provides a rapid scan of the whole tissue and highlights all your stained markers. This helps guide ROI placement to capture single cell-resolution image data using CM. For pixel-clustering analysis of an entire tissue section, users can first identify the placement of tissue using the rapid Brightfield Mode, followed by the novel TM, which 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 workflows with the newly available slide loader for the Hyperion XTi Imaging System streamlines IMC application and makes it a useful resource for high-throughput clinical and translational studies.

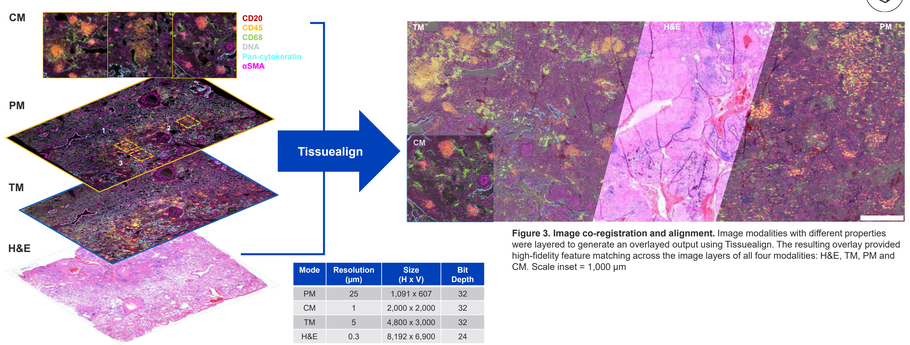
Conclusions

This work demonstrates that the Hyperion XTi Imaging System with its three acquisition modes (PM, TM and CM) can greatly advance the ability of IMC users to obtain answers from complex samples. The interactive capabilities of Phenoplex software allow the user to quickly identify pertinent cell types, find them within a tissue map, define their spatial relationship and analyze their neighbors, leading to valuable biological insights.

Results

IMC images were natively imported with all image overlays, feature segmentations and cellular phenotyping performed utilizing the Phenoplex guided workflow (top adjacent panel). Quantified cell object outputs were further assessed and evaluated for population and phenotypes of interest.

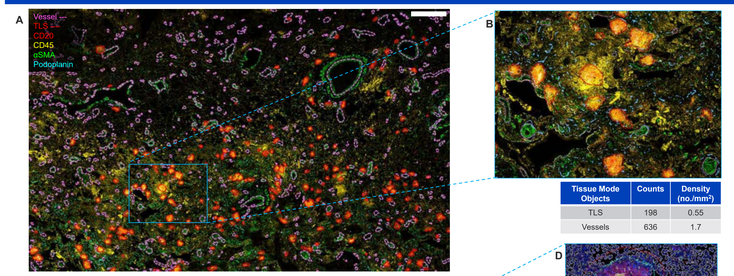
Co-registration of multiple image modes



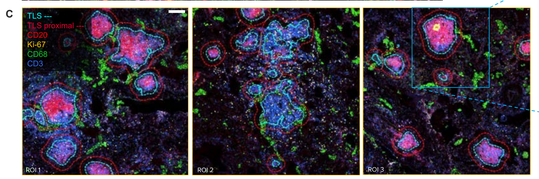
Mode	Resolution (µm)	Size (H x V)	Bit Depth
PM	25	1,091 x 607	32
CM	1	2,000 x 2,000	32
TM	5	4,800 x 3,000	32
H&E	0.3	8,192 x 6,900	24

Figure 3. Image co-registration and alignment. Image modalities with different properties were layered to generate an overlayed output using Tissuealign. The resulting overlay provided high-fidelity feature matching across the image layers of all four modalities: H&E, TM, PM and CM. Scale inset = 1,000 µm

AI-based feature segmentation and designation



Tissue Mode Objects	Counts	Density (no./mm²)
TLS	198	0.55
Vessels	636	1.7



TLS Area (mm²)	TLS Proximal Area	Stromal Area	Cell Object Counts
0.92	1.18	10.16	66,017
7%	9%	84%	

Figure 4. AI-assisted tissue and cell feature segmentation. A) A deep-learning algorithm was trained on tissue features for vessels using αSMA and TLS objects using CD20 markers to calculate B cell-associated TLS structures and vessel densities from TM. Scale = 2,000 µm. Enlarged region (B) of tissue mode shown for clarity.

