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Multi-plexed spatial interaction analysis of the tumour microenvironment in head and neck cancers

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Introduction

Head and neck squamous cell carcinoma (HNSCC) remains a significant clinical burden with poor prognosis and limited therapeutic success, particularly in recurrent or metastatic settings. Although immune checkpoint inhibitors (ICIs) targeting PD-1/PD-L1 have led to improved outcomes in a subset of patients, the overall response rates remain modest, underscoring the need for more refined biomarkers to predict therapeutic efficacy and to unravel the underlying mechanisms of immune evasion and activation. Traditional assays measuring protein expression levels of PD-L1 fail to capture the spatial and functional dynamics of immune regulation within the tumor microenvironment (TME). In this study, we sought to enhance spatial resolution and functional interpretation by employing in situ Proximity Ligation Assay (isPLA) technology to detect three distinct cell-cell interactions that underpin immune activation and suppression pathways in HNSCC tissues. Through the application of NaveniFlex™ and Naveni® Plex assays, we visualized spatial interactions between PD-1/PD-L1, PD-1/PD-L2, MHC class I/CD8, and CD3. Whole-slide imaging was performed using the Vectra® Polaris multispectral platform, followed by image registration and alignment via BigWarp landmarking for spatial comparison across serial sections. Cell segmentation and classification were conducted in QuPath, and spatial data were exported for downstream analysis. Custom Python pipelines and spatial statistics were employed to assess cellular neighborhoods, interaction densities, and distance-based relationships to tumor and stromal boundaries, allowing high-resolution mapping of immune hotspots and suppression zones within the TME.

Methods

Formalin-fixed paraffin-embedded (FFPE) whole-tissue sections derived from treatment-naïve HNSCC patients were analyzed prior to initiation of anti-PD-1 immunotherapy. To achieve spatially resolved detection of immune-regulatory protein interactions within the tumor microenvironment (TME), we applied two in situ Proximity Ligation Assay platforms: Naveni® Flex and Naveni® Plex which are capable of simultaneously detecting three distinct protein-protein interactions using fluorophore-specific probes. These assays utilize Navenibodies, proprietary oligo-conjugated affinity reagents that generate fluorescent signals only upon close molecular proximity, thereby enabling highly specific visualization of interacting protein complexes. The following interactions/markers were detected: PD-1/PD-L1, PD-1/PD-L2, MHC class I/CD8, and CD3. Whole-slide imaging was performed using the Vectra® Polaris multispectral platform, followed by image registration and alignment via BigWarp landmarking for spatial comparison across serial sections. Cell segmentation and classification were conducted in QuPath, and spatial data were exported for downstream analysis. Custom Python pipelines and spatial statistics were employed to assess cellular neighborhoods, interaction densities, and distance-based relationships to tumor and stromal boundaries, allowing high-resolution mapping of immune hotspots and suppression zones within the TME.

Methods

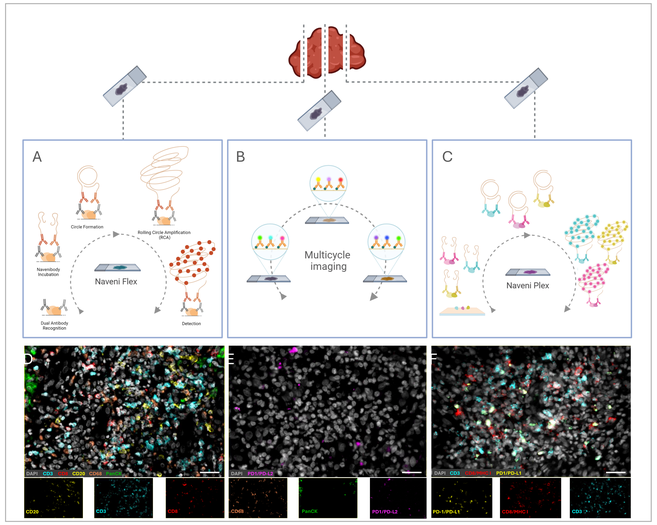


Figure 1: A, C) Naveni Flex and Naveni Plex workflow and **B)** Spatial phenotyping of the tumor serial sections using Phenocycler Fusion system. **D-F)** Three serial sections stained Naveni Flex, Naveni Plex and a multiplex immune panel imaged using the Phenocycler-Fusion. Scale=50 µm **G)** Spatially alignment of images using landmark-based registration in BigWarp (Fiji) to enable cross-panel comparison of spatial features. **H)** Aligned isPLA and multiplex images were analyzed using a deep learning model to identify regions enriched in interactions (hotspots). Proximity edges were then assigned between interacting immune and tumor cells within these hotspots to map cell–cell interactions networks.

