#7672: Automated, Flexible Multiplex Immunofluorescence for Tumor Microenvironment Profiling Using HCR™ Gold IF and Clinically-Relevant Antibody Clones

Wudy Yang[1], Randy Chen[1], Harry Choi[1], Aneesh Acharya[1], Anne Hellebust[2], Will Howat[3]
[1]Molecular Instruments; [2]Indica Labs; [3]Abcam

BACKGROUND AND OBJECTIVES

Multiplex immunofluorescence (mIF) is widely used for tumor microenvironment profiling, but many workflows remain constrained by fixed panels, antibody modification requirements, harsh stripping steps, and assay conditions that can compromise tissue integrity. These limitations can slow panel development and reduce flexibility for custom biomarker studies.

Here, we evaluated an end-to-end workflow combining MI's HCR™ Pro IHC and HCR™ Gold IF assays with Abcam's translationally relevant antibody clones on the Leica Biosystems BOND RX Research Staining System, followed by quantitative image analysis in Indica Labs' HALO platform for flexible tumor microenvironment profiling in FFPE tissues.

WORKFLOW AND METHODS

FFPE sections from healthy colon and colorectal cancer (CRC) tissues were stained on the Leica Biosystems BOND RX Research Staining System using an automated HCR™ workflow. Abcam primary antibodies were first evaluated in single-plex format by comparing HCR™ Pro IHC with the Leica Biosystems BOND Polymer Refine Detection on serial tissue sections, demonstrating that encoded antibody staining performed comparably to a standard IHC method. HCR™ Gold IF was then used for multiplex fluorescence staining, enabling same-species multiplexing without iterative staining.

01 Antibody Selection
Abcam clinically relevant primary antibodies selected for epithelial, proliferation, and immune readouts.

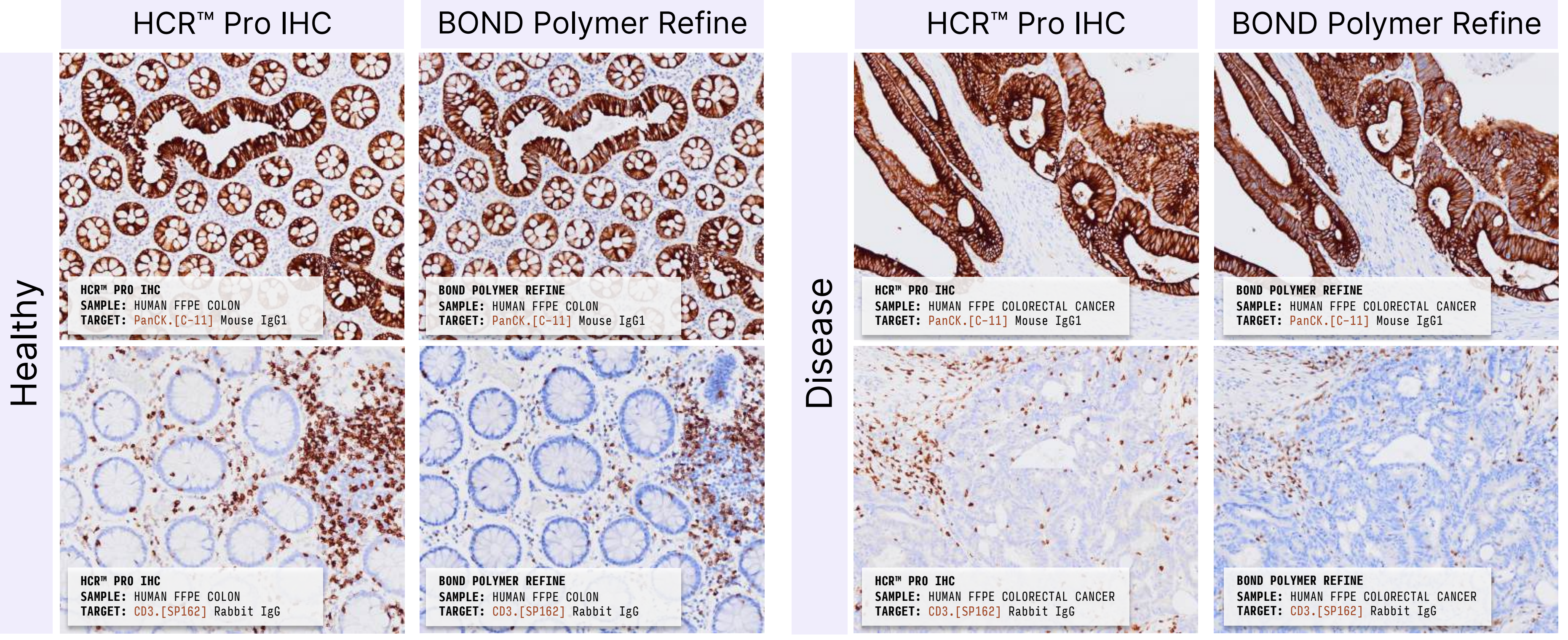
02 1-Plex Validation
HCR™ Pro IHC was compared with BOND Polymer Refine Detection on serial sections to confirm comparable staining to a standard IHC method.

