



Navinci AACR 2024 Poster Collection

1525/12: Integration of High-plex Tumor-immune Phenotyping and Checkpoint Interactions for Deeper Spatial Characterization of Human Cancer Tissues

Sara Bodbin¹, Amelie Viratham², Robert Stad², Ning Ma², Aditya Pratapa², Nadezhda Nikulina², James Monkman³, Rafael Tubelleza³, Niyati Jhaveri², Hampus Elofsson¹, Subham Basu¹, Agata Zieber-Wicher¹, Arutha Kulasinghe³

¹Navinci, Uppsala, Sweden; ²Akoya Biosciences, Menlo Park, California, USA; ³University of Queensland, Brisbane, AU

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 single-plex 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 (Phenolmager[®] 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

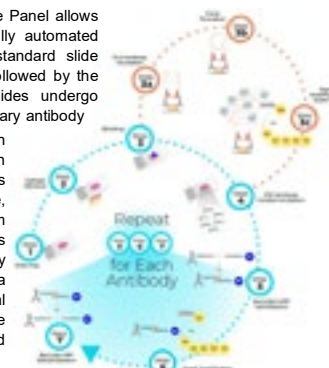
Tonsil tissue and biopsies from patients with mucosal head and neck squamous cell carcinoma (HNSCC) enrolled in immune checkpoint inhibitor therapies were phenotyped using method integration of Naveni[®] PD-1/PD-L1 proximity ligation assay (PLA) with the flexible high-plex PhenoCode[™] Signature Immune Panel (CD3e/CD8/CD20/CD68/Pan-CK) (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 Phenolmager[®] HT 2.0 and inForm software, respectively.

Spectrally unmixed PSP/isPLA 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 Panel combined with Naveni[®] isPLA 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.

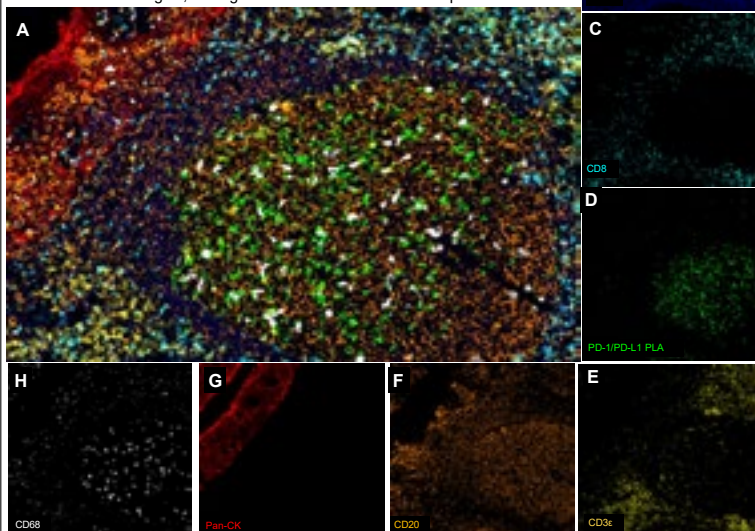
3. Method of Technology Integration

The flexible aspect of the PhenoCode[™] Signature Immune 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 antibody 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.



