

Introduction

The growth in cancer immunotherapy agents requires an understanding of the immune contexture of the tumor microenvironment (TME). This can be aided by high-plex imaging and analysis to obtain phenotypes of specific cells and study their biodistribution and interactions. Imaging Mass Cytometry™ (IMC) is the method of choice for single-step staining and high-plex imaging of tissues, avoiding the complications of autofluorescence and cyclic imaging.

IMC has expanded its capabilities with three distinct imaging modes: Preview, Cell, and Tissue. The Preview Mode is a rapid scanning system that captures a comprehensive overview of the stained tissue, mapping out the distribution of over 40 markers and revealing tissue heterogeneity. This enables researchers to make informed decisions about which areas warrant closer examination on the same. Building on this, Cell Mode offers high-resolution imaging for detailed analysis of the Regions of Interest (ROIs) identified during Preview, all using the same slide. Tissue Mode complements these by providing a fast acquisition of the entire tissue at a lower resolution, which is optimal for quantitative pixel-based analysis of tissue biology. These modes support automated, continuous imaging of more than 40 large tissue samples (400 mm²) weekly. Following Preview Mode, the selection of ROIs for high-resolution imaging is a critical step, enhanced by automated AI algorithms to ensure it is informed by biomarker expression.

Methods and Materials

Tissue sections of colon adenocarcinoma were stained with a 30-marker IMC panel of structural, tumor, stroma, immune cell, and immune activation markers. Images were acquired on Hyperion XTI™ (Standard BioTools), first in Preview Mode, then in Cell Mode with automatic selection of ROIs using Phenoplex™ (Visiopharm). ROIs were automatically selected based on two criteria: 1) actively proliferating tumor regions; 2) cold and hot tumor regions as identified by hotspots of lymphoid and myeloid immune markers within stromal or epithelial tumor regions. A serial section stained with the same panel, was acquired in Tissue mode.

Single cell analysis of the high-resolution images obtained in Cell Mode, was performed as a multi-step workflow using Phenoplex. Tissue segmentation was used to divide the tissue into normal stroma, normal epithelium, tumor stroma and tumor epithelium; cell segmentation was based on Iridium DNA channels; and phenotyping was performed using the guided highplex workflow. These data were used to compare the immune contexture through a series of t-SNE plots partitioned by spatial region and clinical variables.

