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Spatial interaction mapping of PD-1/PD-L1 in Head and Neck Cancer reveals the role of Macrophage-Tumour Barriers associated with immunotherapy response

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INTRODUCTION

Head and neck cancer continues to be a global health challenge, with an estimated 50% mortality rate projected for 2024. While immune checkpoint inhibitors (ICIs), like pembrolizumab and nivolumab, have shown promise in extending survival, their effectiveness remains limited, particularly in PD-1/PD-L1 blockade therapies. Traditional measures, such as PD-L1 protein expression, offer only modest predictive value for ICI therapy success. To address this, we employed a novel approach, mapping PD-1/PD-L1 interactions in pre-treatment tissue samples from head and neck squamous cell carcinoma (HNSCC) patients undergoing ICI therapy. Using a combination of high-plex spatial proteomics and low-plex protein spatial profiling (PSP) panels alongside *in situ* proximity ligation assays (isPLA), we visualized these interactions within the tumor microenvironment (TME).

METHODS

Formalin-fixed paraffin-embedded (FFPE) tissue sections from 36 pre-treatment HNSCC patients who received immune checkpoint inhibitors were analyzed. PD-1/PD-L1 interactions were detected using the Naveni® in situ Proximity Ligation Assay (isPLA), performed as the initial step on whole tissue sections. Following isPLA detection, serial sections from the same tissue blocks were processed using the Akoya Biosciences Phenocycler-Fusion (PCF) platform with a custom 53-plex antibody panel to characterize immune and tumor cell phenotypes. This combined approach enabled spatial co-registration of PD-1/PD-L1 interaction signals with detailed immune context, providing a comprehensive map of functional cell–cell interactions within the tumor microenvironment.

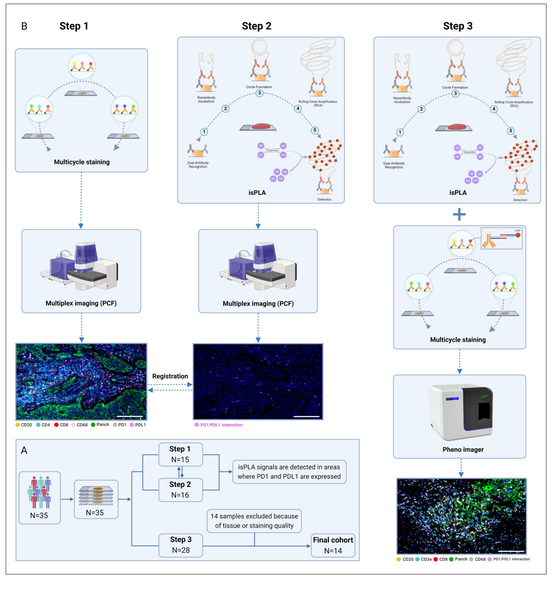


Figure 1. A) A cohort of 35 HNSCC patients was selected for analysis. **B)** Steps 1 and 2 ensure isPLA signals are aligned with PD-1 and PD-L1 expression. In step 3, 28 serial sections were stained with a 6-plex panel.

RESULTS

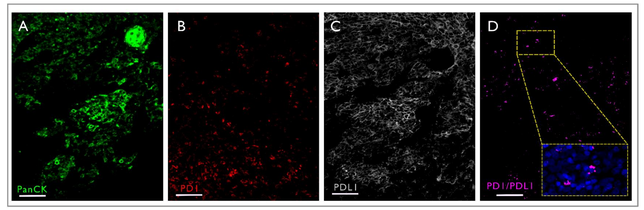


Figure 2. A-C) IF channels for PanCK, PD-1, and PD-L1, compared to panel D, which highlights positive isPLA signals indicating PD-1/PD-L1 interactions. Scale = 50 µm. Green=tumor cells (PanCK), Red=PD-1⁺ immune cells, White=PD-L1⁺ tumor cells, Pink=PD-1/PD-L1 ligation.