03 Multiplex IF
HCR™ Gold IF on the BOND RX Research Stainer enables same-species multiplex fluorescence staining without iterative cycles.

04 Image Acquisition
Whole-slide imaging of healthy colon and colorectal cancer tissues after automated staining.

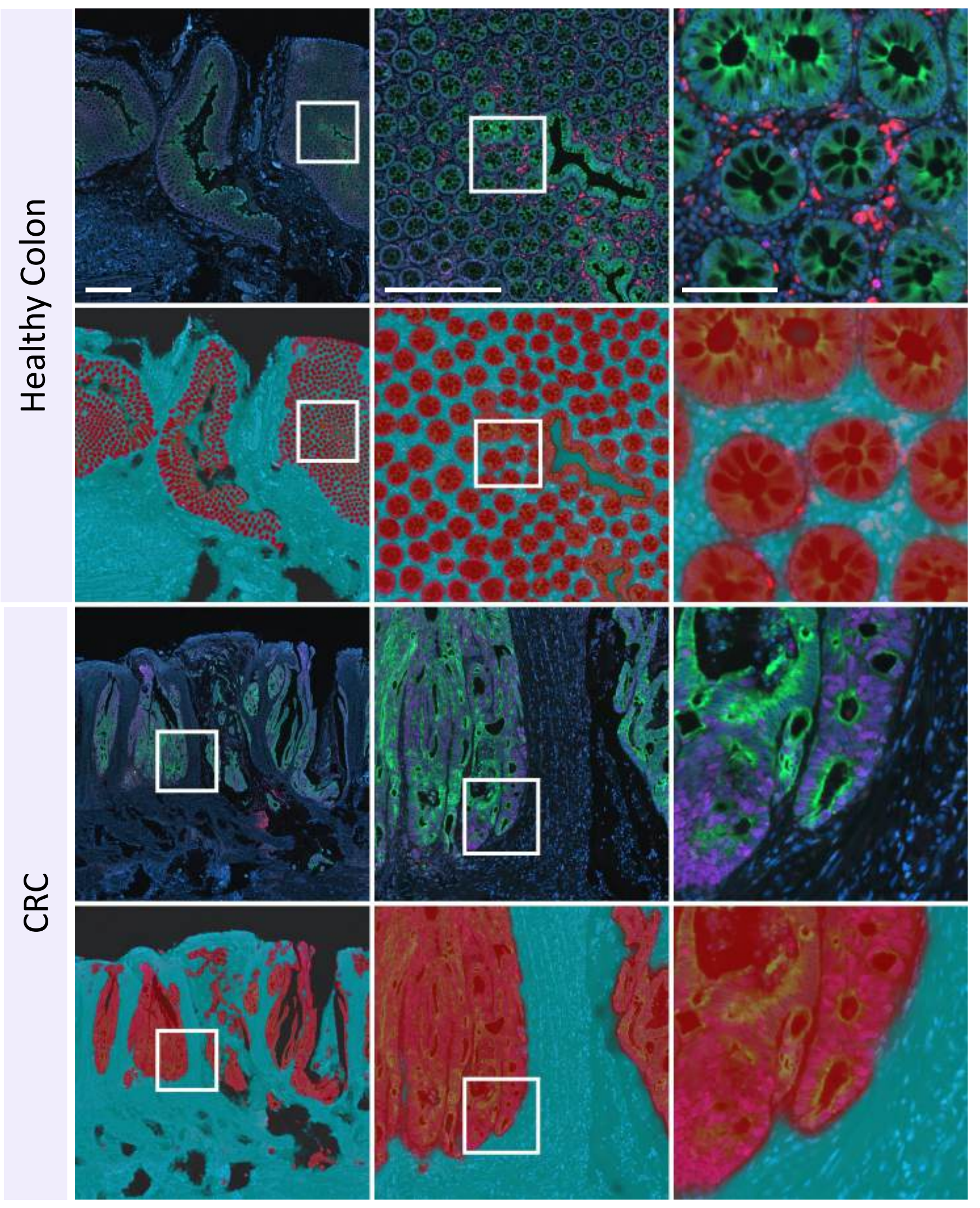
05 HALO Analysis
Segmentation, phenotyping, biomarker quantification, and spatial analysis across tumor and stromal regions.