Results

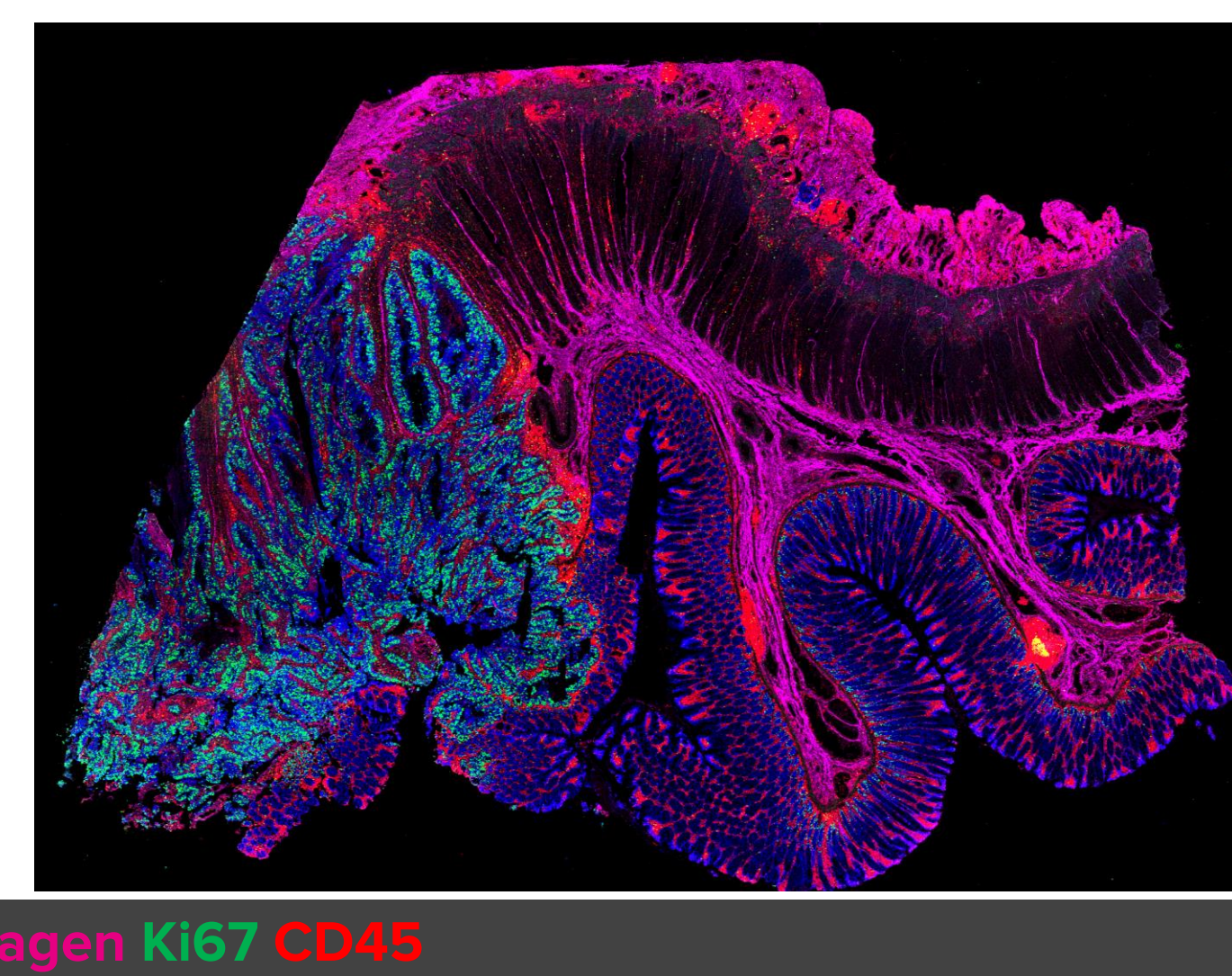
Whole slide imaging with IMC

Here we show whole slide imaging of Colon Adenocarcinoma tissue using Preview and Tissue modes of IMC. Heat maps generated by Phenoplex from Preview mode, were used to guide ROI selection for acquisition in Cell mode. Tissue phenotypic signatures of the TME were uncovered through the determination of immune cell types found in the vicinity of cancerous cells, using Phenoplex.

