1525: Integration of High-plex Tumor-immune Phenotyping and Checkpoint Interactions for Deeper Spatial Characterization of Human Cancer Tissues

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1. Introduction

The introduction of immune checkpoint targeted therapies revolutionized cancer treatment; however, the efficacy of treatment can be variable between patients. This variability is not fully understood but can be partially explained by the weak correlation between PD-1/PD-L1 expression in immunohistochemistry (IHC) assays and expected/observed clinical outcomes. To address this challenge, we created a comprehensive protocol that allows for the visualization of protein-protein interactions within the broader context of the tissue and tumor microenvironment (TME), integrating both cellular and functional data. Proximity ligation assays illuminate active signaling pathways by detecting spatial interactions between ligand-receptor targets. Coupled with multiplex immunofluorescence staining of tumor and immune biomarkers, the resulting integrated protocol can be a powerful tool for improved characterization of the TME in relation to immune checkpoint interactions, deepening the understanding of immune responses and identifying spatial signatures for improved patient stratification. The innovative integration of PhenoCode™ Signature Immune Panel with *in situ* proximity ligation assays (isPLA) using Naveni® PD-1/PD-L1 on high-throughput spatial biology solution (PhenoImager® HT) advances our understanding of the tumor microenvironment, enabling precise analysis of immune responses and biomarker interactions.

2. Integration of PhenoCode Signature Panel with Naveni Proximity Ligation Assay

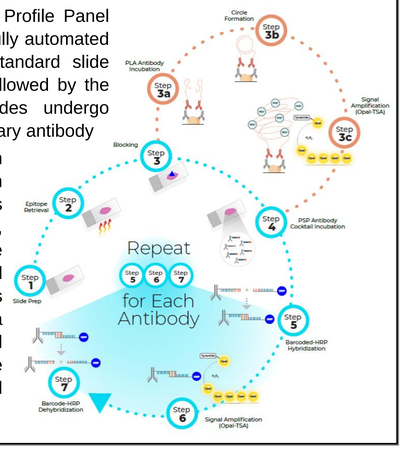
Tonsil tissue and biopsies from patients with mucosal head and neck squamous cell carcinoma (HNSCC) enrolled in immune checkpoint inhibitor therapies were phenotyped using method integration of Naveni PD-1/PD-L1 proximity ligation assay (PLA) with the flexible high-plex PhenoCode Signature Immune Profile Panel (CD3ε/CD8/CD68/CD20/PanCK) (PSP). Formalin-fixed paraffin-embedded (FFPE) tissues were stained using the Leica BOND® RX™ autostainer, with both assays run in one simple merged protocol. Imaging and spectral unmixing were performed on the PhenoImager® HT 2.0 and inForm software, respectively.

Spectrally unmixed PSP/*is*PLA images were imported into QuPath and the DAPI channel was segmented with the Stardist plugin. An ANN pixel classifier was trained on Pan-cytokeratin-positive areas to generate annotations of tumor and non-tumor (stromal) areas. Thus, cells belonged to either a tumor or stroma parent annotation.

The PhenoCode Signature Immune Profile Panel combined with Naveni *is*PLA reveals the localization of PD-1/PD-L1 interaction in relation to immune cell types in the TME on clinical samples, alluding to an improved method for identifying patient responders.

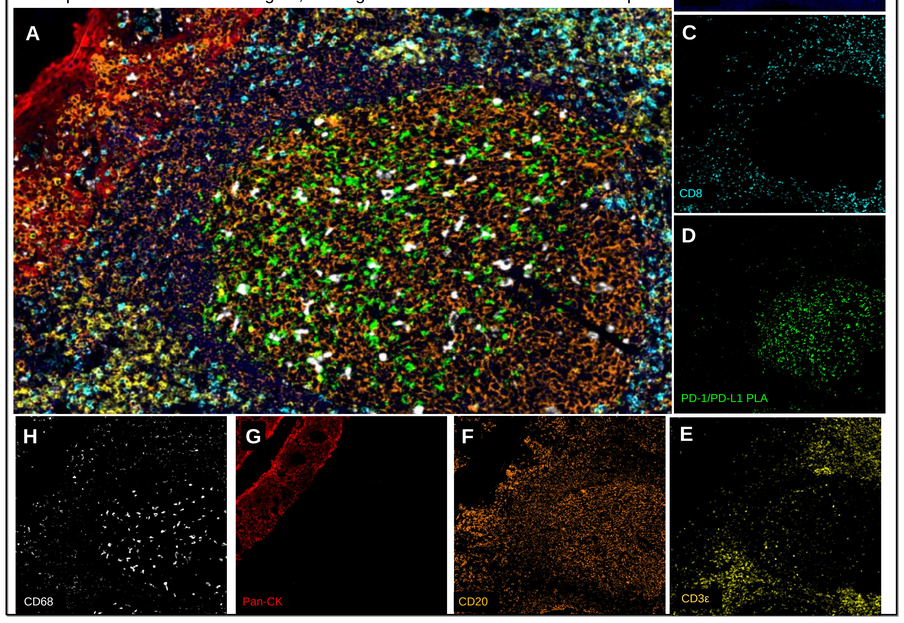
3. Method of Technology Integration

The flexible aspect of the PhenoCode Signature Immune Profile Panel allows Naveni PD-1/PD-L1 to act as the variable marker. Fully automated protocol integration was achieved by undertaking the standard slide preparation steps (baking, dewax and epitope retrieval) followed by the standard Naveni PD-1/PD-L1 protocol. Briefly, the slides undergo standard Naveni blocking, followed by PD-1 and PD-L1 primary antibody incubation. Secondary antibodies attached to oligos, known as Navenibodies, are added which facilitate circle formation and rolling circle amplification. The interaction product is then detected using Opal®-TSA chemistry. Once complete, the PhenoCode Signature Panel protocol resumes from the antibody cocktail incubation step, where antibodies attached to unique oligo barcodes are added. One antibody is revealed at a time through the hybridization of a complimentary reporter conjugated to HRP. Signal amplification occurs via Opal-TSA. Once completed, the HRP conjugate is dehybridized and the process is repeated for the remaining antibodies with different Opal dyes.