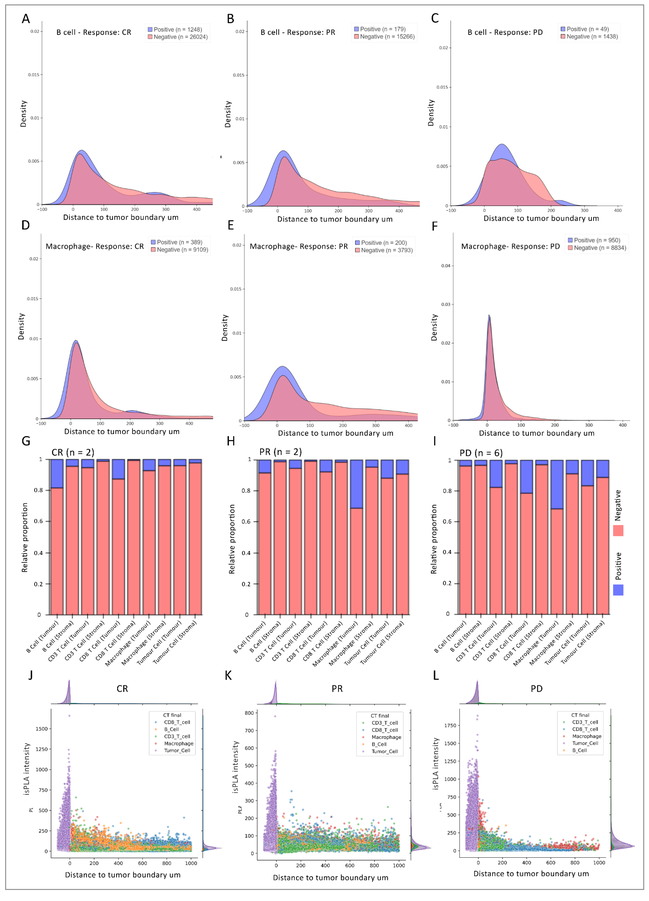


Figure 3. A-F) Display density plots showing the distance to the tumor boundary, cell count, and isPLA status for B cells **(A-C)** and macrophages **(D-F)** across response groups, normalized for comparison. **G-I)** comparison of isPLA-positive cell types per region. **J-L)** Distance to tumor scatter plots against response and isPLA intensity.