A Preview Mode IMC image



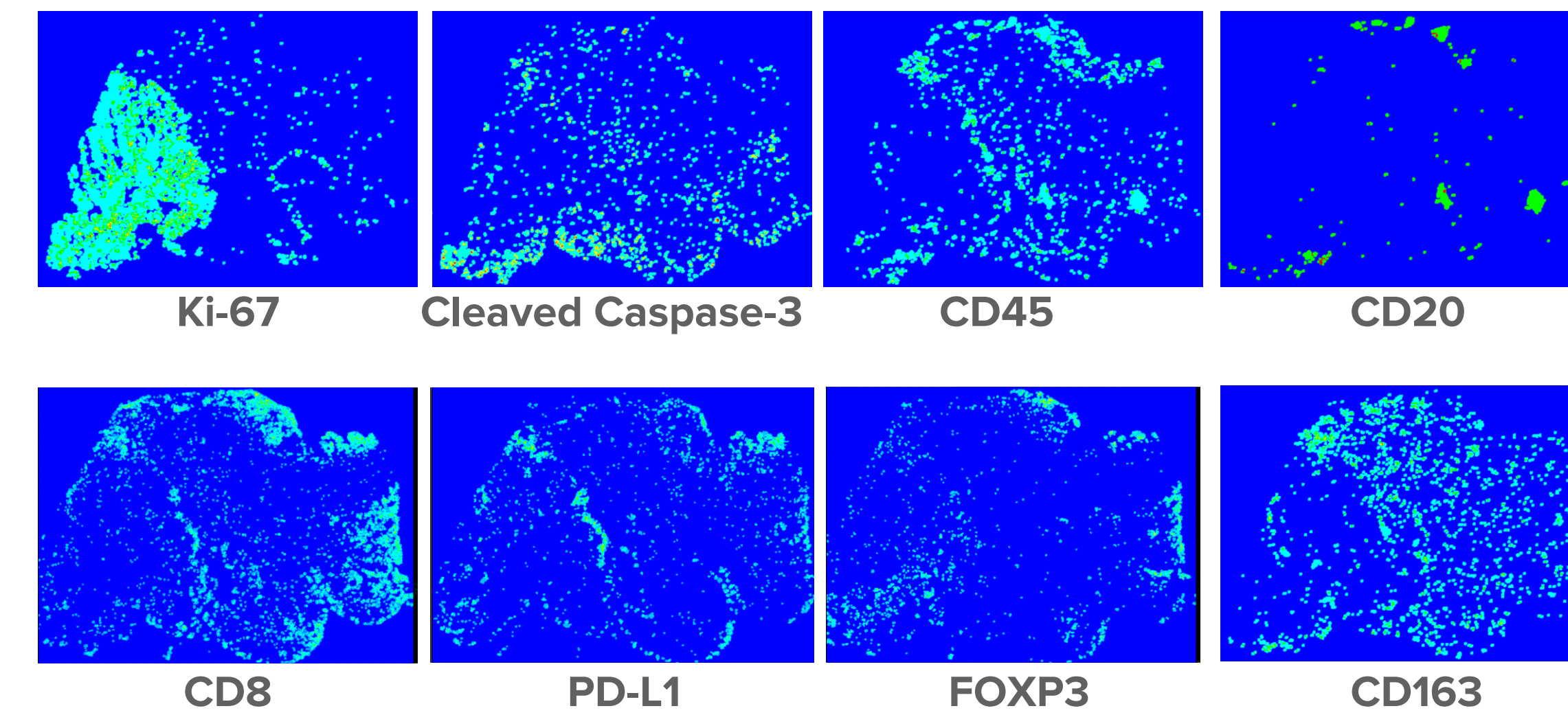
Tissue Mode IMC image



E-Cadherin Collagen Ki67 CD45

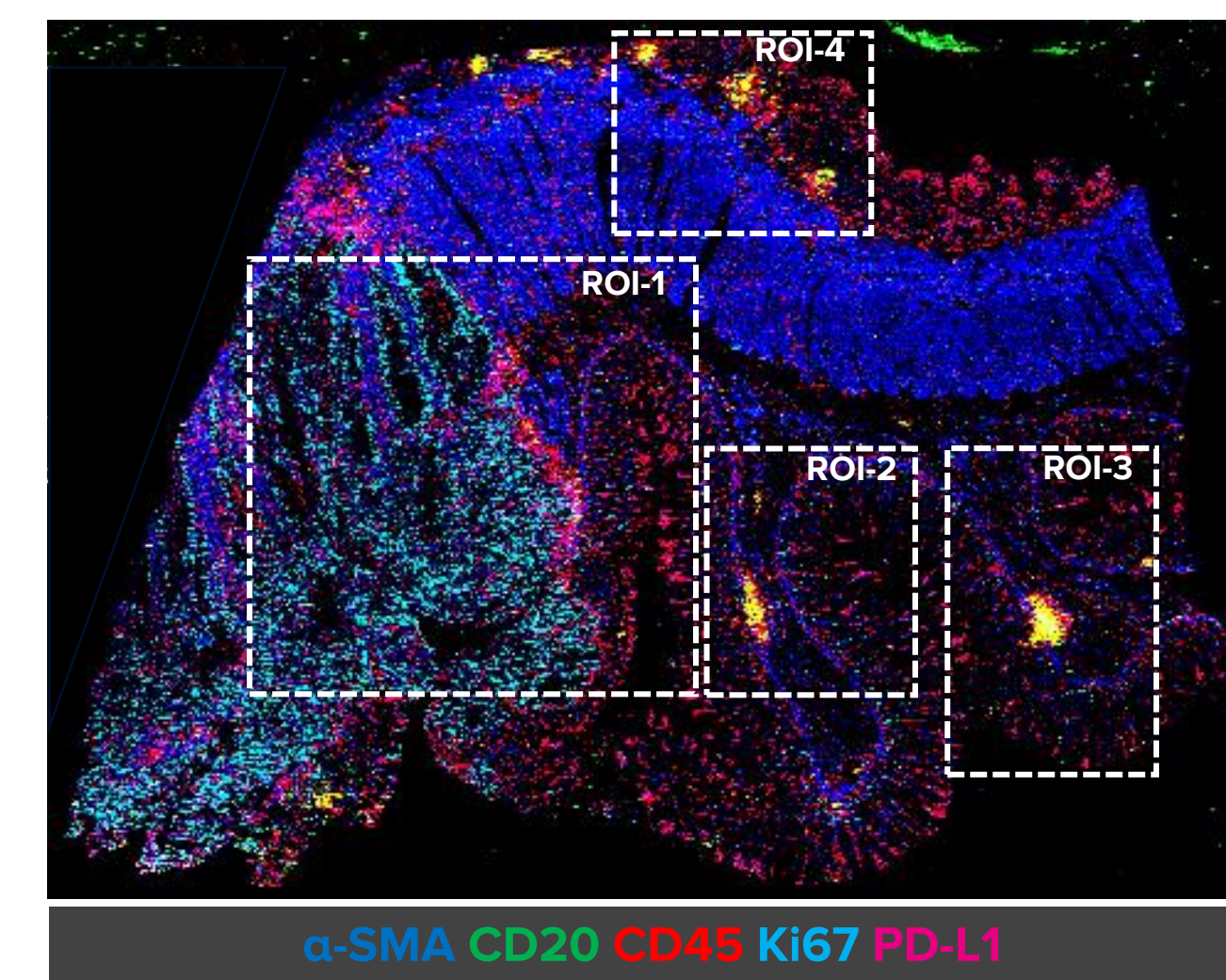
A) Preview mode (1 μm resolution, 25 μm spacing) generates a whole-slide preview of the tissue sample in minutes (using sub-sampling) to visualize all stained markers. It is used to provide guidance on where to place ROIs and ablate the same section using Cell Mode at 1 μm resolution. Tissue mode is to visualize the whole tissue sample at 5 μm resolution within a few hours, the data can be analyzed using pixel-based analyses.

