5503: Ultrahigh-plex Multiomic Spatial Phenotyping of Head and Neck Cancer Tissue Uncovers Protein and RNA Signatures of Immunotherapy Response

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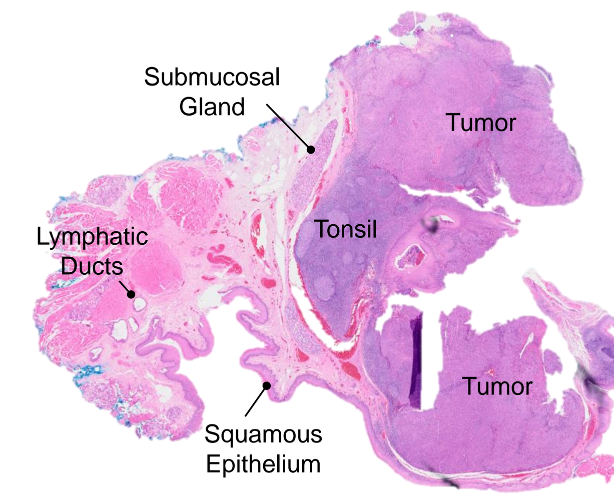
1. Introduction

Immune checkpoint inhibitors (ICI) have proven to be game-changing treatments for mucosal head and neck squamous cell cancer (HNSCC). The tumor microenvironment (TME) composition, contexture, and cellular architecture are key to understanding immune responsive and resistant HNSCC phenotypes. In this study, we performed multiomic spatial phenotyping to characterize a HNSCC tissue from a patient with a partial response to Pembrolizumab/Nivolumab treatment (H&E-stained section shown below). Pathology annotations indicate a large tumor mass, a regionally intact tonsil, as well as an Esophageal Submucosal Gland, Lymphatic Ducts and normal Squamous Epithelium

Here, we demonstrate how **Ultrahigh-plex Protein Spatial Phenotyping with Targeted Spatial RNA analysis** can produce a uniquely comprehensive analysis of the patient's TME and how it may explain the patient's partial response to ICI therapy.



2. Multiomic (Protein & RNA) Spatial Phenotyping on PhenoCycler-Fusion 2.0

Ultrahigh-Plex Protein Spatial Phenotyping

Targeted Spatial RNA Detection

PhenoCycler-Fusion 2.0

The **PhenoCycler®-Fusion 2.0** system is a spatial biology solution that enables multiomic imaging of whole tissues at single-cell resolution. The high-speed solution coupled with molecular barcoding chemistry enables deep characterization of whole-slide tissue sections, which often contain millions of cells. Along with ultrahigh-plex protein spatial phenotyping, the PhenoCycler workflow can be used to detect RNA targets via the **ViewRNA™ tissue assay**.

In this study, we first analyzed HNSCC tissue with **101-plex protein panel** that comprehensively labeled both immune and metabolic markers. We then imaged **2 panels of 4-plex RNA probes** that label TME cytokines and chemokines on a serial section from the same tissue.

3. Ultrahigh-Plex Spatial Phenotyping and Targeted RNA Spatial RNA Analysis

101-plex Antibody panel

We combined markers from **the pre-designed PhenoCode™ Discovery Panel modules and created a 56-plex custom panel from the Akoya antibody database** to label immune cell lineages, checkpoints, metabolic, and cell stress markers.