1-PLEX VALIDATIONS

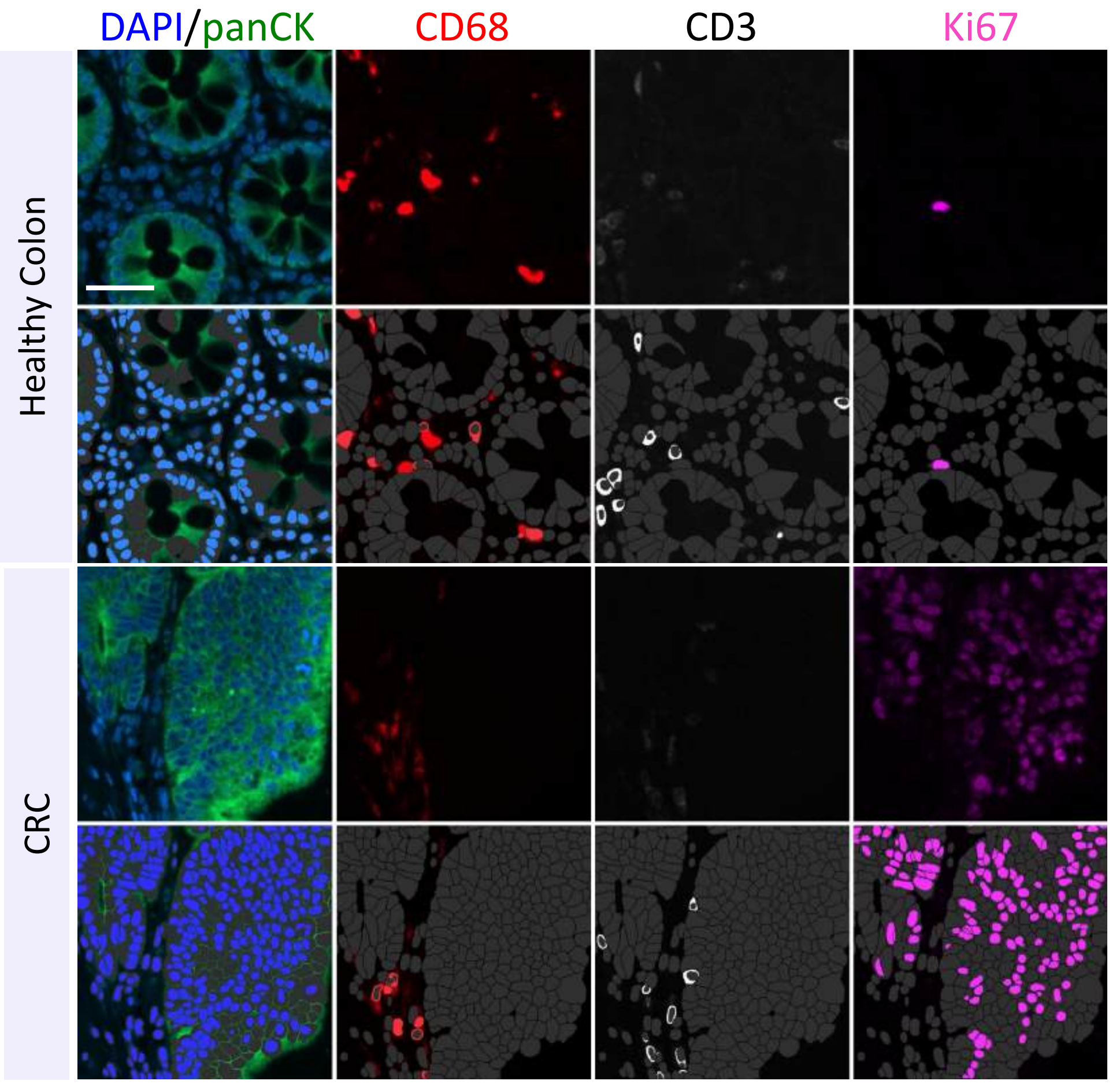


Representative single-plex staining of Abcam antibody targets using HCR™ Pro IHC and Leica Biosystems BOND Polymer Refine Detection on serial tissue sections demonstrates comparable signal pattern and tissue localization across both healthy and disease tissue. These results support the use of HiFi Encoder-based detection for antibody staining and establish a single-plex foundation for subsequent HCR™ Gold IF multiplex panel development.