B Heat maps from Preview Mode to guide ROI selection

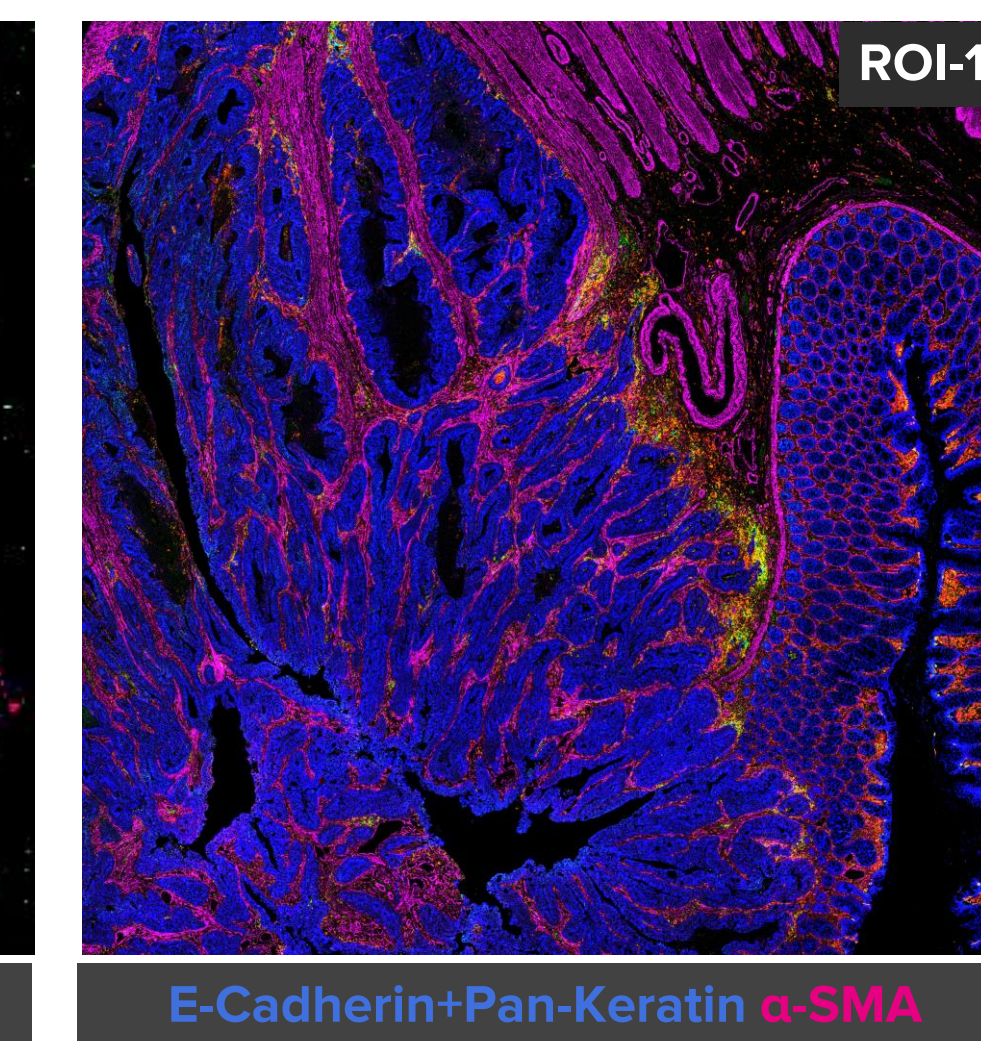


B) Preview Mode of the Hyperion XTI was used to generate a whole-slide overview IMC image of a Colon Adenocarcinoma tissue section. Heat maps of individual biomarkers were generated by Phenoplex. These heatmaps were used to guide the ROI selection for high-resolution acquisition in Cell mode. Heatmaps for Proliferation (KI-67), cell death (CC3), and immune phenotypic signatures (CD45, CD8, CD20, PD-L1, FOXP3, CD163) of the TME were generated to guide the ROI placement.

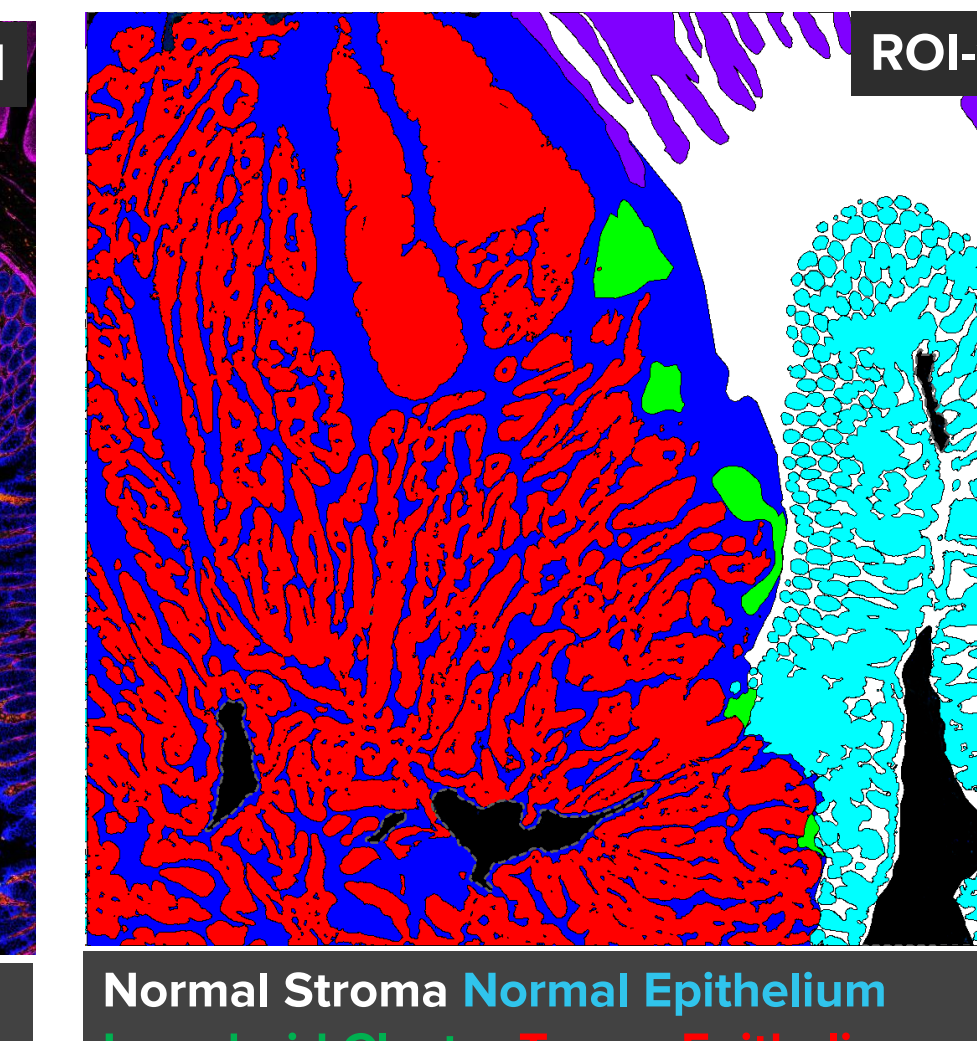
C Heatmap-guided ROI selection in preview mode