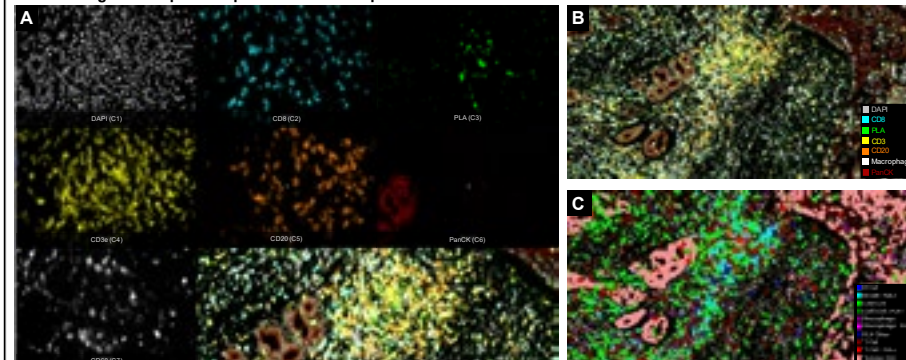
4. Validation of Technology Integration

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 Panel enables the visualization of the immune landscape in relation to the isPLA 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 isPLA signal. For example, within the germinal center, CD20, CD68 and CD3e positive cells overlap with the interaction signal, hinting their involvement in the checkpoint activation.



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

5. Pathological complete responder vs non-responders



(A) Close up of an immune rich region. Here, PD-1/PD-L1 isPLA is visualized in the tissue and tumor microenvironment. There is strong overlap in isPLA signal with cells positive for CD3e, 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 isPLA 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-isPLA Approach

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



High-resolution analysis of immune checkpoint activation utilizing a combined PD1/PD-L1 *in situ* proximity ligation assay (isPLA) and multiplex immunofluorescence (mIF) imaging approach

Arne Christians¹, Jannik Boog¹, Charles E. Jackson², Matthew H. Ingalls², J. Spencer Schwarz², Sara Bodbin³, Agata Zieba Wicher³, Subham Basu³, Oliver Braubach²
Canopy Biosciences® - A Bruker Company, ¹St. Louis, Missouri, USA; ²Hannover, Germany; ³Navinci Diagnostics, Uppsala, Sweden

CellScape™ Precise Spatial Multiplexing



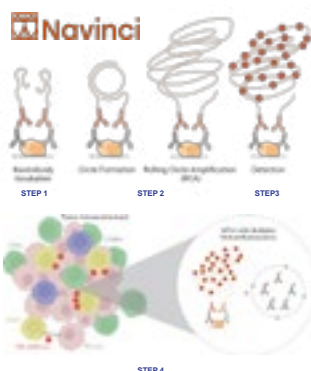
The CellScape platform enables automated cyclic mIF for quantitative spatial phenotyping.

VistaPlex Spatial Immune Profiling Biomarkers			isPLA probes	
CD3	CD68	KI-67	PD-1	PD-L1
CD4	CD45	PD-L1	Additional Biomarkers	
CD8	CD45RA	Pan-CK		
CD20	CD45RO	Gm B	CD11c	CD27
FoxP3	PD-1	DNA	Vimentin	

VistaPlex™ Multiplex Assay Kits are ready-to-use validated antibody panels for comprehensive phenotyping of the tumor microenvironment. The kits are modular and can be combined with other commercially available and custom antibodies.

The Navinci *in situ* Proximity Ligation Assay (isPLA) was used to visualize protein-protein interactions between PD-1 and PD-L1 along with the Spatial Immune Profiling kit and three additional antibodies to identify cell populations.

isPLA on the CellScape Platform



1. FFPE tissues were prepared for the CellScape workflow and stained with anti-PD-1 & PD-L1; primary antibodies were labeled with oligo-modified Navenbody probes
2. Oligos in close proximity formed a circular DNA strand that acted as a template for rolling circle amplification
3. HRP-labeled probes were used to visualize the interaction through fluorescent TSA (AF488-Tyramide)
4. After imaging the isPLA signal, mIF was performed with the standard CellScape workflow

