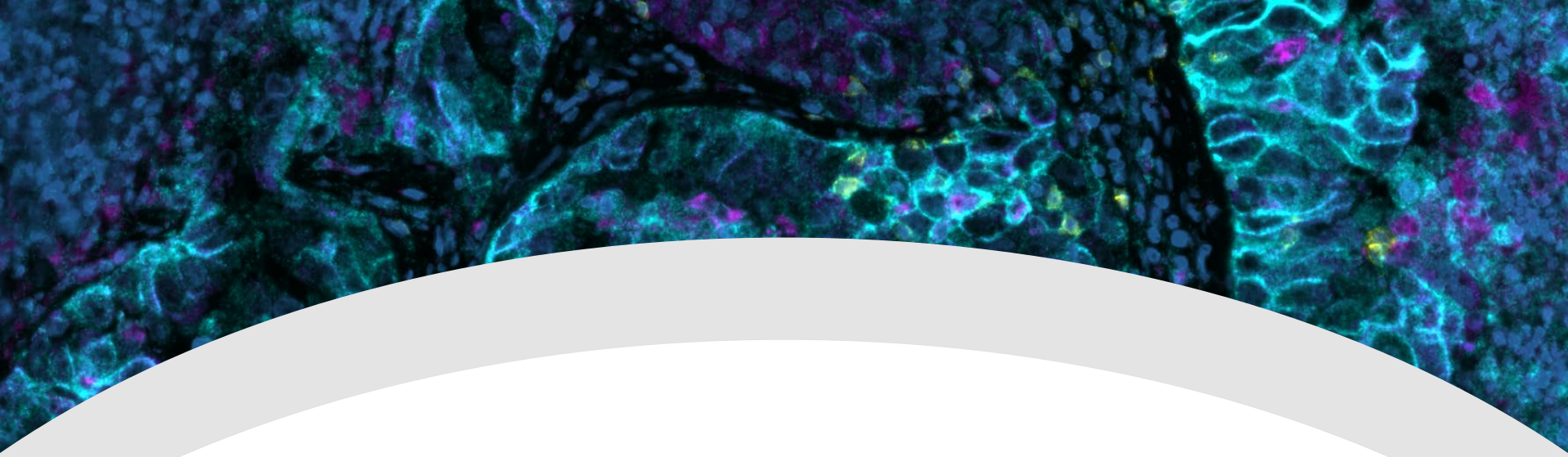

Depicting the cellular architecture of the tumor microenvironment by integrating hyperplex immunofluorescence and automated image analysis

Ines Nearchou, Joanna Kowal, François Rivest, Amber Ortiz, Doug Bowman, Benjamin Pelz, Diego Dupouy

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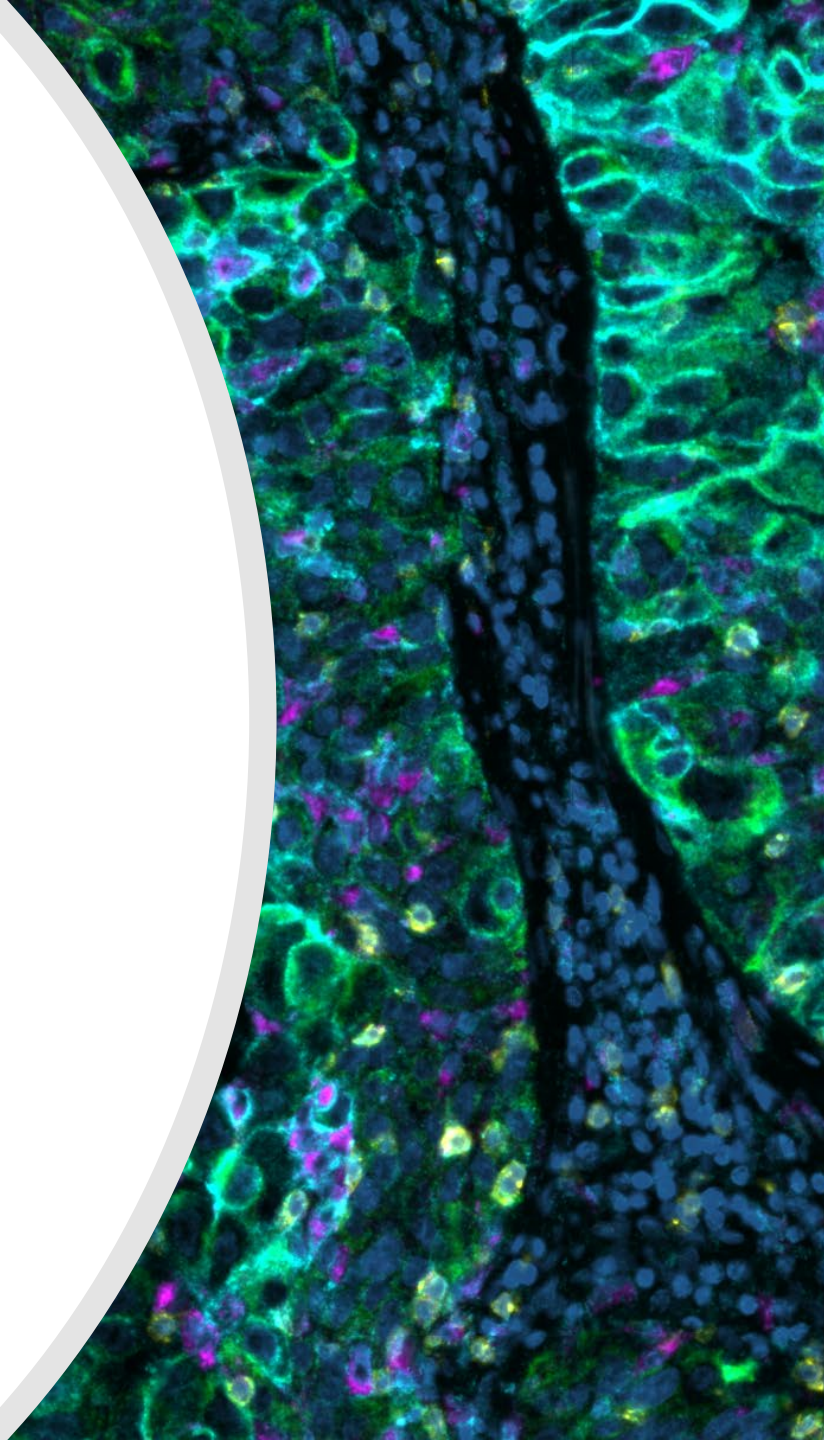
INTRODUCTION

The tumor microenvironment (TME) is emerging as an important factor that shapes the dynamic of tumor growth, heterogeneity, and response to therapies. Thus, efforts are underway to understand better the biology of cells within the TME and to provide spatial mapping of TME components and their interactions. In this study, we focus on the phenotyping of cells across different tumor types on a tissue microarray (TMA) with an immuno-oncology panel encompassing 20 biomarkers. We interrogated their TME with the use of the COMET™ automated staining and imaging system, and HALO® and HALO AI image analysis platforms.

20-plex panel includes: FOXP3, CD68, αSMA, CD31, CD38, IDO-1, s100, CD11c, PD-L1, Ki-67, CD8, PD-1, CD4, CK, CD3, CD20, CD16, HLA-DR, Vimentin, CD45