D Cell Mode IMC image

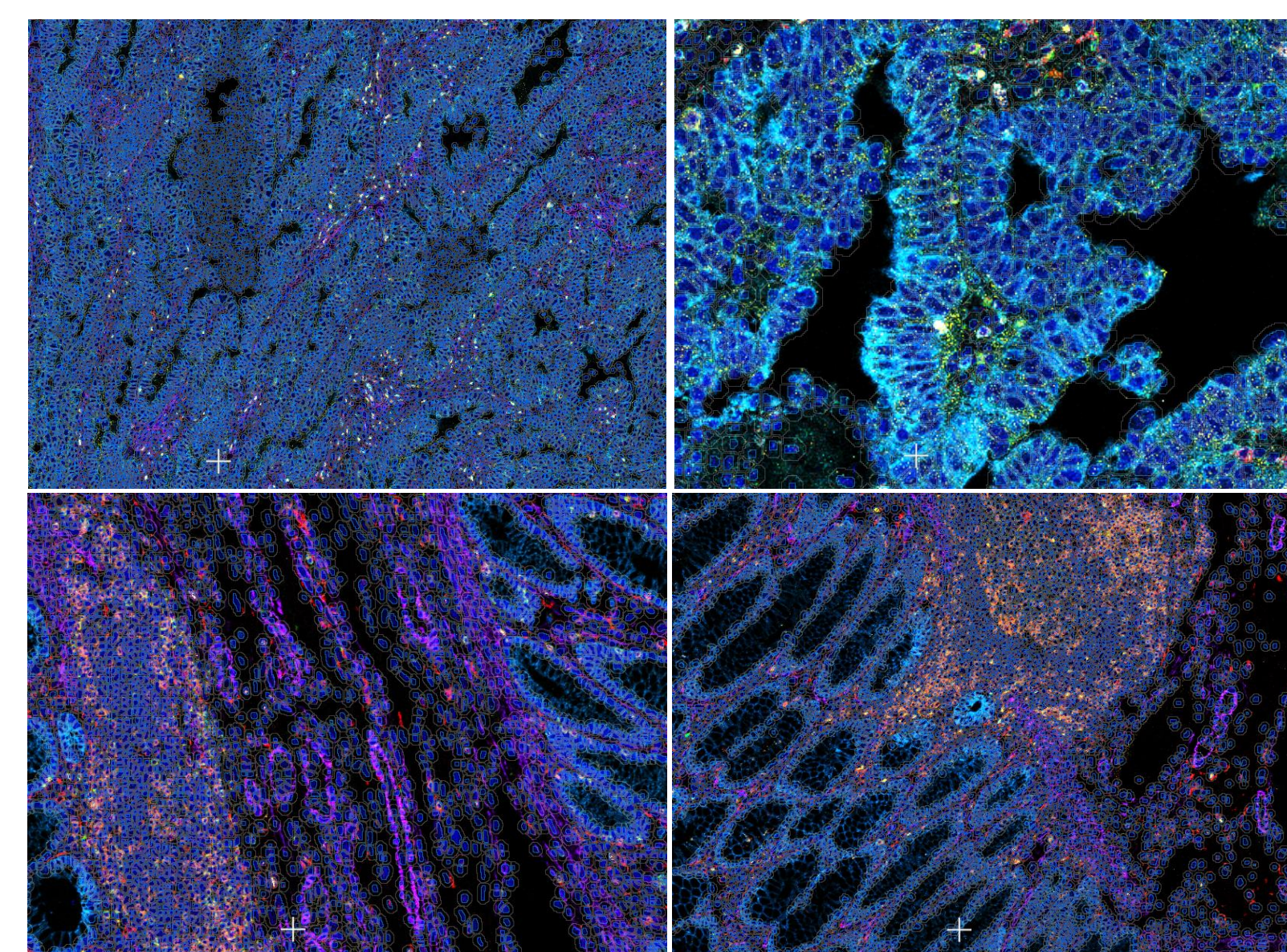


E AI-based tissue segmentation

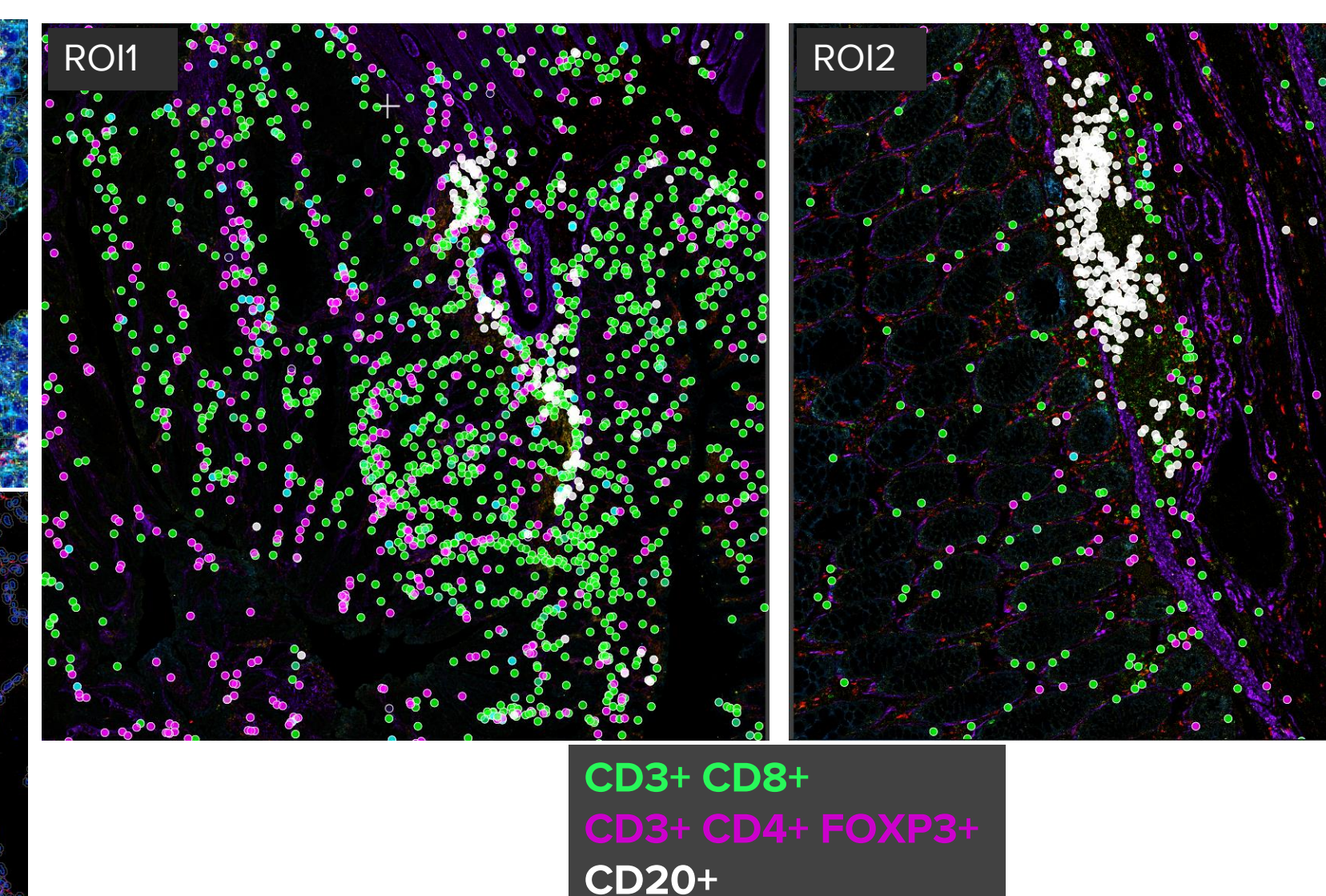


C) Whole slide imaging of a Colon Adenocarcinoma in Preview Mode with overlaid ROIs (1 – 4) guided by biomarker heatmaps as generated in Figure B, using Phenoplex. **D)** ROI-1 acquired on the same section in Cell Mode at 1 μm resolution is depicted here **E)** AI-based tissue segmentation of ROI-1 for differentiation of stroma and epithelial areas, both tumor and normal, was trained using E-Cadherin, DNA1, and COL1 channels as input for the deep learning network. Smooth muscle segmentation was guided by α-SMA positivity, and lymphoid clusters were added as additional segmentations for more detailed analysis options.