	Immune Cell Profiling Core	*Tissue Architecture*	*Immune Activation & Proliferation*	*Lymphocyte Profiling*
PhenoCode Discovery Panel Modules	CD4,CD68, CD20, CD11c, CD8, CD3ε, HLA-DR, CD44, CD45, HLA-A, CD14, CD56, Ki67, CD45RO, PanCK	E-cadherin, SMA, Vimentin, Collagen IV, CD34, CD31, β-actin, β-catenin, Podoplanin, Caveolin	PD-1, PD-L1, ICOS, PCNA, LAG3, IDO1, CD40, VISTA, HLA-E, IFNG	T-bet, FOXP3, Granzyme B, CD21, CD39, CD79α, CD107a, CD38, TOX, TCF-1
Custom Panel	ASCT2, BAD, BAK, BAX, Beclin-1, CPT1A, Cyt. C, Cyc D1, HK1, LDHA, IDH2, GLUT1, pNRF2, ATP5A, MMP9, MPO, NA/K ATPase, C1Qa, AXL, CCR6, Citrate Synthetase, HIF1α, iNOS, HLA-DPB1, LEF1, GAL9, MC-Tryptase, OX40, G6PD, IDHA, TP63, GP100, CK17, GATA3, SDHA, S100A4, CD163, Pax5, CD2, CD1a, CD163, CD141, CD19, CD57, CD69, CD227, CD138, CD7, CD15, ZAP70, ZEB1			

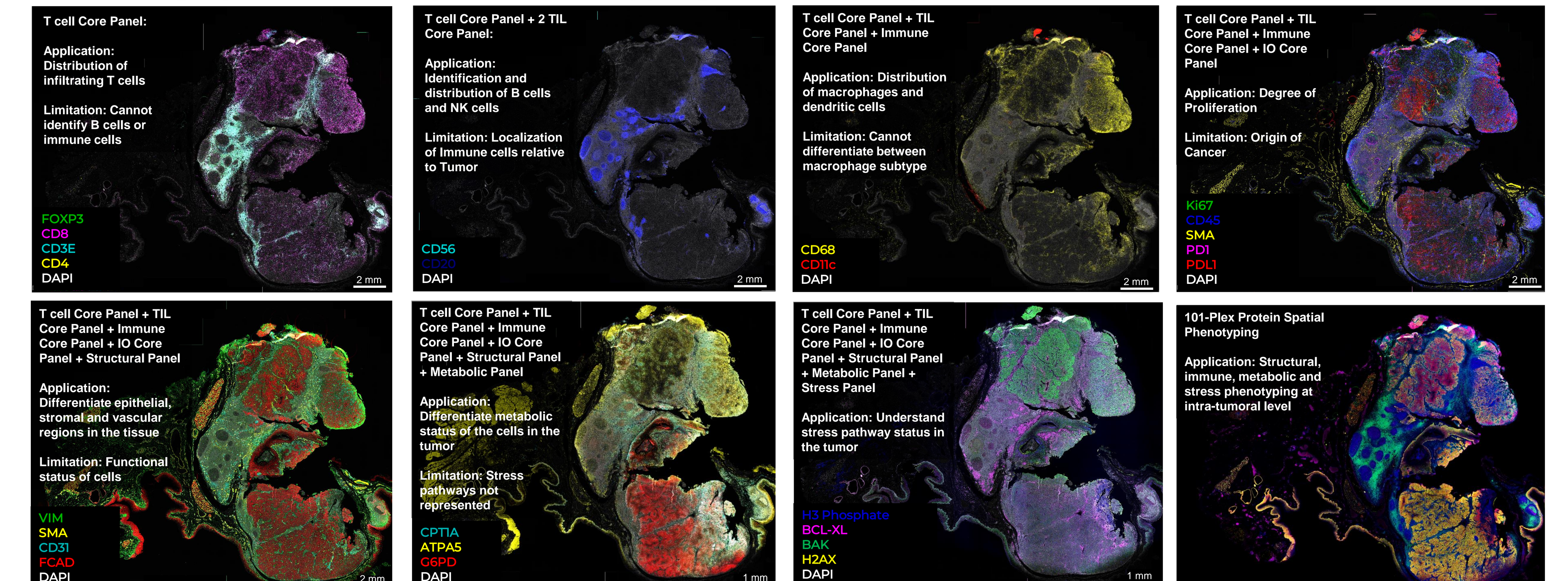
4-plex ViewRNA panel

We selected 4 house keeping genes to serve as internal controls for the ViewRNA assay. We also tested two different 4-plex ViewRNA panels on two serial sections of the HNSCC tissue to detect cytokines and chemokines molecules that may modulate lymphocyte activation and immune activation in the TME .