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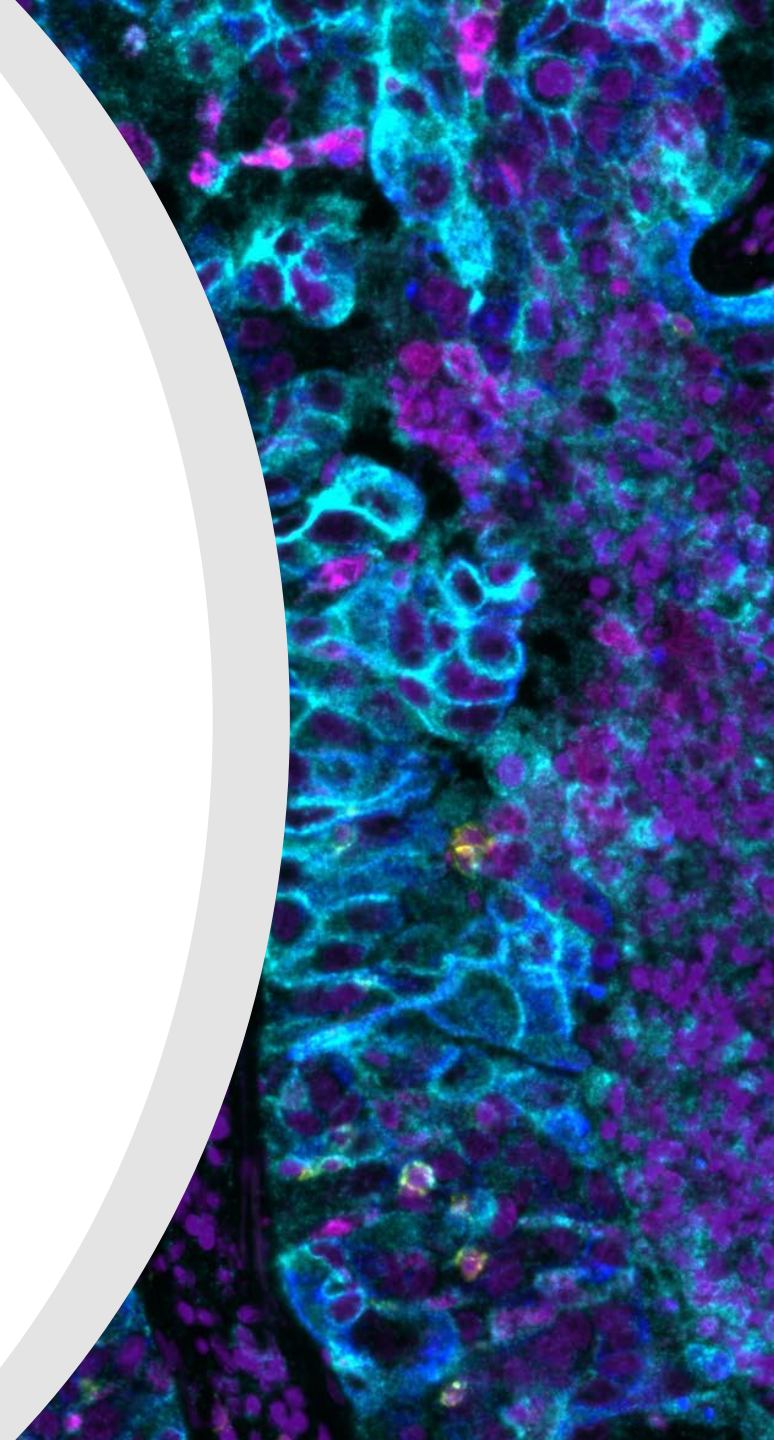
METHODS: STAINING AND IMAGING

A sequential IF (seqIF™) protocol was executed in fully automated fashion on a Lunaphore COMET™ device. A formalin-fixed, paraffin-embedded (FFPE) slide of TMA containing multiple tumor indications including invasive breast carcinoma cores and colon adenocarcinoma cores was preprocessed with the use of a PT module with dedicated reagents at pH9 (Epredia). Subsequently, an automated COMET™ protocol was created by the user. Primary and secondary antibodies were prepared offline and loaded on the machine together with proprietary buffers enabling all the steps of seqIF™ protocols: washing, imaging, quenching and elution buffers. At each cycle signal, from 2 markers and DAPI, was acquired by the integrated fluorescent microscope. A single multichannel ome.tiff image was produced as the result and delivered to the user.



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METHODS: COMET™ PLATFORM

COMET™ is an automated platform providing a slide in, data out-workflow for spatial biology. COMET™ system includes COMET™ instrument and its Control Station, which hosts the COMET Control Software and the Viewer by Lunaphore. Images are delivered as multi-stack ome.tiff files, ensuring compatibility with different image visualization and analysis tools.

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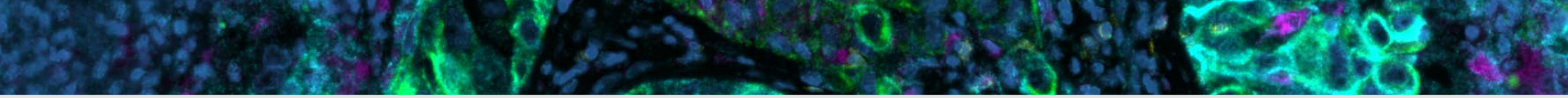
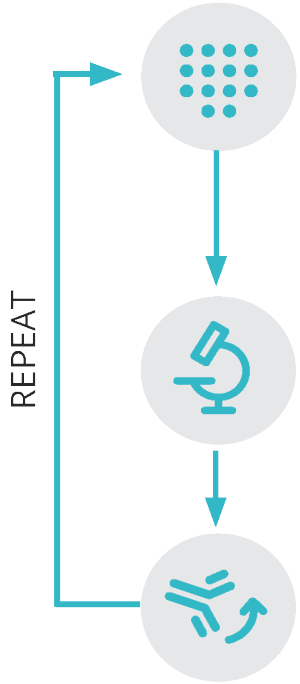


METHODS: COMET™ MULTIPLEXED IMAGING WORKFLOW



1. STAIN Controlled by an automated protocol, primary and secondary antibodies are delivered onto the tissue. User can choose to deliver **1 antibody or a mix of 2 antibodies from different species.**

2. IMAGE The **integrated microscope** then acquires the image through the window of the imaging chip.

3. ELUTE The fluorescent signal is erased by removing the primary-secondary antibody complex with a **dedicated elution buffer and temperature-controlled cycle**.

The COMET™ workflow applies the sequential immunofluorescence principle, where tissues undergo subsequent cycles of staining, imaging and antibody elution in an automated manner without the need of user intervention. COMET™ provides the possibility to perform 20 cycles per automated run, yielding a 40-plex immunostaining image. Final images are delivered ready to be analyzed and there is no need of data postprocessing from the user.

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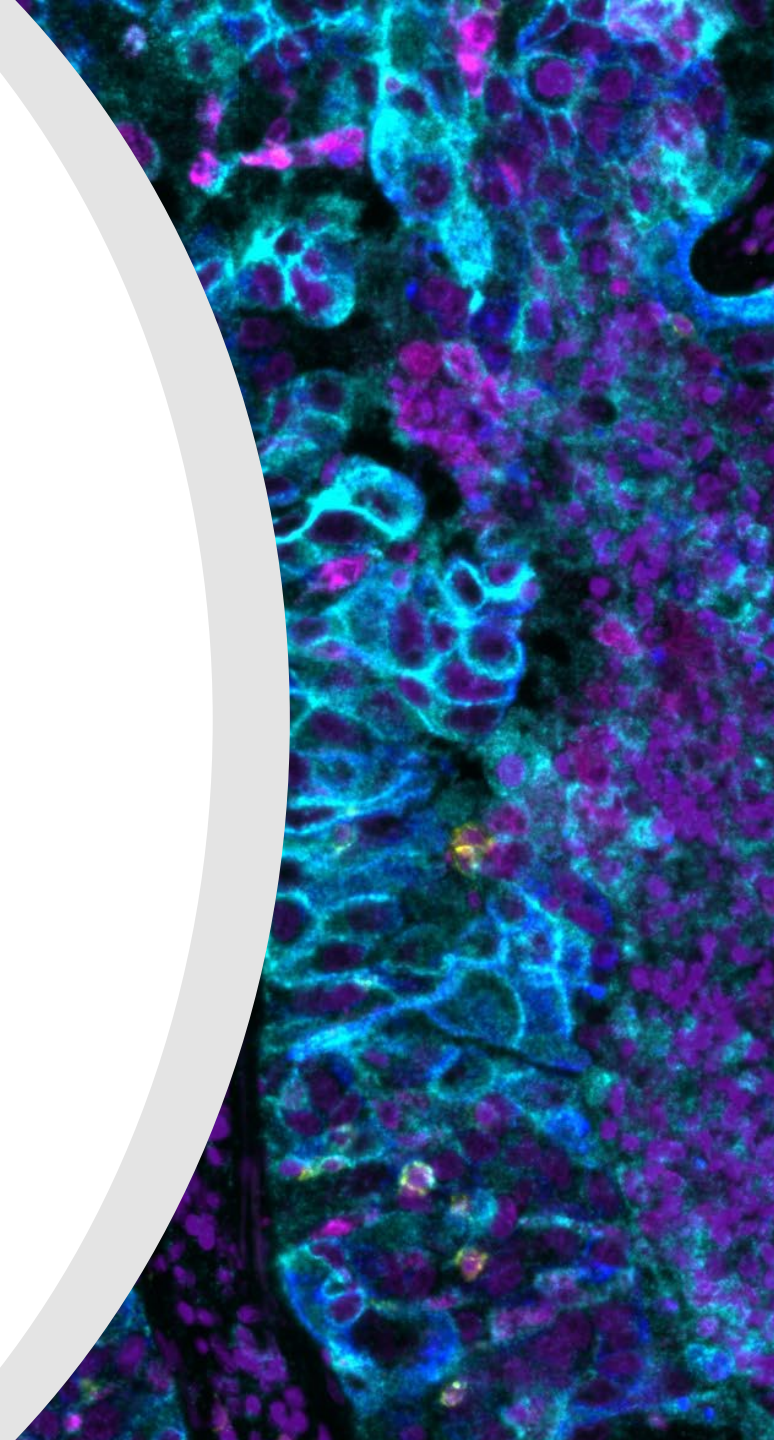
METHODS: IMAGE ANALYSIS

The image analysis workflow was performed by HALO and HALO AI. The HALO TMA add-on was used to segment cores and mark those judged invalid. Subsequently, a HALO AI-based Tissue Classifier was developed for each tissue type and was embedded in the HALO HighPlex FL module for cell-based phenotypic characterization reported by tissue class, proximity analysis, and infiltration analysis. User defined thresholds were applied to each of the biomarkers to define positivity for the appropriate subcellular localization (nuclear, cytoplasmic, and/or membrane) for phenotypic analysis.