F Cell segmentation

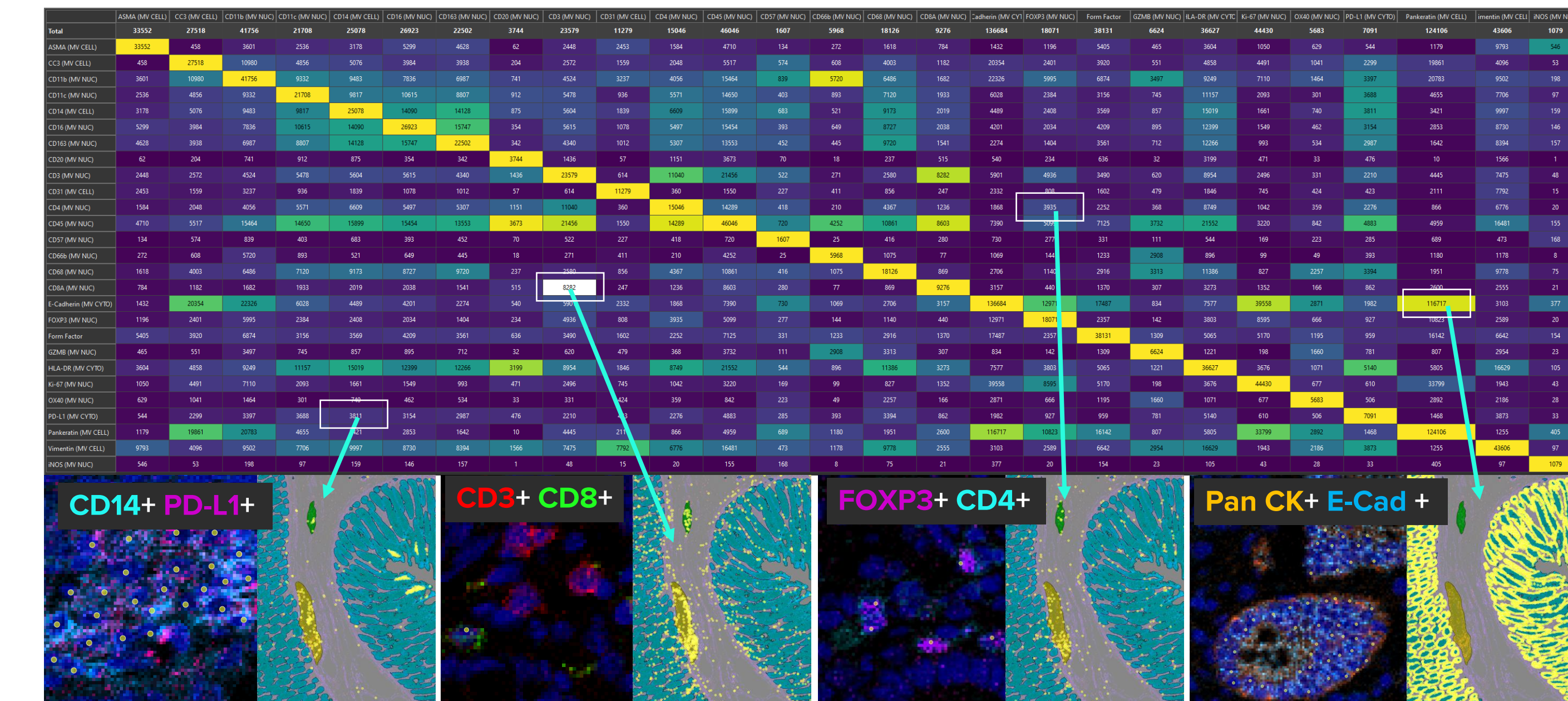


G Phenotyping



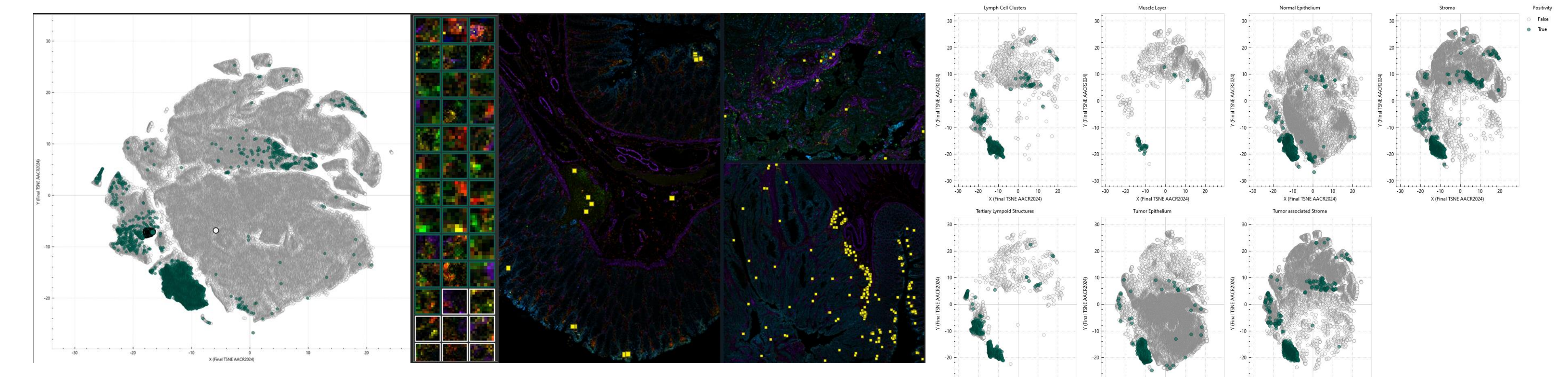
F) Visiopharm's pretrained Deep Learning based Cell Segmentation algorithm for IMC, trained on DNA1 and DNA2 as input channels, was applied to all cell mode images. A postprocessing step added a cytoplasmic compartment to the nuclei. This generates three distinct outputs for phenotyping, providing users the option to select nuclear, cytoplasmic/membrane, and whole-cell object-based outputs for determination of object positivity (e.g., phenotype). **G)** Examples of generated phenotypes are shown as overlays on ROI-1 and ROI-2. CD3+CD8+ (green), CD3+CD4+FOXP3+ (magenta), CD20+ (white). Phenotypes can be visualized directly from the Phenoplex workflow allowing users to investigate distribution of cell populations across multiple samples simultaneously.