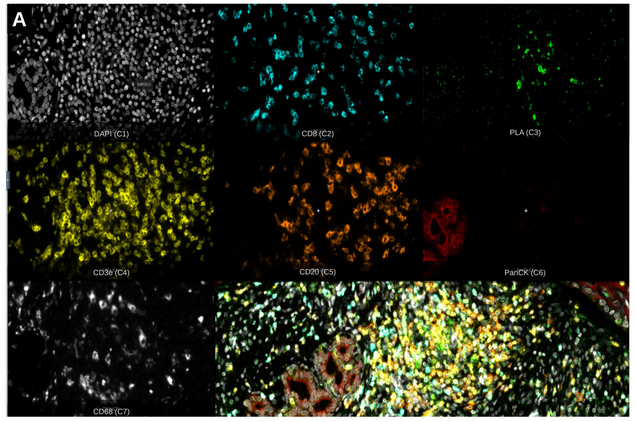
4. Validation of Technology Integration

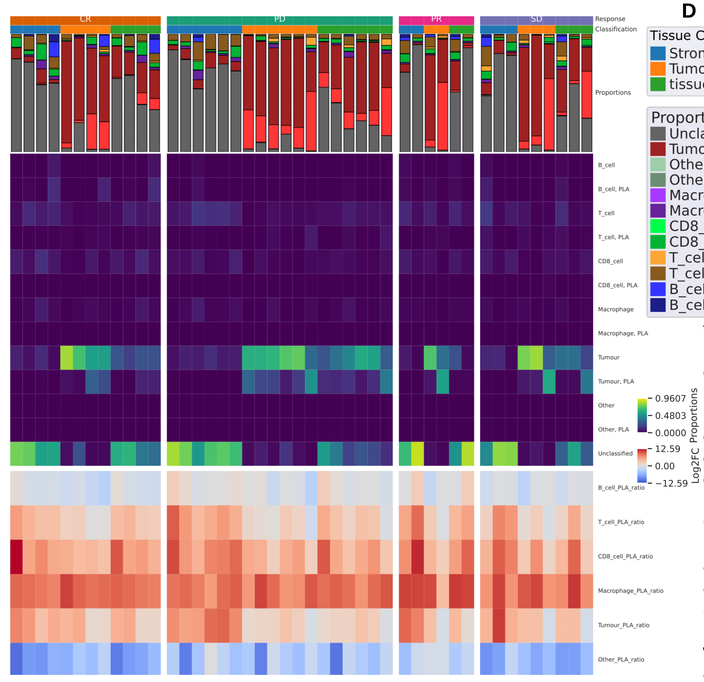
The combined assay was initially validated on tonsil tissue to assess assay compatibility. **(A)** Depicts a tonsil germinal center and surrounding tissue. PD-1/PD-L1 interaction is detected strongly in the germinal center, as expected. The addition of the PhenoCode Signature Immune Profile Panel enables the visualization of the immune landscape in relation to the *is*PLA interaction. **(B-H)** Individual channel breakdowns permit the view of each marker and provide a clear depiction that correlates certain immune cell localization to the *is*PLA signal. For example, within the germinal center, CD20, CD68 and CD3ε positive cells overlap with the interaction signal, hinting their involvement in the checkpoint



5. Functional Proteomics Unveils Novel Insights into HNSCC with Combined PhenoCode-PLA

5. Pathological complete responder vs non-responders





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Tissue Class
- Stroma
- Tumor
- tissue

Proportions
- Unclassified
- Tumour
- Other, PLA
- Other
- Macrophage, PLA
- Macrophage
- CD8_cell, PLA
- CD8_cell
- T_cell, PLA
- T_cell
- B_cell, PLA
- B_cell

(A) Close up of an immune rich region. Here, PD-1/PD-L1 *is*PLA is visualized in the tissue and tumor microenvironment. There is strong overlap in *is*PLA signal with cells positive for CD3ε, CD20, CD8 and CD68. **(B&C)** Using the selected region, cell segmentation was performed for deeper phenotypic analysis. A training image composed of representative areas of each tissue was generated by selecting two 1mm x 1mm areas per tissue. An ANN cell classifier was trained on the training image for each marker to generate positive predictions for cell markers. This classifier was applied to each image and cells were preferentially assigned labels corresponding to the phenotype dictated by their unique marker expression. **(D)** Cell type proportions and *is*PLA positivity in HNSCC immunotherapy cohort according to immunotherapy response and tissue compartment location. Samples are grouped by immunotherapy response, and proportions within the whole tissue, tumor only, or stroma only are shown independently.

6. Multiplexed Immunophenotyping and Functional Interactions: A PhenoCode-*is*PLA Approach

Combining Akoya's PhenoCode Signature Immune Profile Panel with Navinci's *in situ* PLA assay has the potential to reveal novel insights into the intricate cell-to-cell interactions between immune cells, and between immune cells and tumor cells. This combination approach can broaden our understanding of the HNSCC TME by deciphering immune cell composition, spatial interaction, and functional proteomics of these interactions at a molecular level.

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