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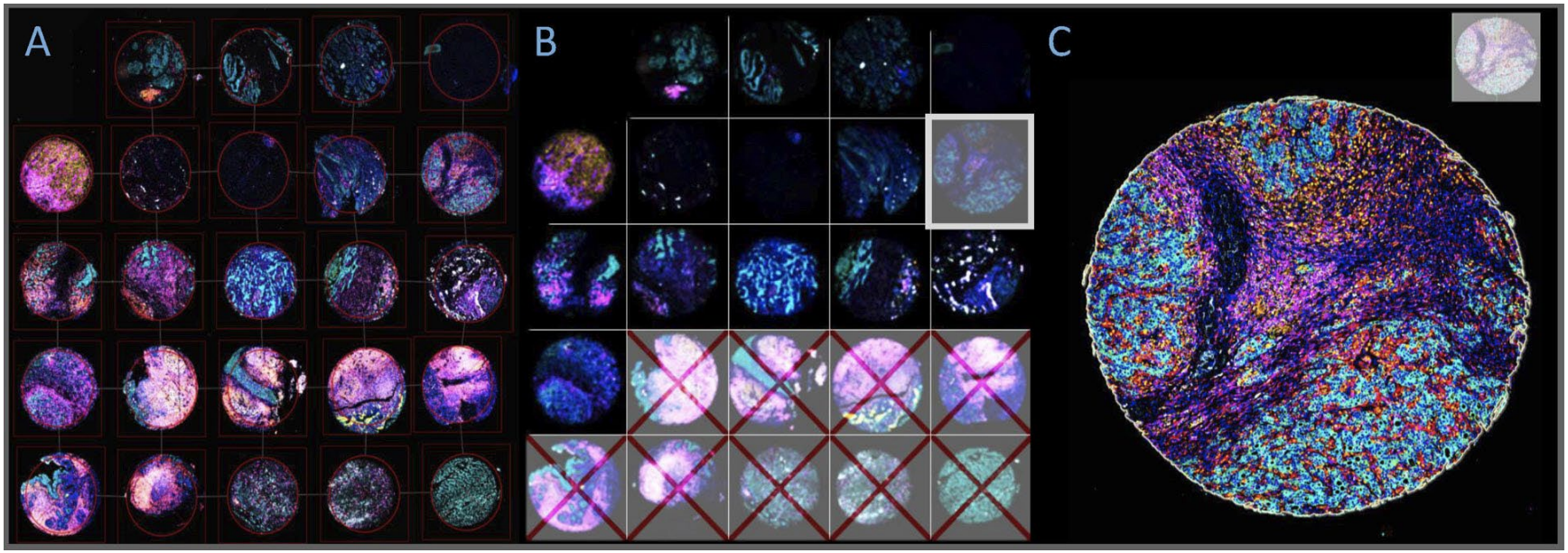
METHODS: TMA SEGMENTATION & TISSUE CORE ANNOTATION



(A) The cores are automatically detected and cropped into square boxes with the tissue core centered (red). Cores can be marked as valid/invalid. Invalid cores are demarcated by a red X through the spot overlay as depicted here for the non-tumoral tissue cores. (B) Spot array with segmented cores. (C) The individual tissue cores can be automatically annotated using the flood tool. Manual edits as well as additional exclusion annotations can be made.

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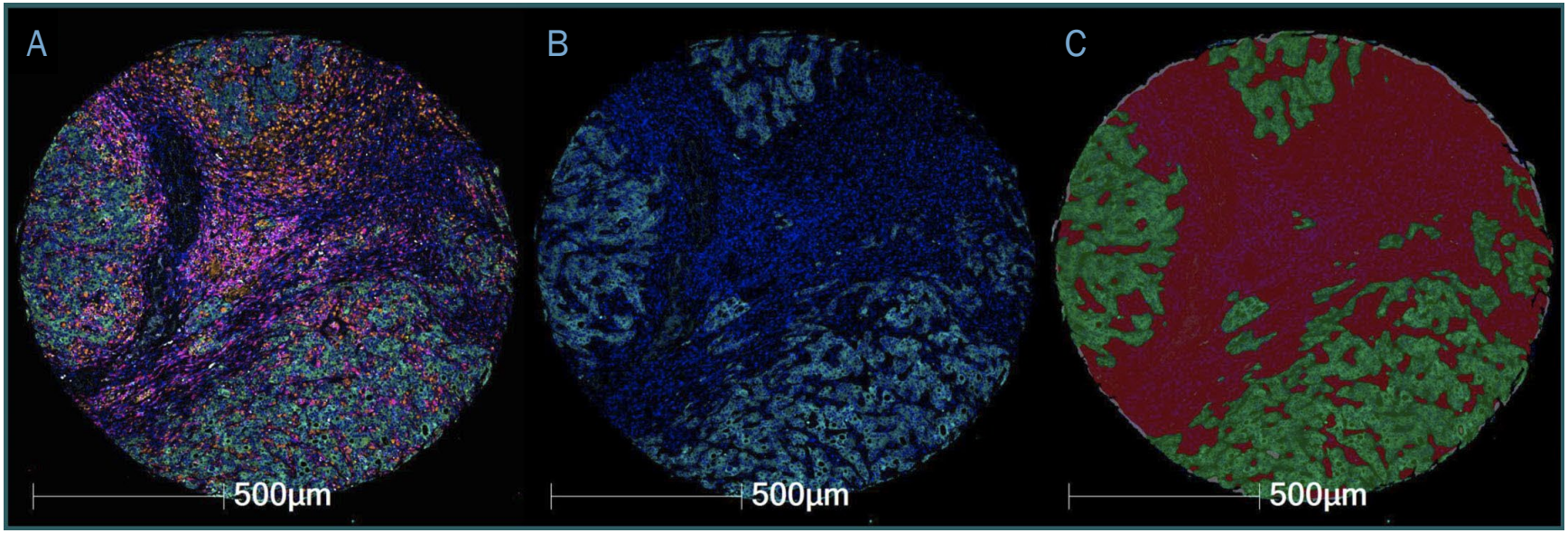
METHODS: TISSUE CLASSIFICATION



Tumor and stroma tissue classification. (A) Raw multiplex immunofluorescence image; (B) nuclei in blue and cytokeratin positive cells (tumor) in cyan; (C) tumor segmentation using HALO AI (tumor in green and stroma in red).

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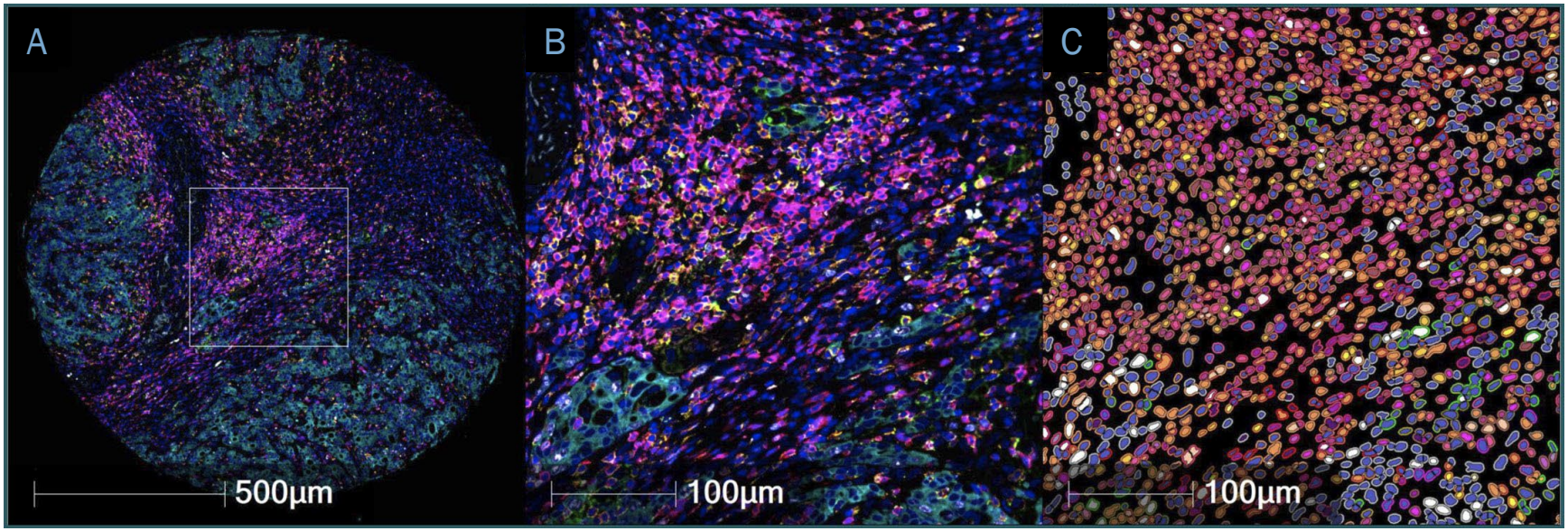
METHODS: IMMUNE CELL SUBTYPE CLASSIFICATION



Detection and classification of immune cell subtypes. (A) Raw multiplex immunofluorescence image; (B) closeup multiplex immunofluorescence image (nuclei in blue, tumor cells in cyan, CD45 in orange, CD3 in red, CD4 in pink, CD8 in yellow, Ki-67 in white, PD-L1 in green, FOXP3 in purple); (C) cell classification/analysis mark-up (colocalization) using the Highplex FL module.