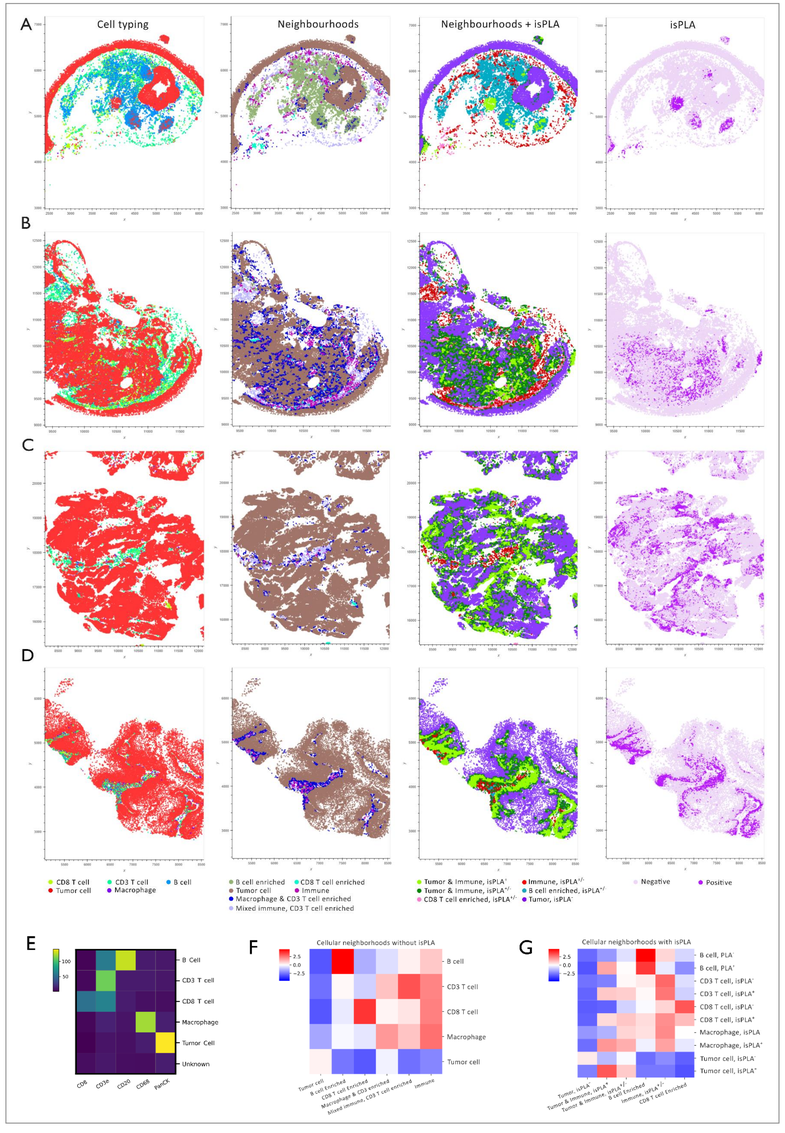


Figure 4. A-D) Cell typing and cellular neighbourhoods across response groups (CR **(A)**, PR **(B)**, SD **(C)**, PD **(D)**). Comparison of cellular neighbourhoods with and without including isPLA+ clusters showing a different isPLA+ neighbourhoods distribution across different response groups. **E)** The cell typing heatmap shows the expression markers' intensity vs. proposed cell types. **F, G)** Cell types were clustered into unsupervised cellular neighbourhoods according to their nearest neighbours. The heatmap shows an abundance of cell types in each neighbourhood with and without including isPLA positivity.

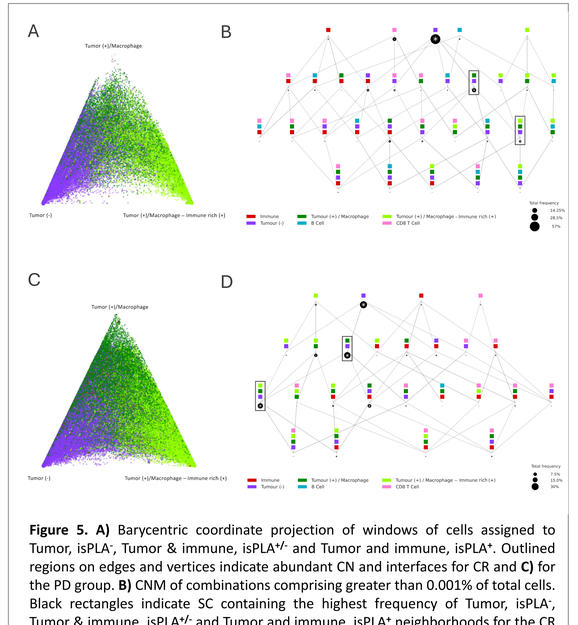


Figure 5. A) Barycentric coordinate projection of windows of cells assigned to Tumor, isPLA⁻, Tumor & immune, isPLA$^{+/-}$ and Tumor and immune, isPLA⁺. Outlined regions on edges and vertices indicate abundant CN and interfaces for CR and **C)** for the PD group. **B)** CNM of combinations comprising greater than 0.001% of total cells. Black rectangles indicate SC containing the highest frequency of Tumor, isPLA⁻, Tumor & immune, isPLA$^{+/-}$ and Tumor and immune, isPLA⁺ neighborhoods for the CR group and **D)** for the PD group.

CONCLUSION

This study presents the first in situ spatial mapping of PD-1/PD-L1 interactions in pre-treatment head and neck squamous cell carcinoma (HNSCC) tissues using a combination of proximity ligation assay (isPLA) and high-plex spatial proteomics. By integrating these two platforms, we identified spatially resolved PD-1/PD-L1 interactions and linked them to the underlying immune architecture. Our findings reveal that the distribution and intensity of PD-1/PD-L1 interactions, particularly in the form of isPLA+ tumor-macrophage interfaces at the tumor-stroma boundary, are associated with resistance to immune checkpoint inhibitor (ICI) therapy. In contrast, complete and partial responders were characterized by isPLA+ aggregates of B and T cells forming organized immune neighborhoods at tumor margins. These distinct spatial configurations highlight the importance of tissue context in determining immunotherapy outcomes. While further validation across larger cohorts and cancer types is warranted, this approach lays the groundwork for improving patient stratification, guiding therapeutic decision-making, and advancing biomarker development through spatially resolved functional assays.

ACKNOWLEDGEMENT

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