House Keeping Genes
PPIB, LDHA, POLR2A, UBC
Lymphocyte Activation and Recruitment Panel
CXCL9, CCL22, CXCL13, CD20
Immune Activation and Response Signature Panel
IFNG, IFI44L, STAT1, CD3

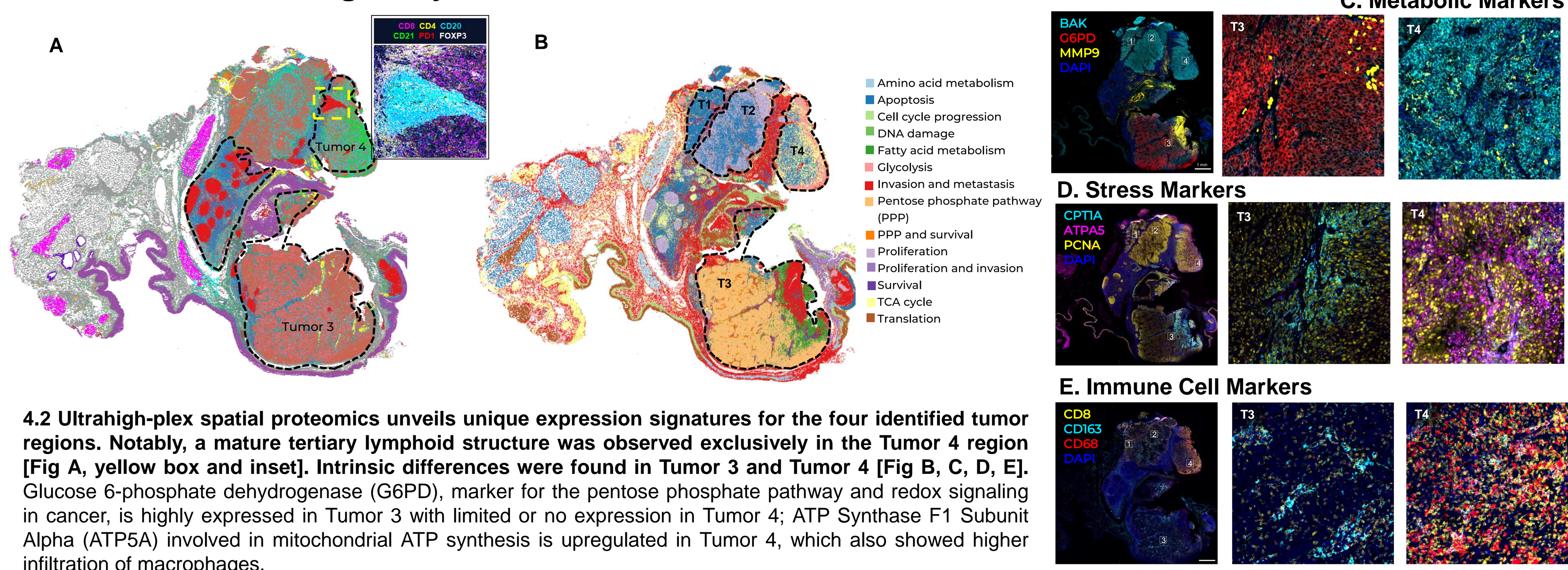
4. Ultrahigh-Plex Protein Spatial Phenotyping Reveals Distinct Immune , Stress and Metabolic Signatures in Different Tumor Regions

4.1. Ultrahigh-plex Protein Spatial Phenotyping Enables Deeper Characterization of the Tumor



4.1. Ultrahigh-plex spatial protein phenotyping of HNSCC enabled deeper characterization of the sample. While the T-cell core panel, TIL panel, Immune Core Panel, and IO core panel offered valuable insights into the immune status of the tumor, additional layers of characterization were achieved through the inclusion of the structural panel, metabolic panel, and stress panel. Moreover, by expanding the analysis beyond a specific region of interest (ROI) or a small field of view (FOV), we uncovered intra-tumoral heterogeneity, providing crucial insights into the partial immune therapy response observed in the patient.