H Cellular phenotypes in the Interactive Co-occurrence matrix



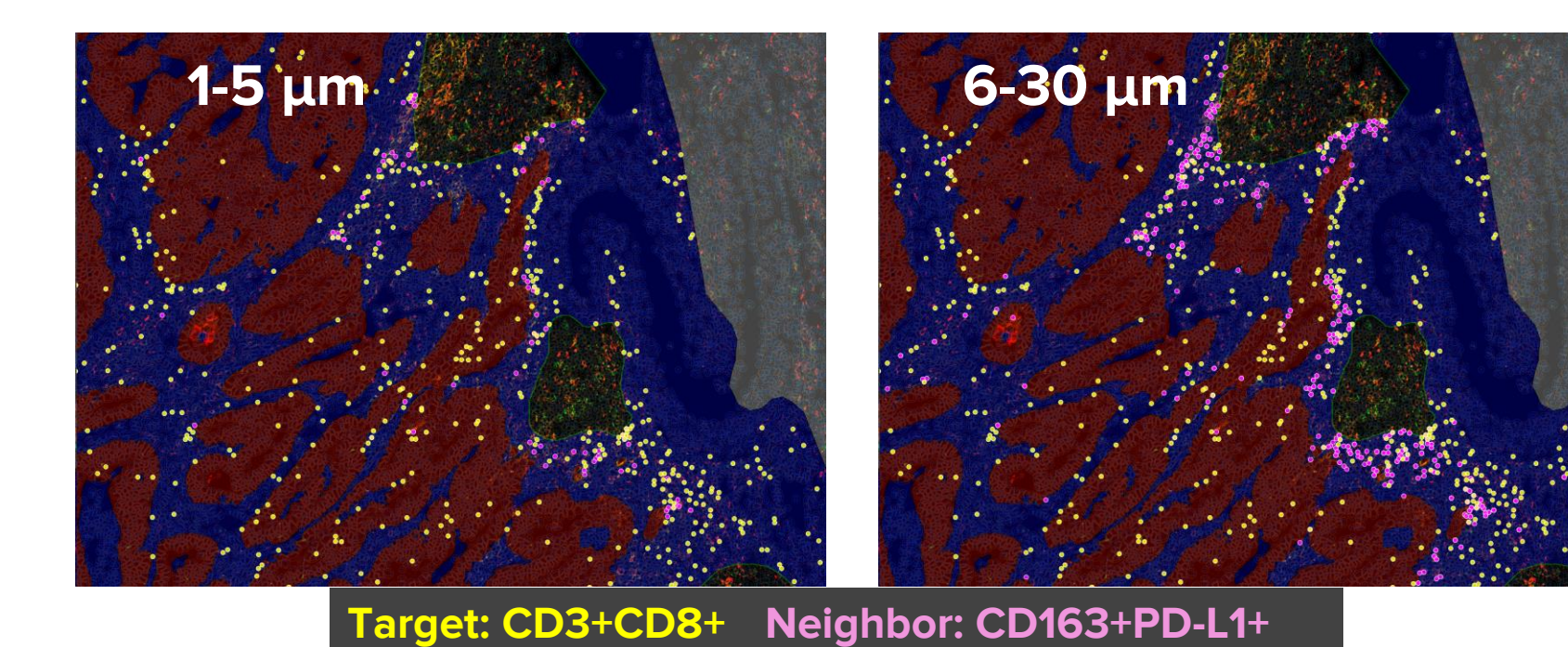
H) The Co-Occurrence matrix provides users a means to overview paired biomarker combinations. Each biomarker is represented as a column and a row. Co-occurrences are normalized to each column's total count of positive objects. Users can investigate interactively the co-localization of the gated biomarker pairs by selecting the relevant table cells. The double positive cell objects are highlighted on all images and can be investigated by the users. Here we show examples for immune populations and epithelial cells in the adjacent normal ROI-2. Yellow dots represent double-positive objects on the images.

I Interactive data exploration using t-SNE plots and cell object gallery



I) Generated cell objects were plotted as a tSNE using the built-in Data plotting capabilities of Phenoplex. All data visualization plots are fully interactive and allow users to color objects (green = positive objects for CD3+CD8+), review selected objects in an object gallery, and display selected objects across all open images. Additional options allow to split (facet) generated plots by any variable or metadata; here data were split by ROI names.

J Spatial analysis tools



J) Phenoplex Neighbor Count analysis enables users to define specific targets as center points and neighbors based on the positivity gates of the objects and quantify the number of neighbors around targets. Here we show the analysis of targets CD3+CD8+ (yellow dots) and neighbor counts for immune-suppressive macrophages CD163+PD-L1+ double positive cell objects (magenta dots) specifically within the tumor microenvironment (blue and red regions). Analysis was performed at two distinct distance bands left: 1-5 μm and right: 6-30 μm distance from target cell objects.



Here we show region-specific analysis and visualization options of the Neighbor Count analysis for FOXP3+CD4+CD3+ (Tregs, magenta dots) in the vicinity of CD8+CD3+ (Cytotoxic T-cells, yellow dots) within the Tumor microenvironment (left image) and the adjacent normal tissue (right image). The data table provides counts of the number of adjacent neighbor cell objects as mean with standard deviation.

Conclusions

- The multi-modal features of Hyperion XTI can greatly accelerate the ability of IMC users to gain useful insights from complex biological samples.
- Phenoplex enables a comprehensive workflow for the analysis of this complex data, providing automated ROI selection, phenotyping and spatial analyses of high-resolution IMC images for biological assessment.