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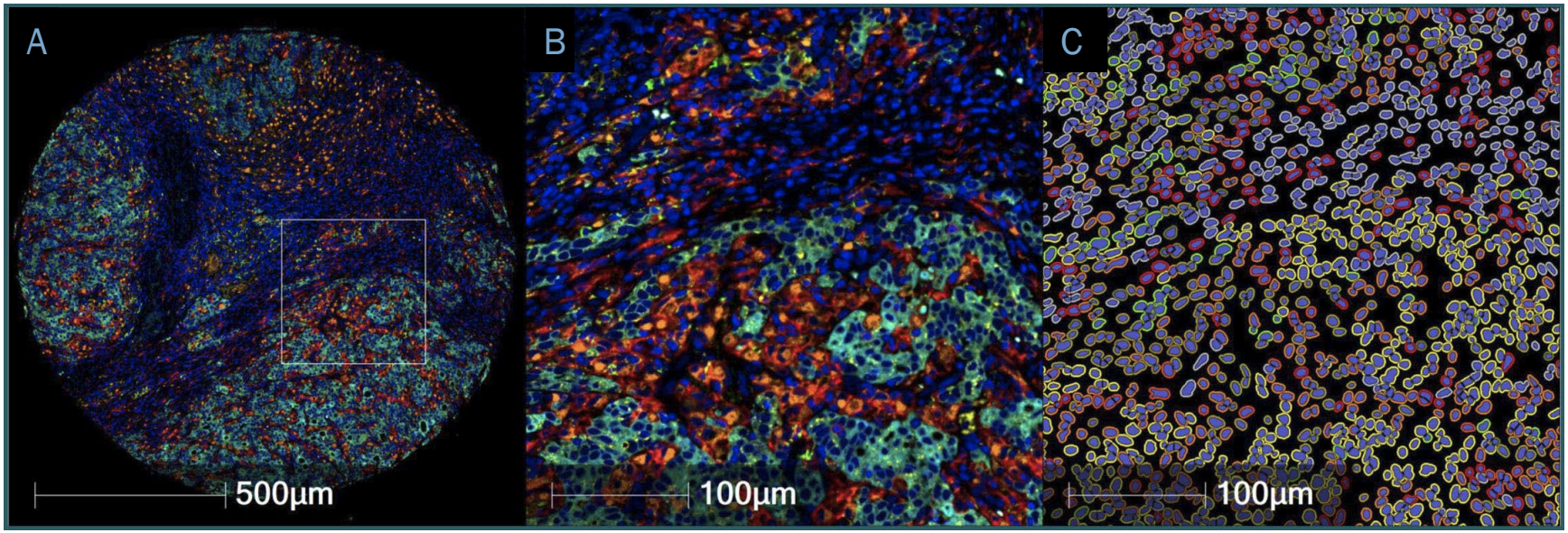
METHODS: MACROPHAGE SUBTYPE CLASSIFICATION



Detection and classification of macrophage subtypes. (A) Raw multiplex immunofluorescence image; (B) closeup multiplex immunofluorescence image (nuclei in blue, tumor cells in cyan, CD68 in orange, CD16 in red, HLA-DR in yellow PD-L1 in green); (C) cell classification mark-up (colocalization) using the Highplex FL module.

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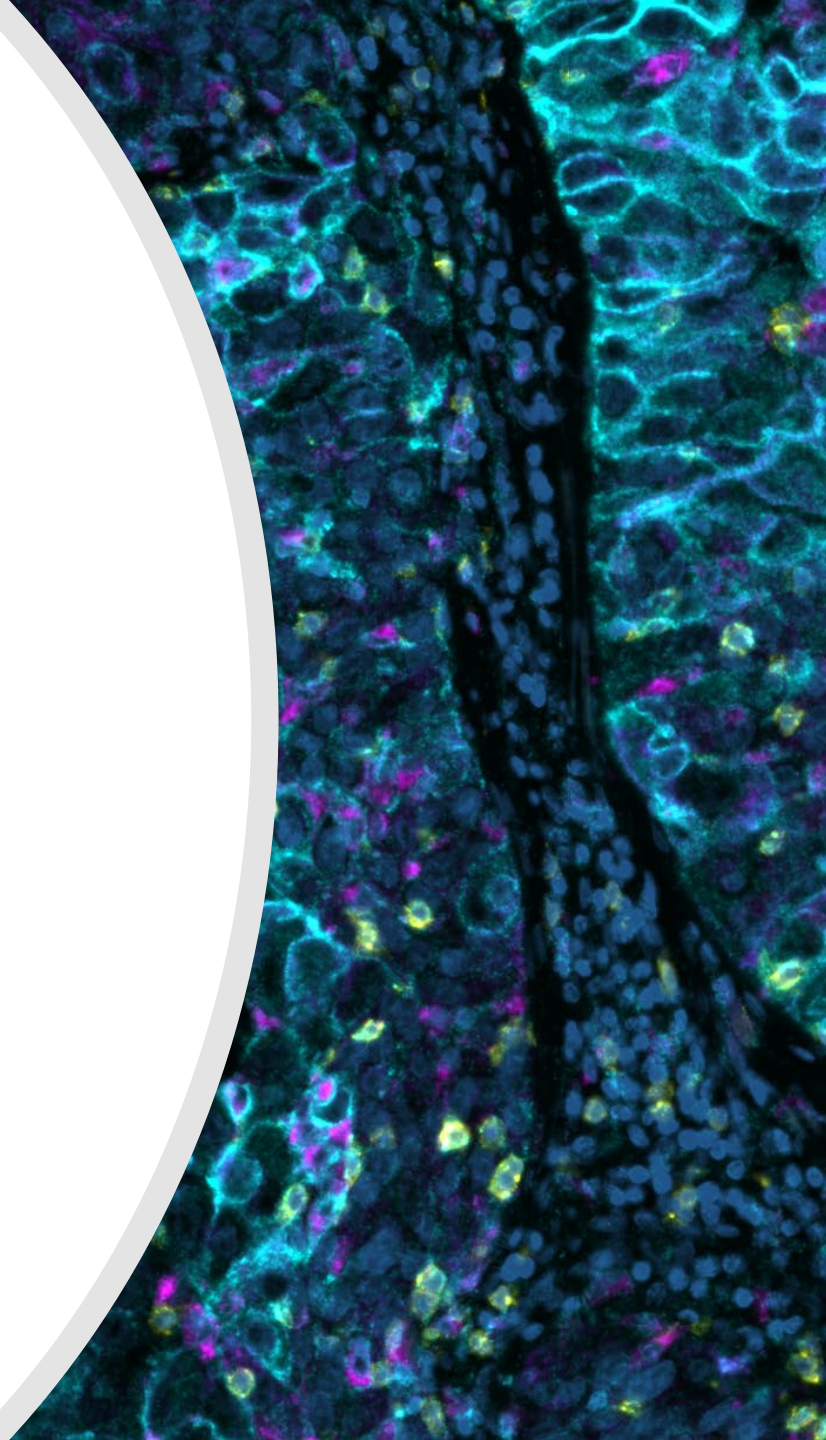
RESULTS

The 20-plex panel was leveraged to define 17 unique immune cell subtypes based on binned expression and co-expression of biomarkers. The automatic classification of stroma and tumor facilitated the spatial analysis of these immune phenotypes within the TME in invasive breast carcinoma cores and colon adenocarcinoma cores. The results demonstrate differences in the densities of immune cells between tumor and stroma and between cancers, as well as varied proximity of subtypes relative to both tumor cells and the tumor boundary.