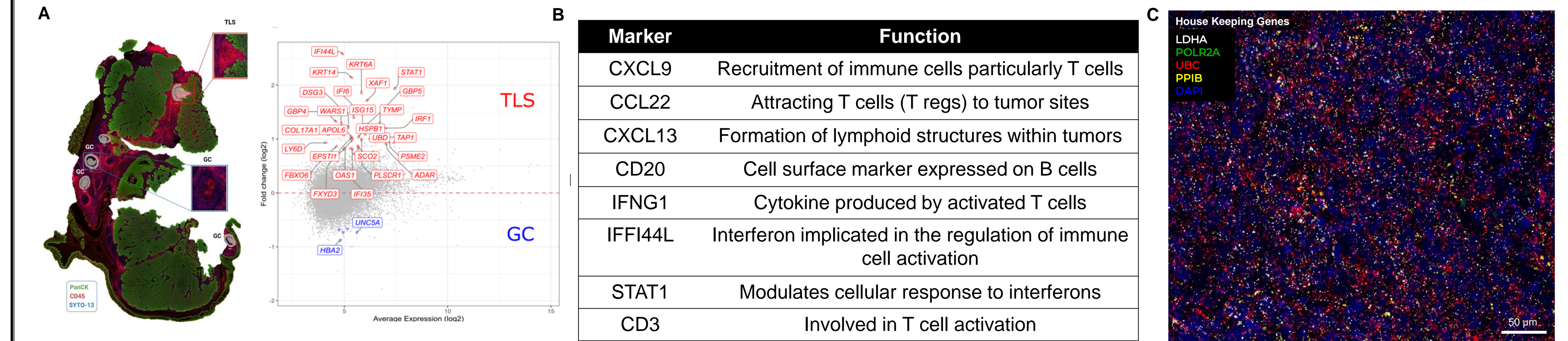
4.2. Ultrahigh-plex Protein Spatial Phenotyping Reveals Presence of Mature TLS in Tumor 4 and Intra-Tumoral Heterogeneity in Metabolic and Stress Profiles in Tumor 3 vs Tumor 4



4.2 Ultrahigh-plex spatial proteomics unveils unique expression signatures for the four identified tumor regions. Notably, a mature tertiary lymphoid structure was observed exclusively in the Tumor 4 region [Fig A, yellow box and inset]. Intrinsic differences were found in Tumor 3 and Tumor 4 [Fig B, C, D, E]. Glucose 6-phosphate dehydrogenase (G6PD), marker for the pentose phosphate pathway and redox signaling in cancer, is highly expressed in Tumor 3 with limited or no expression in Tumor 4; ATP Synthase F1 Subunit Alpha (ATP5A) involved in mitochondrial ATP synthesis is upregulated in Tumor 4, which also showed higher infiltration of macrophages.