IMAGE ANALYSIS: SEGMENTING & PHENOTYPING



Tissue Segmentation: Healthy colon and colorectal cancer tissue shown without (row 1,3) and with (row 2,4) HALO AI tissue segmentation overlay at 1X (left column), 5X (middle column), and 20x (right column). Scale bars are 1 mm, 500 µm, and 100 µm, left to right. Stromal area is cyan and epithelial and tumor regions are red.



Cell Segmentation and Thresholding: HALO AI cell segmentation and protein expression quantification is shown in healthy colon and colorectal cancer tissue. Input images for healthy and cancer colon tissue are shown in rows 1 and 3, respectively. The DAPI and panCK channels were leveraged to create custom nuclear and membrane segmentation networks. Input channels for CD68, CD3, and Ki67 are displayed above individual channel overlays for each respective channel. Negative cells are represented as grey and positive cells for each biomarker are represented as their respective color overlay. Scale bar is 50 µm.

Phenotyping: Once segmented, individual cells were designated to specific cell phenotypes based off biomarker expression. Phenotypes were created based off the definitions in the table below.

Phenotype	Definition	Biologic focus
Tumor cells	panCK+	Epithelial compartment
Proliferating tumor	panCK+ Ki67+	Ki67 index
T cells	CD3+ panCK-	Immune infiltration
Macrophage signal	CD68+	Myeloid compartment