Validation of Combined VistaPlex mIF and isPLA Staining

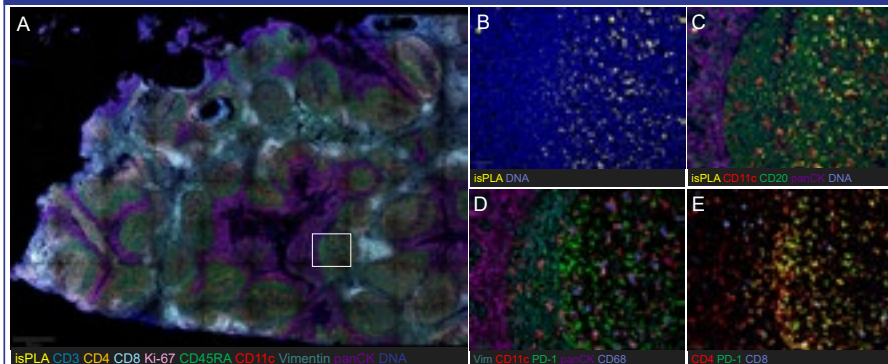


Figure 1: Combined isPLA and VistaPlex staining on FFPE tonsil tissue. (A) Whole slide overview image. The isPLA signal is most prevalent in germinal center regions. The marked germinal center is shown at higher magnification in B-E. (B - E) The isPLA signal is strongest in germinal center areas with CD11c+CD68+Vim+ follicular dendritic cells and PD-1-expressing follicular CD4+ Helper T cells.

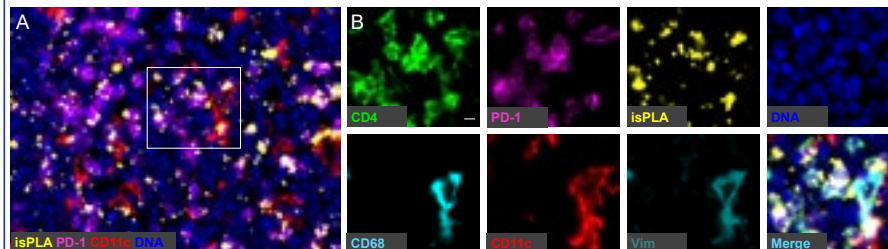


Figure 2: CellScape high resolution imaging allows precise identification of cells involved in PD-1/PD-L1 protein-protein interactions. Image (A) shows the same region as Fig. 1 B-E. The marked area is expanded in subcellular magnification in B. (B) Expression of CD11c, CD68 & Vimentin is a hallmark of follicular dendritic cells. FDCs interact with follicular helper T & B cells to regulate immune responses and prevent autoimmunity.

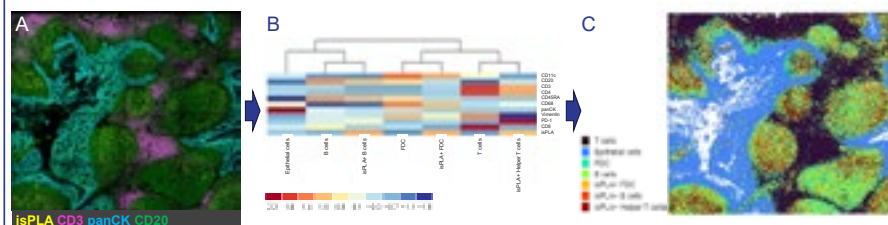


Figure 3: Bioinformatic analysis of isPLA / mIF data. (A) Images are exported as 32-bit OME TIFF files compatible with image analysis platforms, like Enable Medicine that was used here. Cell segmentation was performed with StarDist and followed up with unsupervised clustering analysis for cell phenotyping. (B) Cluster analysis shows high correlation of isPLA signal with CD4 & PD-1 and CD11c & CD68. (C) Precise spatial mapping of cell populations of interest with the isPLA signal shown in orange to red.