5. Multiomic Spatial Phenotyping Provides Mechanistic Insights into Partial Immune Response of a HNSCC Patient

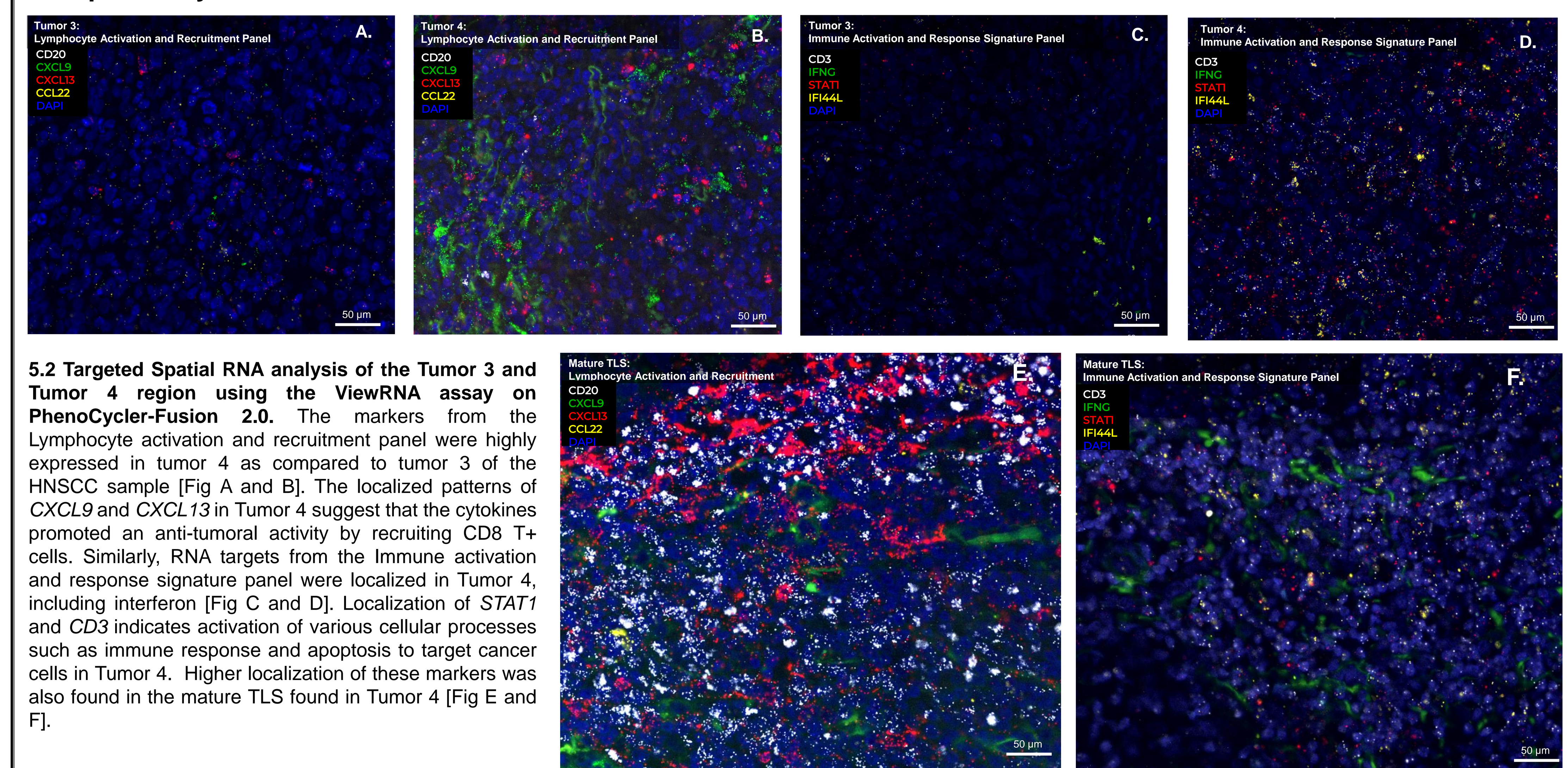
5.1. Design and Development of the Targeted Spatial ViewRNA Panel



Marker	Function
CXCL9	Recruitment of immune cells particularly T cells
CCL22	Attracting T cells (T regs) to tumor sites
CXCL13	Formation of lymphoid structures within tumors
CD20	Cell surface marker expressed on B cells
IFNG1	Cytokine produced by activated T cells
IFFI44L	Interferon implicated in the regulation of immune cell activation
STAT1	Modulates cellular response to interferons
CD3	Involved in T cell activation