C) A similar deep-learning algorithm was separately trained for general TLS structures using CD45+ cells to delineate TLS zones (cyan dashed outline) in CM. A TLS proximal zone dilated 50 µm from the TLS zone boundary was established (red dashed outlines). Calculated areas and corresponding percentages are pooled across all CM regions (ROI 1, 2, 3). These designated zone compartments were then used to further investigate the cellular identities and phenotypes associated with these structures based upon the robust cell segmentation generated by the trained algorithm, even in densely packed clusters (D). Scale = 200 µm

QC marker expression

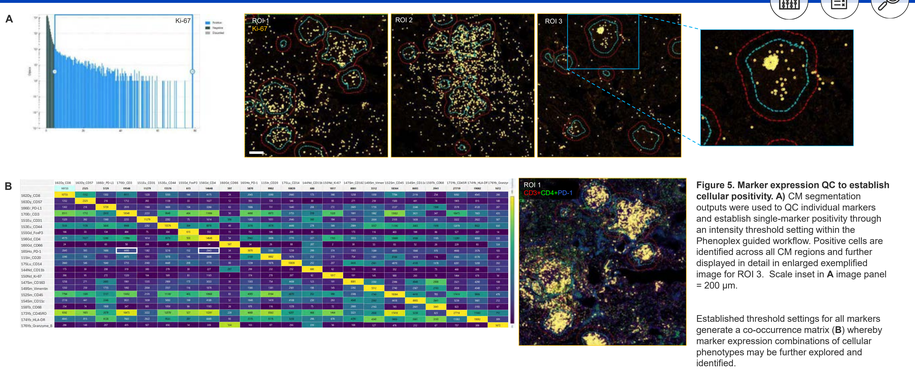


Figure 5. Marker expression QC to establish cellular positivity. A) CM segmentation outputs were used to QC individual markers and establish single-marker positivity through an intensity threshold setting within the Phenoplex guided workflow. Positive cells are identified across all CM regions and further displayed in detail in enlarged exemplified image for ROI 3. Scale inset in **A** image panel = 200 µm.

Established threshold settings for all markers generate a co-occurrence matrix (**B**) whereby marker expression combinations of cellular phenotypes may be further explored and identified.

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Phenotype cluster exploration

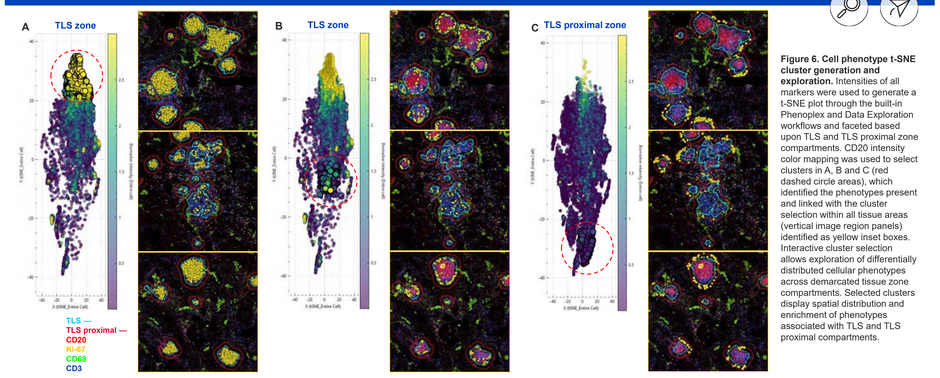

Figure 6. Cell phenotype t-SNE cluster generation and exploration. Intensities of all markers were used to generate a t-SNE plot through the built-in Phenoplex and Data Exploration workflows and faceted based upon TLS and TLS proximal zone compartments. CD20 intensity color mapping was used to select clusters in A, B and C (red dashed circle areas), which identified the phenotypes present and linked with the cluster selection within all tissue areas (vertical image region panels) identified as yellow inset boxes. Interactive cluster selection allows exploration of differentially distributed cellular phenotypes across demarcated tissue zone compartments. Selected clusters display spatial distribution and enrichment of phenotypes associated with TLS and TLS proximal compartments.