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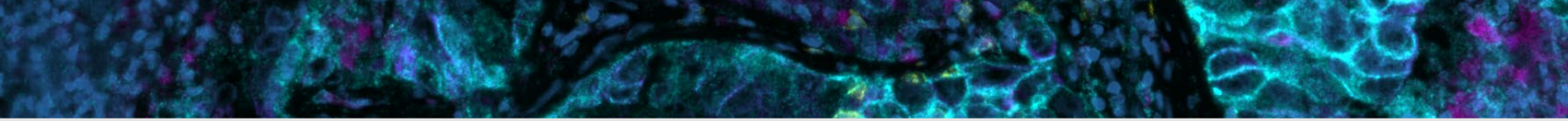
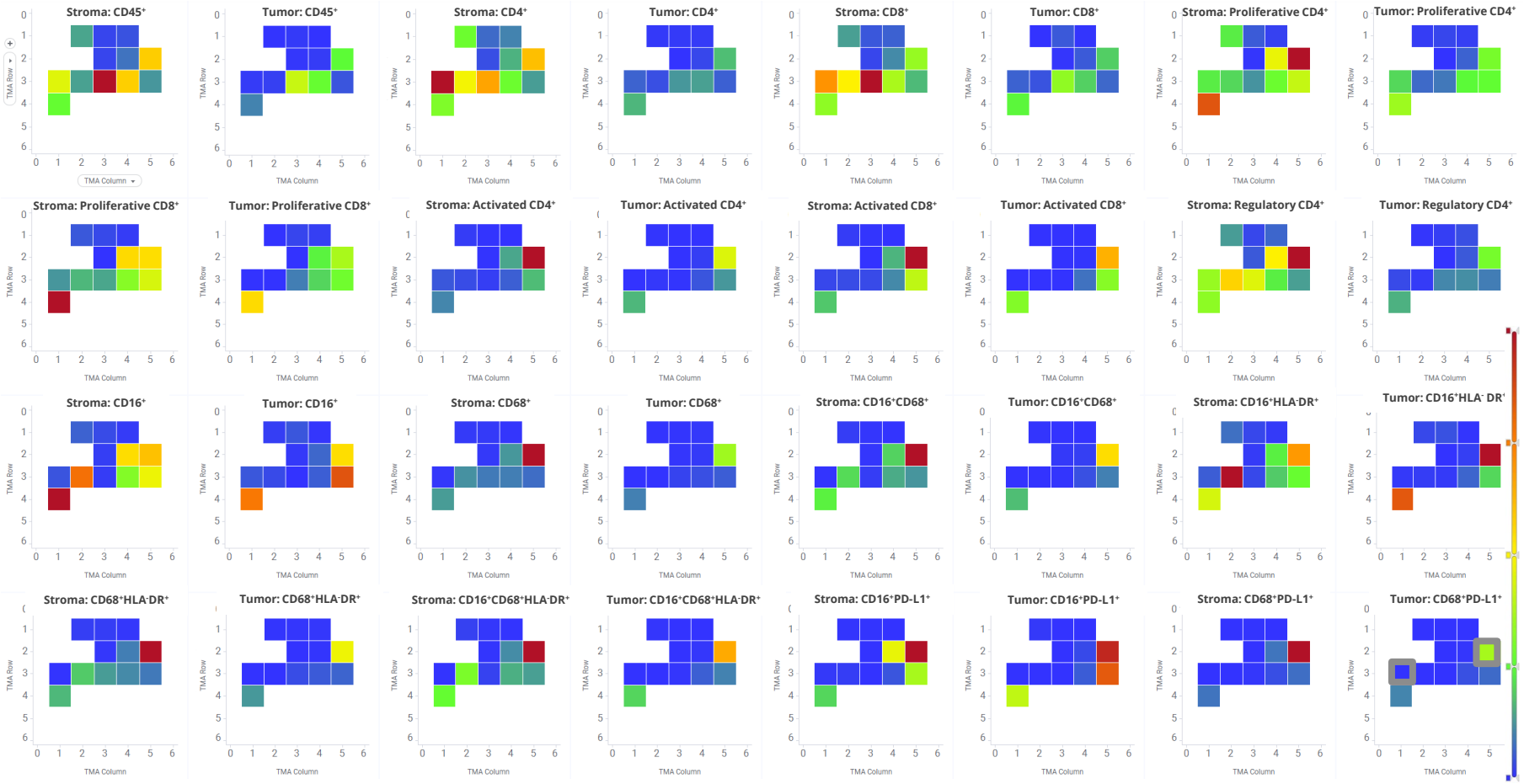


RESULTS: TMA DENSITY HEATMAP GENERATION FOR DIFFERENT PHENOTYPES

TMA Heatmaps for the density of each phenotype of interest in the automatically classified stroma and tumor regions. Each square represents a TMA core.



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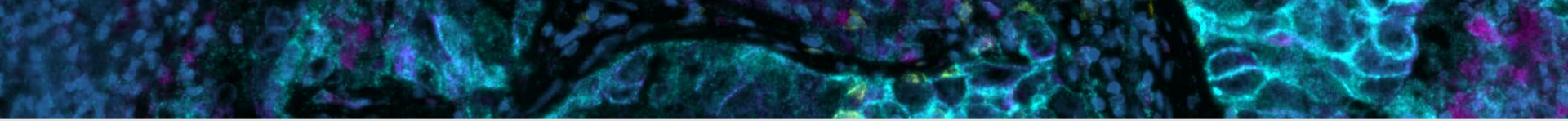
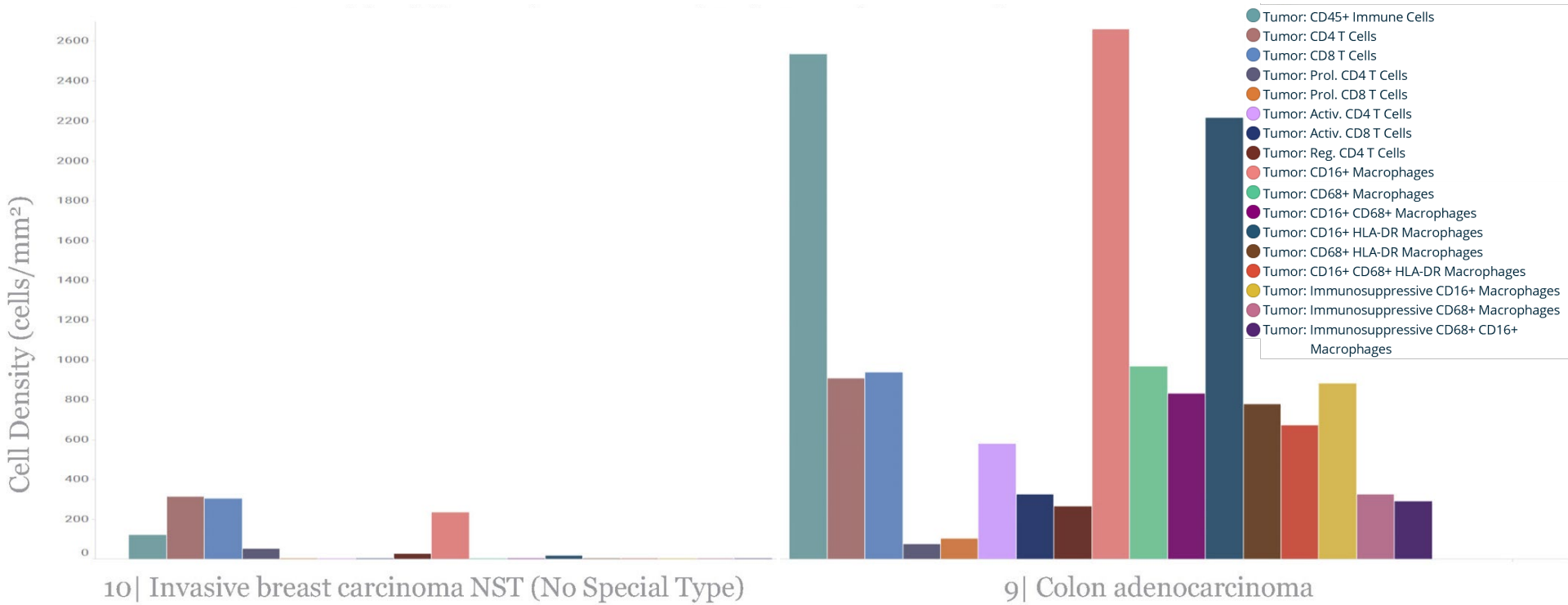


RESULTS: DENSITY OF DIFFERENT PHENOTYPES IN TUMOR REGIONS



Histogram of the density of the different phenotypes within the tumor regions of cores 10 (invasive breast carcinoma) and 9 (colon adenocarcinoma).