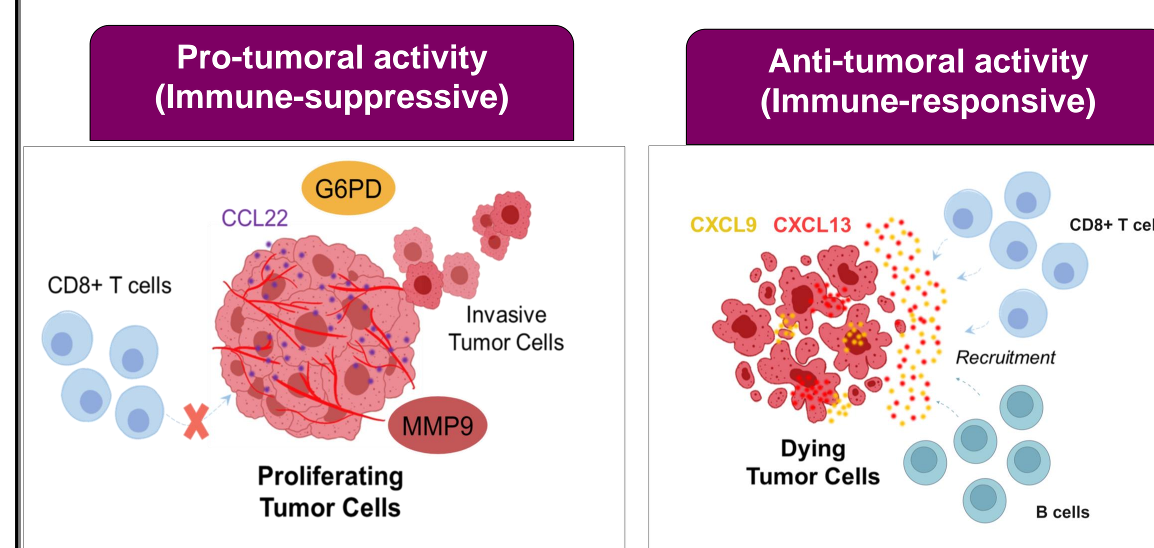
5.1. To delve deeper into tumorigenic mechanisms of Tumor 3 vs Tumor 4 and the structured and mature TLS, two 4-plex RNA panels were designed based on literature review and whole transcriptome assay. These panels, tailored for lymphocyte activation, recruitment *[CXCL9, CCL22, CXCL13, CD20]*, and immune activation/response *[IFNG1, IFI44L, STAT1, CD3]*, were analyzed using the ViewRNA assay on the PhenoCycler-Fusion 2.0 platform [Table B] . Before investigating Tumor 3 and Tumor 4, we first tested housekeeping genes as positive controls for ViewRNA assay on the PhenoCycler-Fusion 2.0 platform [Fig C].

5.2. Targeted Spatial RNA Analysis Unveils Distinct Patterns of Lymphocyte Activation, Recruitment and Immune Response Dynamics in Tumor 3 vs Tumor 4



5.2 Targeted Spatial RNA analysis of the Tumor 3 and Tumor 4 region using the ViewRNA assay on PhenoCycler-Fusion 2.0. The markers from the Lymphocyte activation and recruitment panel were highly expressed in tumor 4 as compared to tumor 3 of the HNSCC sample [Fig A and B]. The localized patterns of *CXCL9* and *CXCL13* in Tumor 4 suggest that the cytokines promoted an anti-tumoral activity by recruiting CD8 T+ cells. Similarly, RNA targets from the Immune activation and response signature panel were localized in Tumor 4, including interferon [Fig C and D]. Localization of *STAT1* and *CD3* indicates activation of various cellular processes such as immune response and apoptosis to target cancer cells in Tumor 4. Higher localization of these markers was also found in the mature TLS found in Tumor 4 [Fig E and F].

6. The Power of Ultrahigh-Plex Protein Spatial Phenotyping and Targeted Spatial RNA Detection



Our study underscores the value of ultrahigh-plex protein resolution and targeted spatial analysis. By utilizing ultrahigh-plex protein capability on PhenoCycler-Fusion 2.0, we can effectively resolve crucial anatomical structures pivotal for sustained antibody production, such as TLS (tertiary lymphoid structures).

Additionally, targeted RNA analysis provided insights into the intricate cytokine and chemokine signaling pathways operating within these regions highlighting the synergistic power of multi-modal approaches in cancer research

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