Cell population analysis

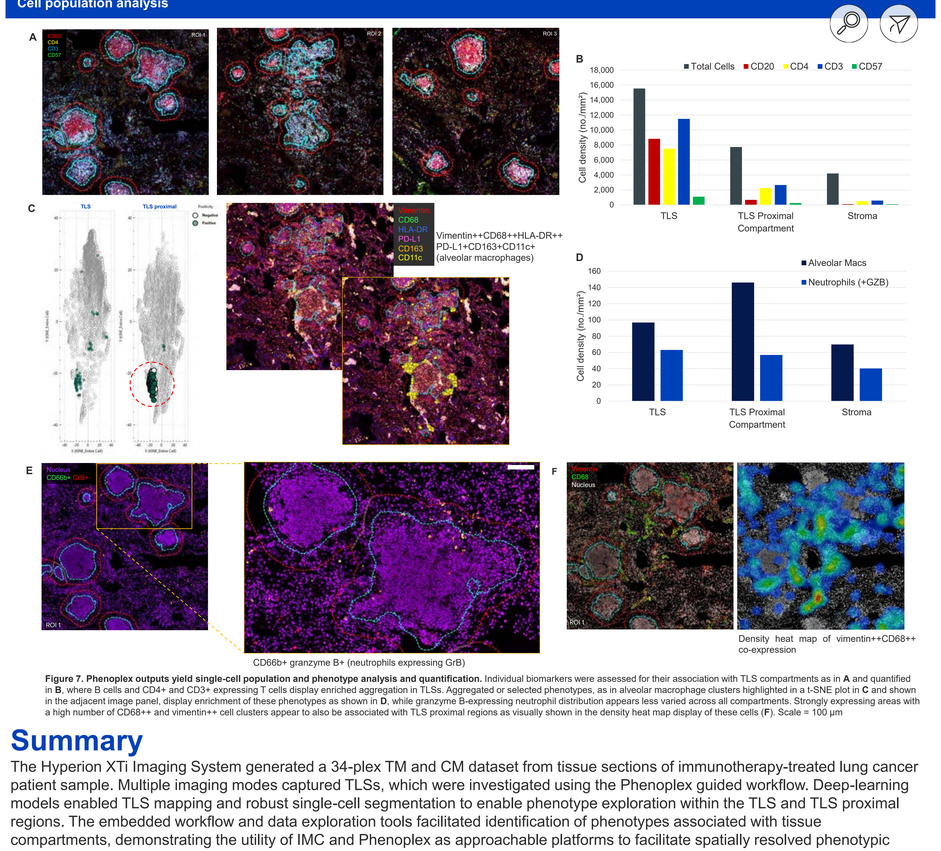

Figure 7. Phenoplex outputs yield single-cell population and phenotype analysis and quantification. Individual biomarkers were assessed for their association with TLS compartments as in **A** and quantified in **B**, where B cells and CD4+ and CD3+ expressing T cells display enriched aggregation in TLSs. Aggregated or selected phenotypes, as in alveolar macrophage clusters highlighted in a t-SNE plot in **C** and shown in the adjacent image panel, display enrichment of these phenotypes as shown in **D**, while granzyme B-expressing neutrophil distribution appears less varied across all compartments. Strongly expressing areas with a high number of CD68++ and vimentin++ cell clusters appear to also be associated with TLS proximal regions as visually shown in the density heat map display of these cells (**F**). Scale = 100 µm

Summary

The Hyperion XTi Imaging System generated a 34-plex TM and CM dataset from tissue sections of immunotherapy-treated lung cancer patient sample. Multiple imaging modes captured TLSs, which were investigated using the Phenoplex guided workflow. Deep-learning models enabled TLS mapping and robust single-cell segmentation to enable phenotype exploration within the TLS and TLS proximal regions. The embedded workflow and data exploration tools facilitated identification of phenotypes associated with tissue compartments, demonstrating the utility of IMC and Phenoplex as approachable platforms to facilitate spatially resolved phenotypic studies in complex human tissue samples

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