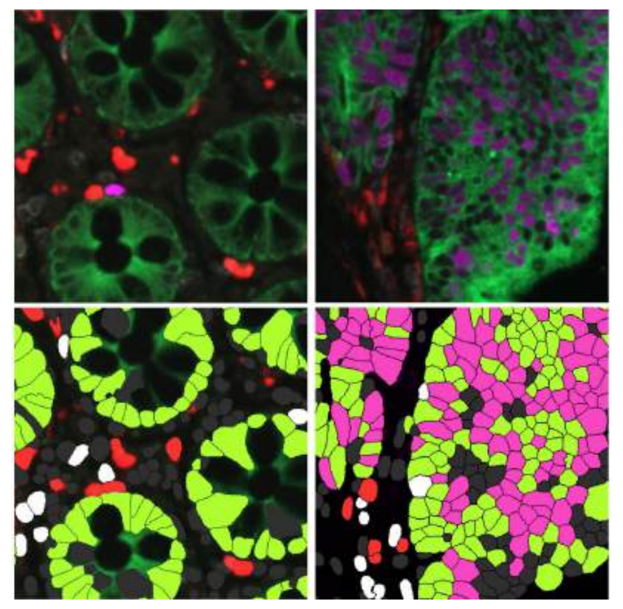
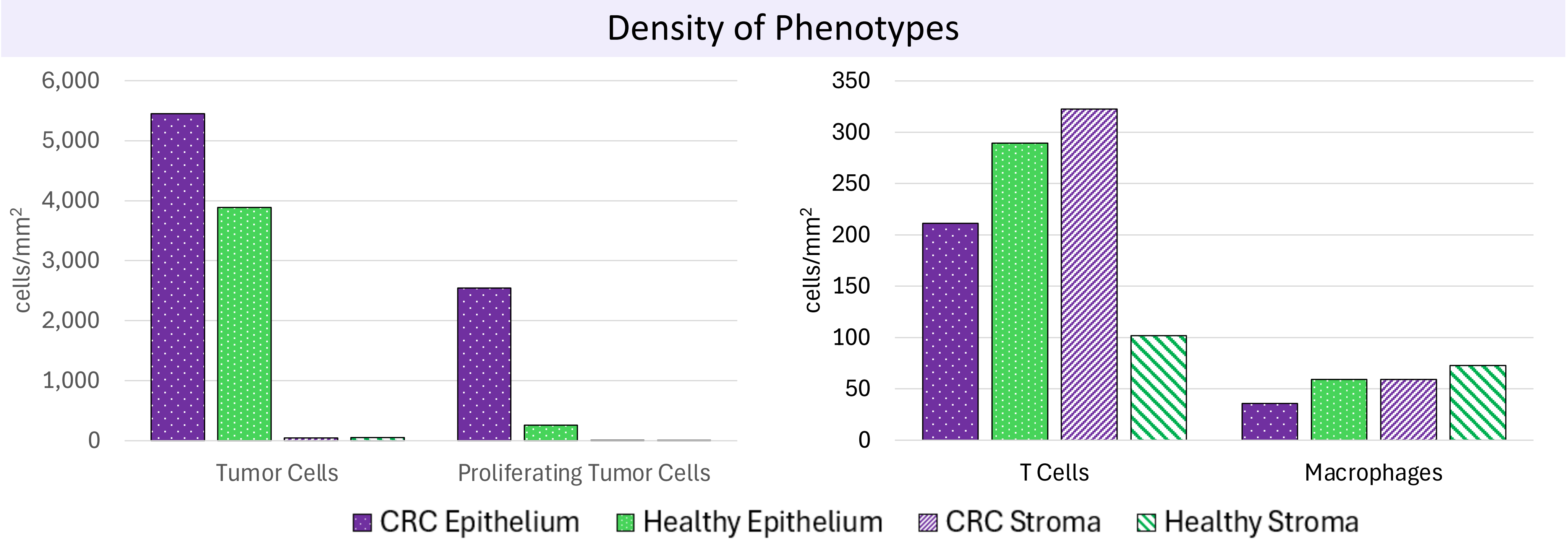
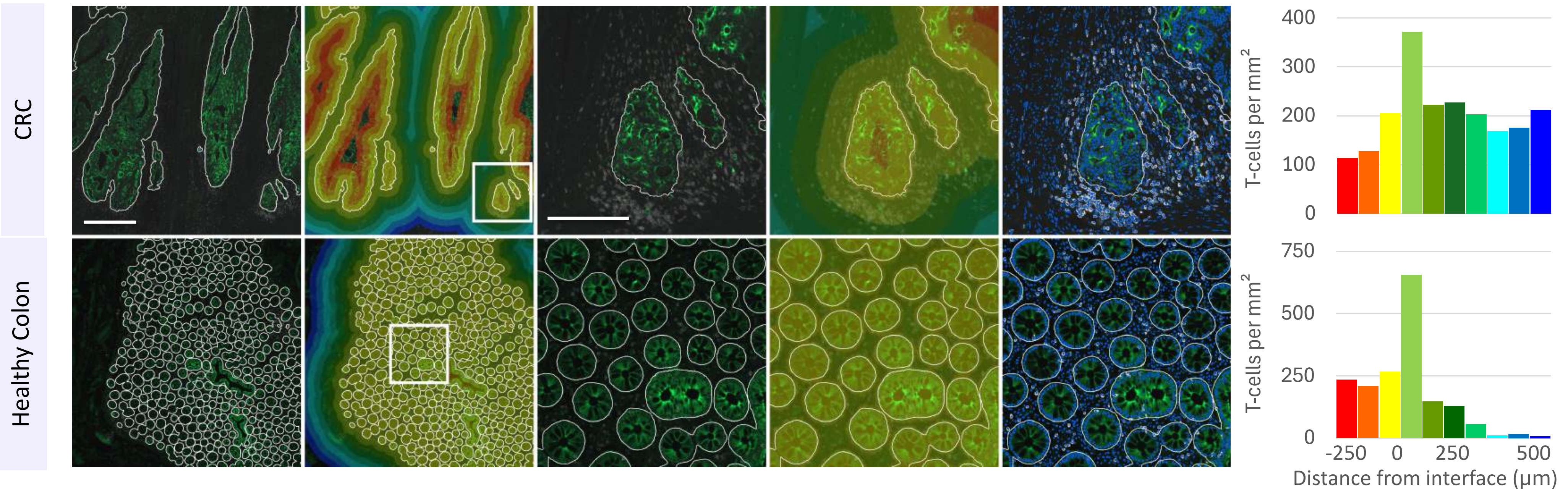