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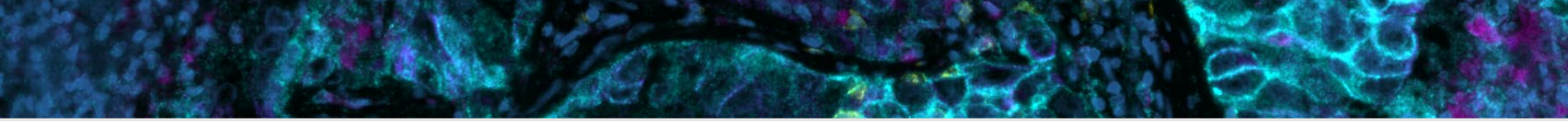
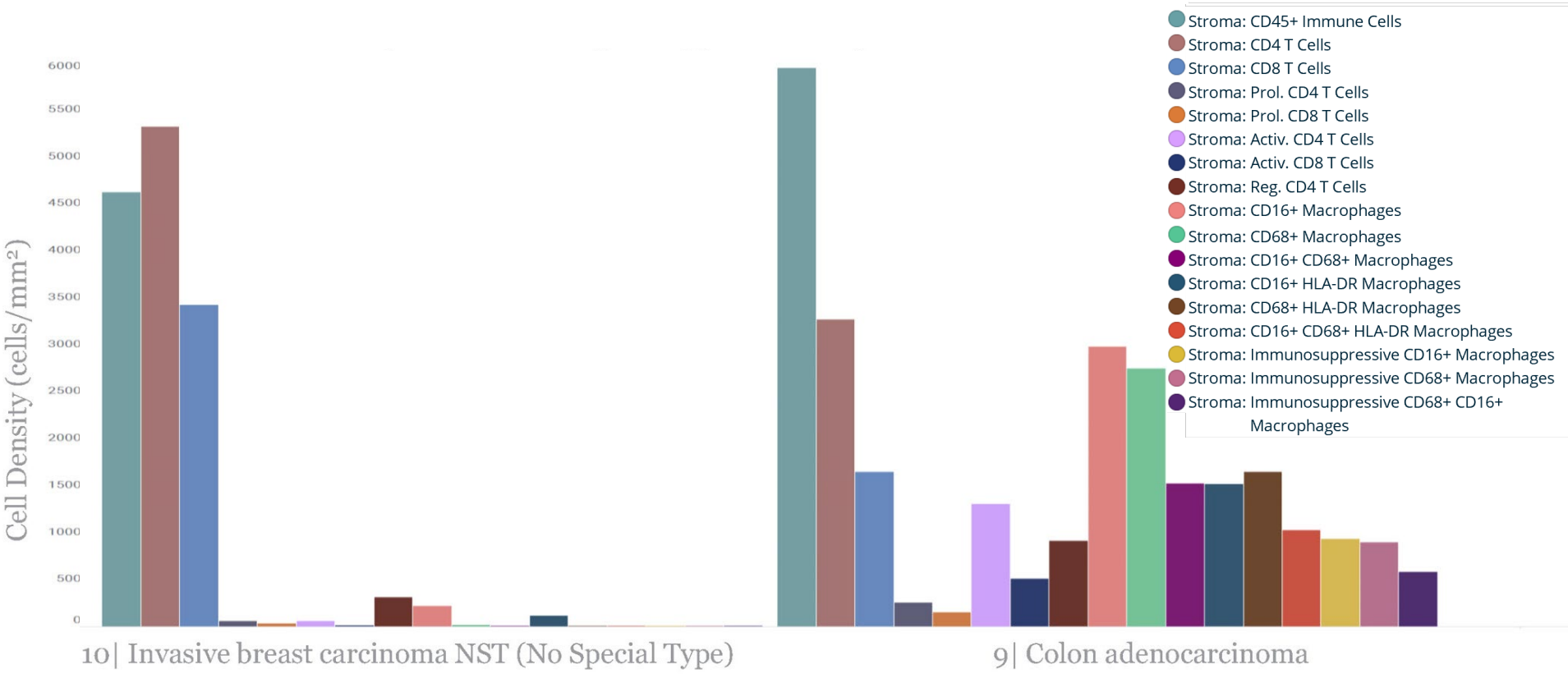


RESULTS: DENSITY OF DIFFERENT PHENOTYPES IN STROMAL REGIONS



Histogram of the density of the different phenotypes within the stromal regions of cores 10 (invasive breast carcinoma) and 9 (colon adenocarcinoma).

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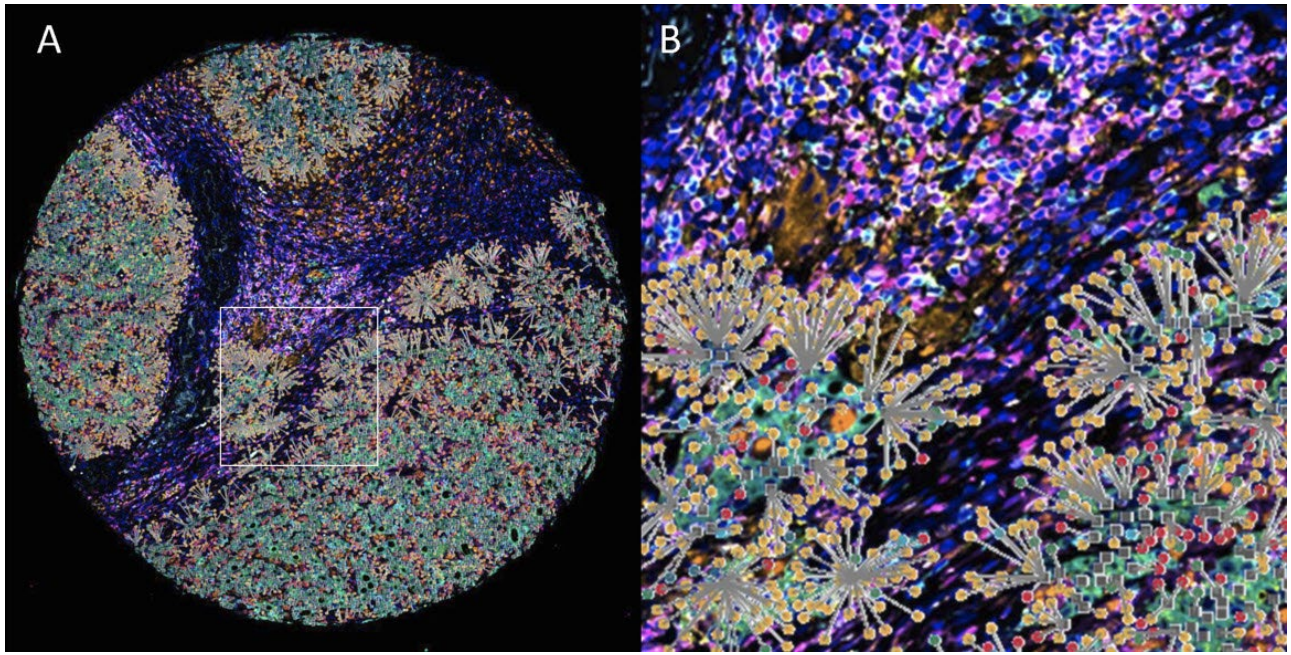
RESULTS: PROXIMITY ANALYSIS



The number of all the different cell phenotypes within a specified radius of a tumor cell (in this case 50 µm) is calculated. Full core image (A) and zoom-in into tumor border area (B).