Results

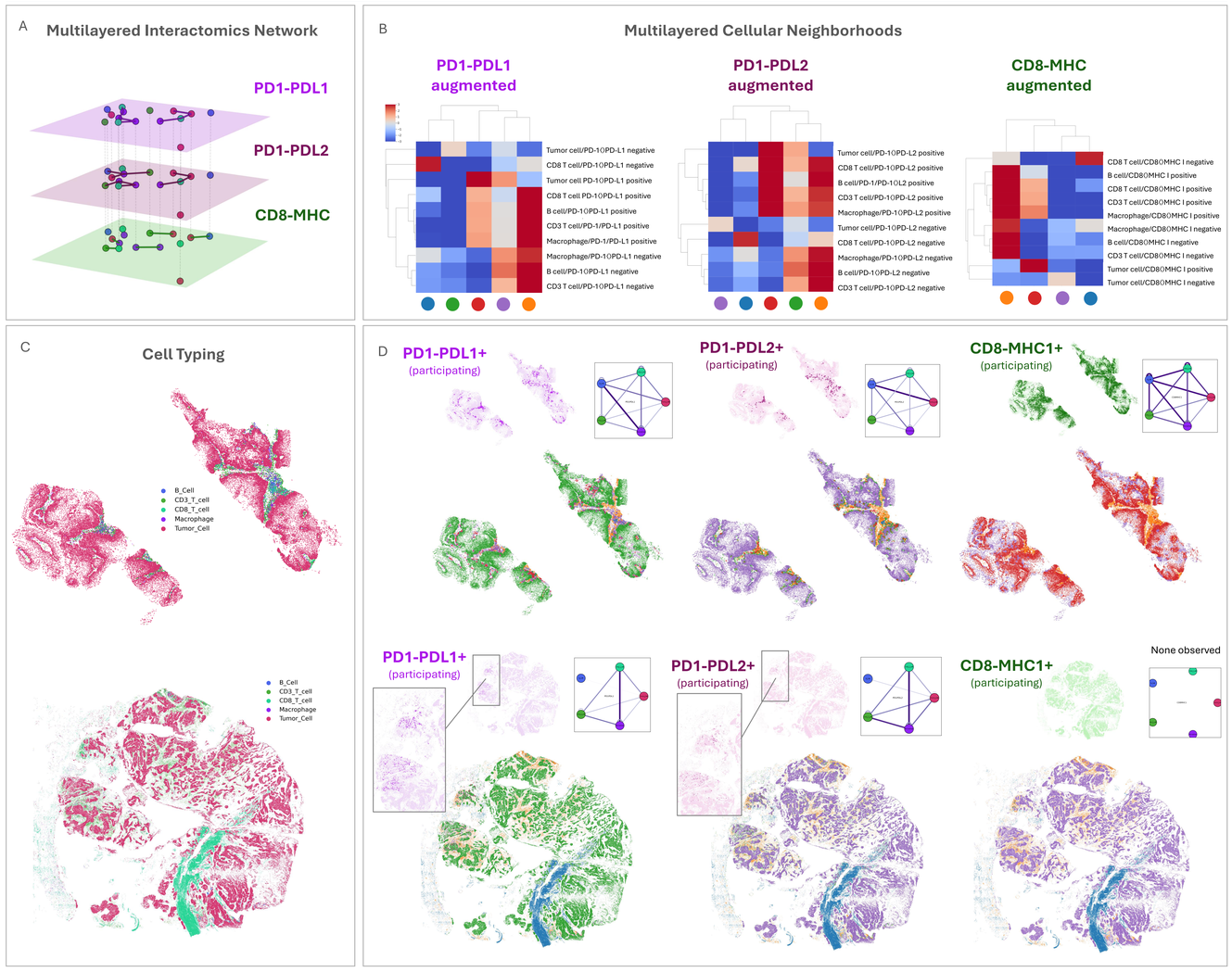


Figure 2: **A)** By identifying the same cells across registered serial sections using multilayered interactomics network, we integrated spatial data from isPLA and multiplex panels to uncover multiple interaction modalities. **B)** A neighborhood clustering heatmap derived from the spatial distribution patterns of various cell types. **C)** Cell typing analysis **D)** Cellular neighborhoods in relation to the clustering algorithm across three different interaction modalities.

Conclusion

By integrating three spatial assays across serial HNSCC tissue sections, we mapped distinct immune activation and suppression interactions within the tumor microenvironment. This multilayered spatial approach reveals immune interaction patterns that may support improved patient stratification and guide personalized immunotherapy strategies.

Acknowledgement

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