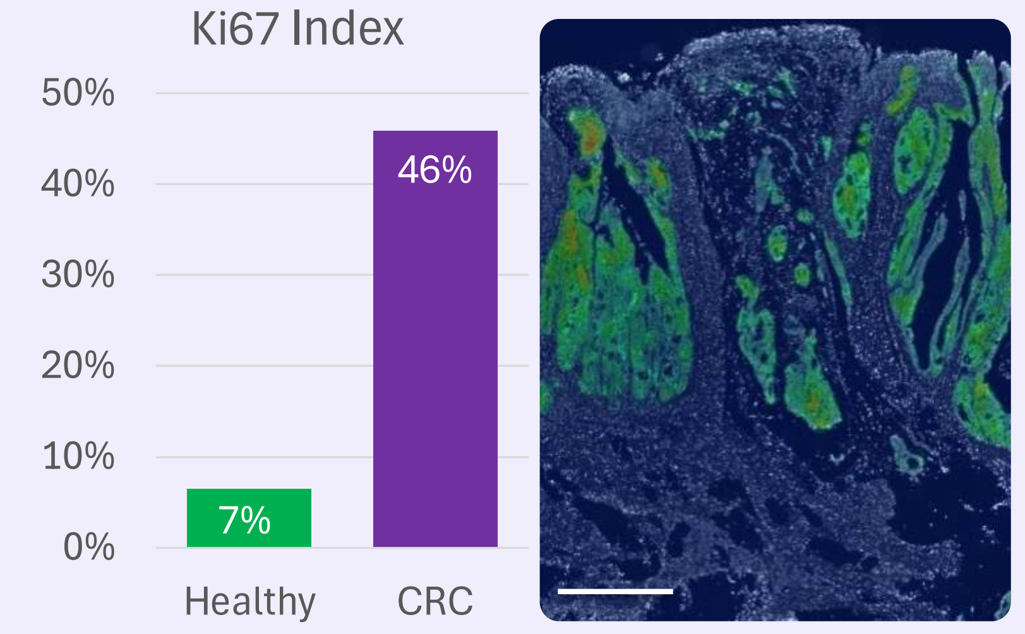
IMAGE ANALYSIS: HEALTHY VS. TUMOR



Phenotype Density: After segmenting and phenotyping cells in the tissue samples, cell densities per tissue type and per tissue compartment were calculated. Tumor and proliferating tumor cells are enriched in CRC epithelium compared to healthy epithelium, while stromal compartments show minimal tumor cell presence. In contrast, immune populations (T cells and macrophages) are more abundant in stromal regions, particularly in CRC stroma for T cells, highlighting compartment-specific differences in tumor and immune cell distribution.



Ki67 Index: The Ki67 index measures the percentage of actively dividing cancer cells in tissue. The Ki67 index was much higher in the CRC tissue (46%) compared to the healthy tissue (7%). The HALO density heatmap shows the heterogeneous distribution of proliferating tumor cells in the CRC tissue. Scale bar is 1mm.



Spatial Analysis: HALO's infiltration spatial analysis feature was used to measure the distance of T cells from the epithelial/tumor boundary (shown in white). The tumor boundary was drawn by the tissue segmentation mask in a previous step. Representative images show tissue architecture (panCK and CD3 staining only) at 2X and 10X views to highlight differences in cellular organization between tissue types. In colorectal cancer, epithelial structures are irregular and heterogeneous, with disrupted distribution and increased immune cell infiltration at the tumor–stroma interface. In contrast, healthy colon displays well-organized crypt architecture and consistent T-cell spatial distributions. Histograms of T cell density as a function of distance from the epithelial–stromal interface highlight the altered immune localization and broader spatial dispersion in cancer compared to healthy tissue. Scale bars are 500 µm and 100 µm, respectively.

CONCLUSIONS

This study demonstrates an automated, flexible mIF workflow with Abcam antibodies that combines HCR™-based staining on the Leica Biosystems BOND RX Research Staining System with quantitative analysis in Indica Labs' HALO platform. In single-plex format, HCR™ Pro IHC produced staining patterns comparable to BOND Polymer Refine Detection, supporting the use of HiFi Encoder-based detection with biologically meaningful Abcam antibody clones. Building on that validation, HCR™ Gold IF enabled same-species mIF without iterative staining, simplifying panel development while preserving tissue morphology. Together, these results support a practical end-to-end approach for scalable tumor microenvironment profiling in FFPE tissues.

CONTACT: info@molecularinstruments.com