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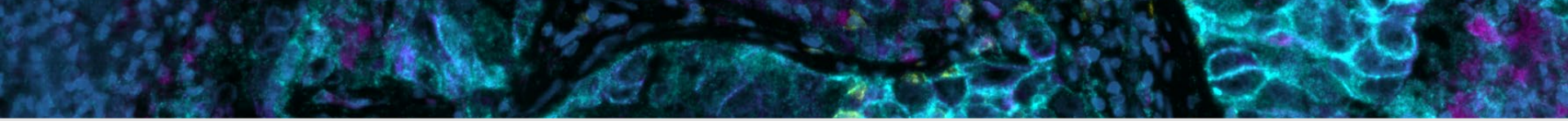
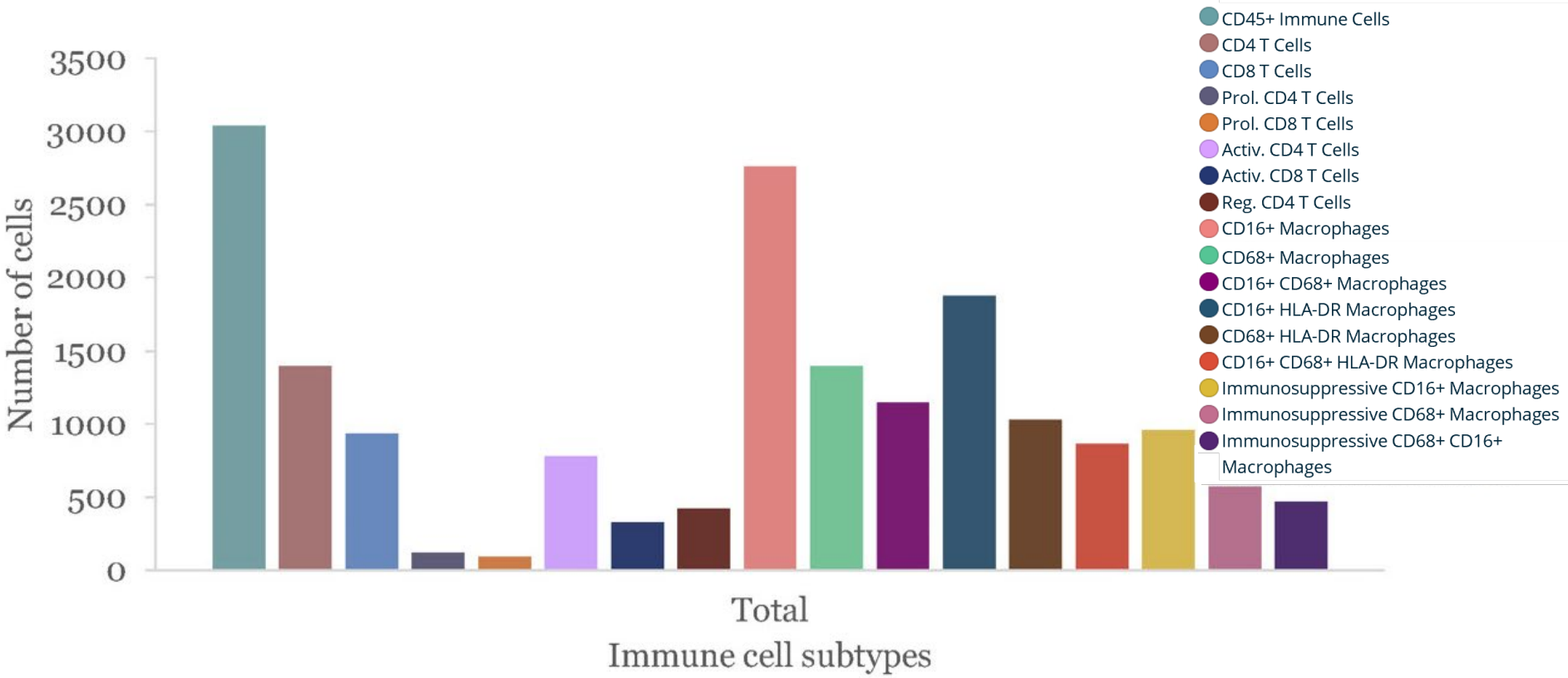


RESULTS: PROXIMITY ANALYSIS



Histogram of the number of the different phenotypes within a 50 μm radius from any tumor cell of core 9 (colon adenocarcinoma).

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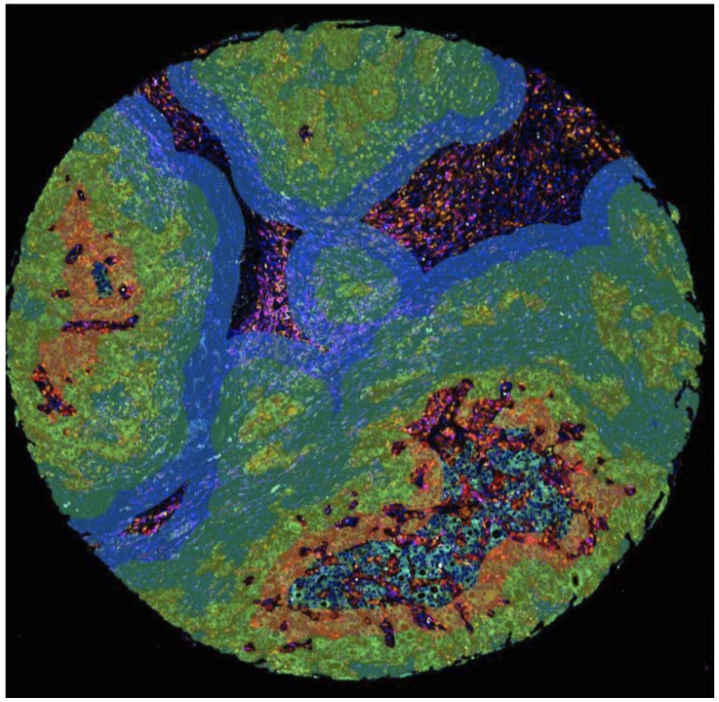
RESULTS: INFILTRATION ANALYSIS



The density/ number of all the different cell phenotypes are calculated within a 50 and 100 µm distance from the tumor boundary (inside and outside).

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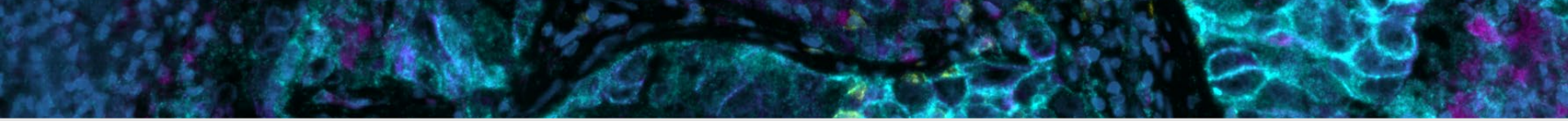
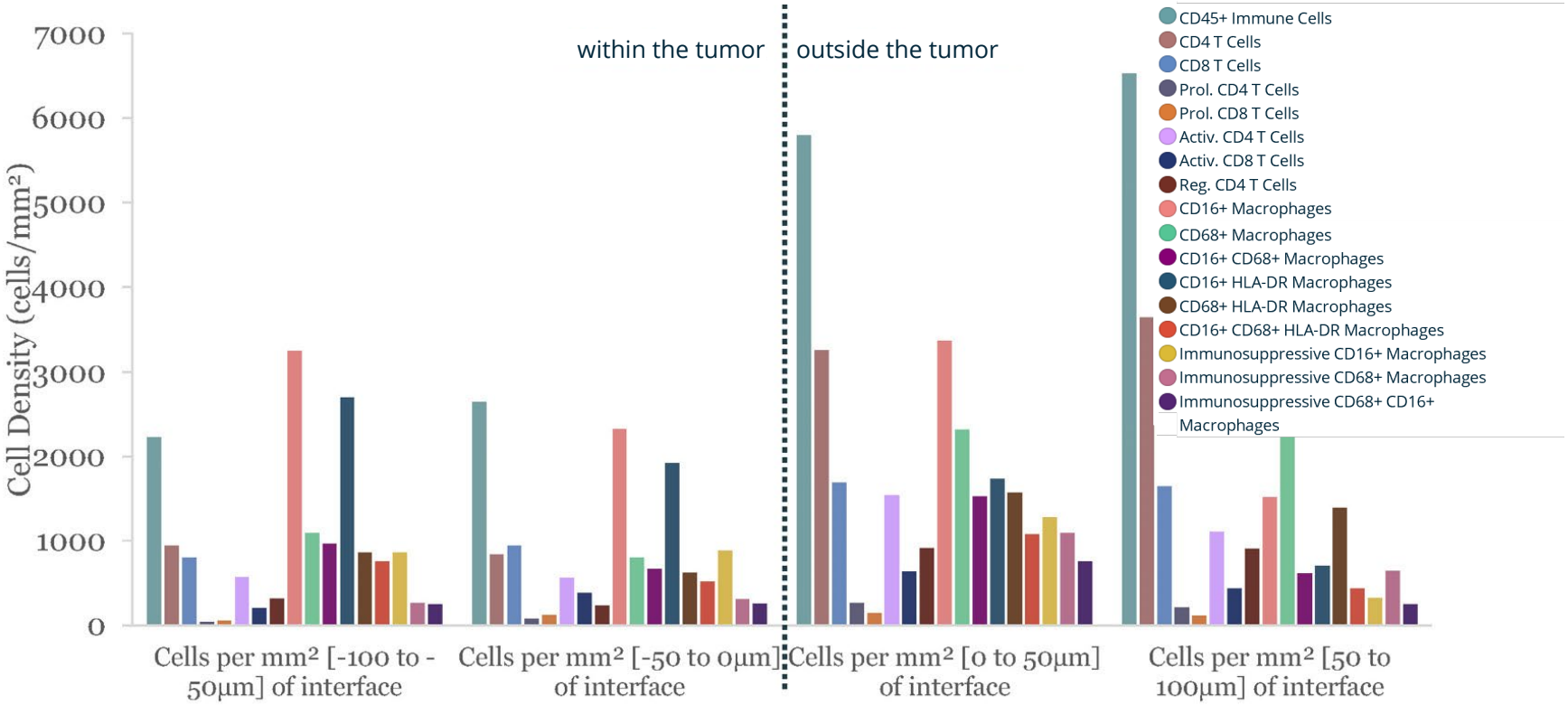


RESULTS: INFILTRATION ANALYSIS



Histogram of the density of the different phenotypes within a 100 µm range from the tumor interface of core 9 (colon adenocarcinoma).

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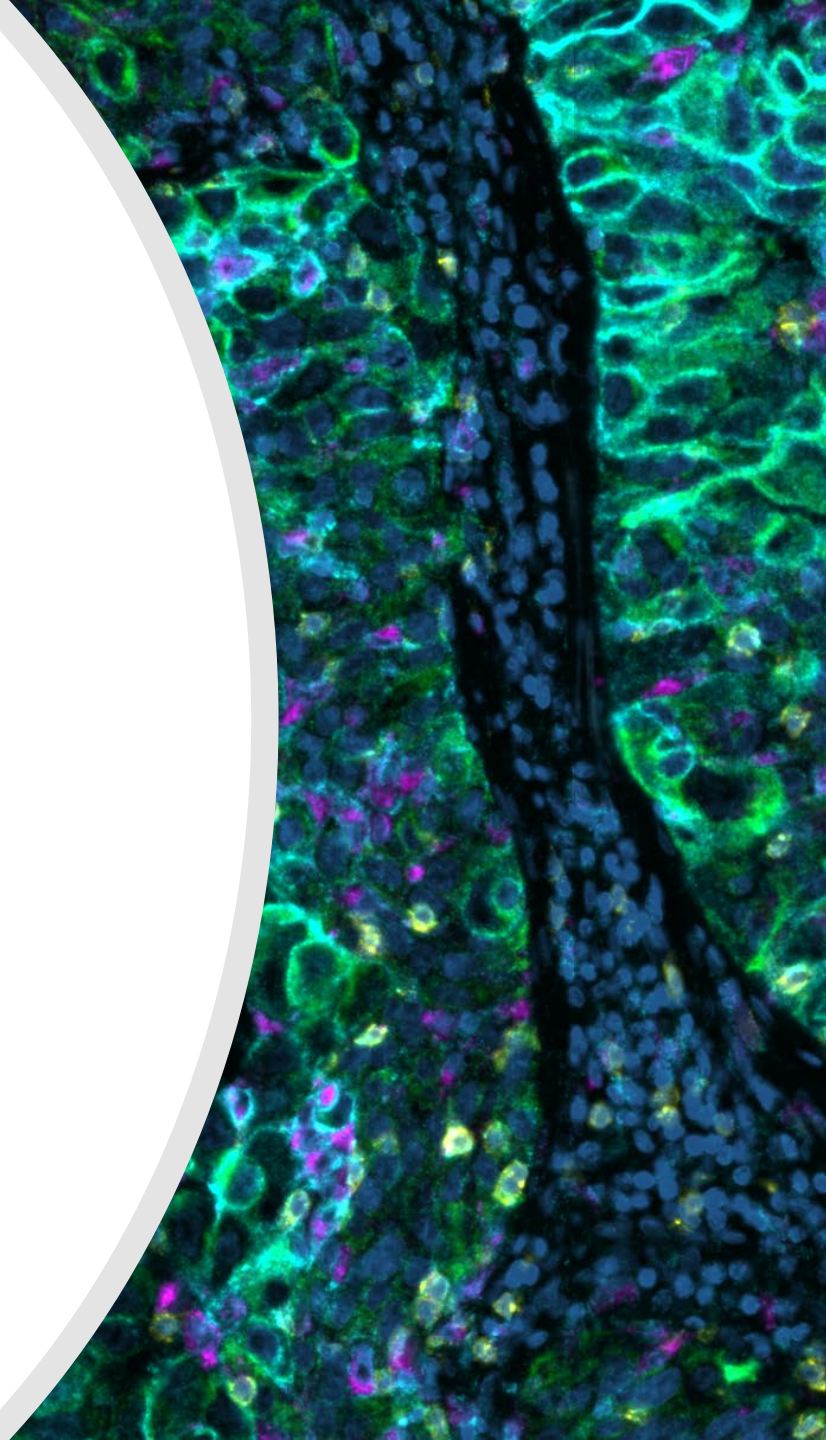
PRODUCT INFORMATION

Learn more about the **COMET™ spatial biology platform** on the Lunaphore website, or email **info@lunaphore.com**.

Learn more about **Pharma Services** and the **HALO® image analysis platform** at **https://indicalab.com**, or email **info@indicalab.com**.



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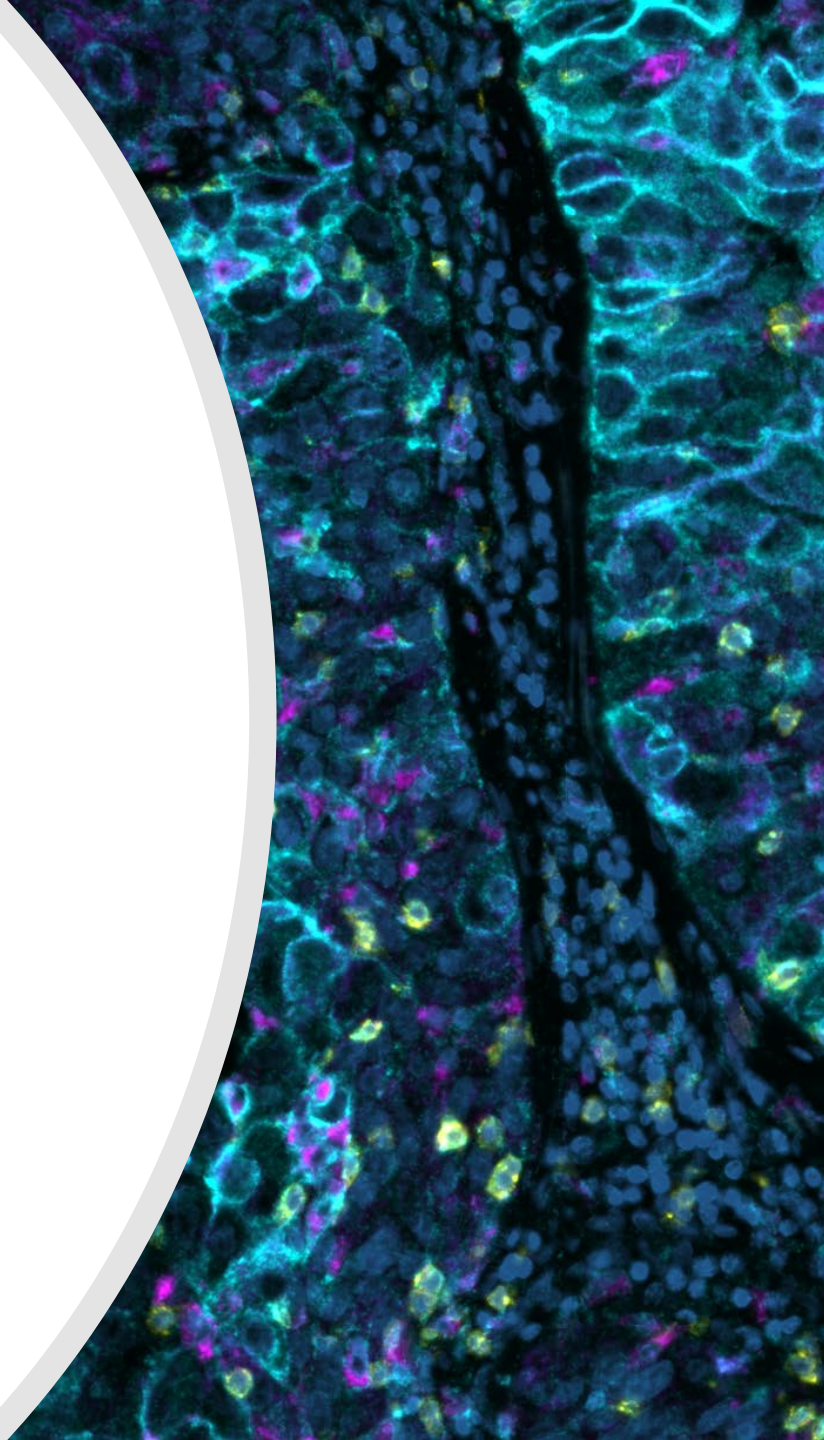
CONCLUSIONS

The workflow presented here highlights the easiness of adoption of the seqIF™ protocol and a supervised image analysis pipeline. We demonstrate how the combination of COMET™ hyperplex images with the HALO and HALO AI image analysis platform results in an easy and straightforward workflow for interrogating heterogeneous TME and depicting tissue architecture on the single-cell level.

Our analysis of a 20-plex immuno-oncology panel on a TMA containing invasive breast carcinoma cores and colon adenocarcinoma cores yielded several insights into the cellular phenotypes and complex spatial relationships in the TME including T lymphocytes and macrophages. We were able to demonstrate distinct tumor infiltration patterns and characterize the accumulation of immune cells in the stroma and tumor compartment.



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