Combined VistaPlex mIF and isPLA Staining on Tumor Tissue

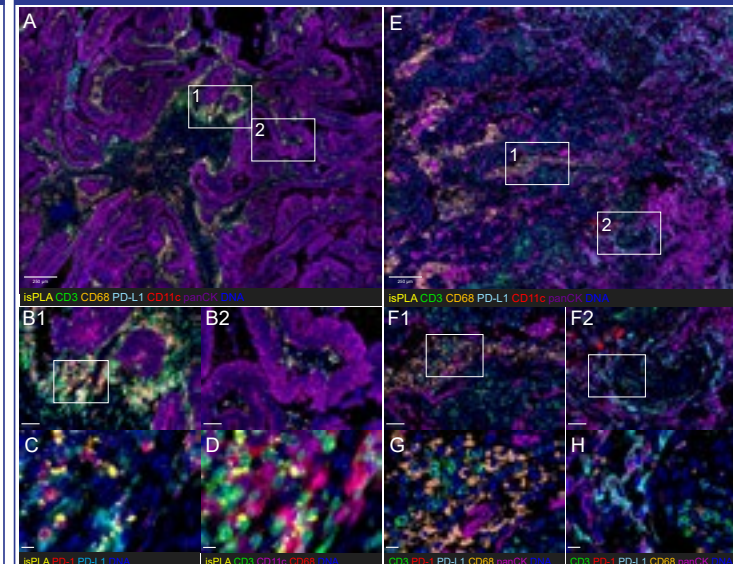


Figure 4: Differential isPLA signal in cancer tissues. (A) mIF immune profiling combined with isPLA on colorectal carcinoma. (B1-B2) PD-L1 staining is not present on tumor cells, but on certain immune cell populations in the stroma. These regions also show isPLA signal, suggesting PD-1/PD-L1 interaction and checkpoint activation. (C) Marked region in B1 shows an immune cell cluster with staining of PD-1 and PD-L1, correlating with an isPLA signal. (D) PD-1+ cells are mostly CD3+, while PD-L1+ cells show staining for CD11c and CD68. This suggests that a potential T-cell anti-tumoral response is suppressed by tumor-associated macrophages. (E) mIF/isPLA dual staining on a PD-L1+ lung cancer. (F1-F2) Approximately 40% of tumor cells are PD-L1+ and CD68+ cells were abundant. (G-H) No PD-1 staining was observed in different tumor regions and, consistently, no isPLA signal. This suggests lacking PD-1/PD-L1 interactions and no checkpoint activation, despite tumor PD-L1 positivity.

Conclusions

- High resolution imaging of immune checkpoint activation utilizing an *in situ* Proximity Ligation Assay and Precise Spatial Multiplexing on the CellScape platform uncovers differential tumor-immune cell regulation in cancer tissues.
- The isPLA signal was consistent with both fluorescent antibody-mediated biomarker localization and known immune cell interactions.
- isPLA enables an additional "interactome" readout for CellScape that provides comprehensive insight into specific interactions of biomarkers and cell populations in both tumor and non-tumor contexts.

References

1. Heesters, B. A., et al. (2021). Characterization of human FDCs reveals regulation of T cells and antigen presentation to B cells. *The Journal of experimental medicine*. 218(10).
2. Sage, P. T., et al. (2016). Dendritic Cell PD-L1 Limits Autoimmunity and Follicular T Cell Differentiation and Function. *Journal of Immunology* (Baltimore, Md. : 1950), 200(8), 2592-2602.

Contact

For more information, contact
hello.canopy@bruker.com
Or visit CanopyBiosciences.com



Contact us or
request a quote

A spatial interactomics approach reveals interplay between the tumor microenvironment and T cell activation

Authors
Desirée Edén, Jonas Vennberg, Tanja Büsgen, Doroteya Raykova, Agata Zieba-Wicher

Navinci Diagnostics, Research and Development, Uppsala, Sweden. contact@navinci.se



Introduction

T cells play a fundamental role in immune response to tumors. However, the tumor microenvironment (TME) – a complex network of cells, signaling molecules, and extracellular matrix components – employs various strategies to evade immune surveillance and suppress T cell activity. Among the many molecular interactions taking place in the TME, the role of CD8/MHC-I, LAG3/MHC-II and PD1/PDL1 is pivotal in modulating anti-cancer response and the escape routes that cancer in turn uses to withstand immune defense. LAG3/MHC-II and PD1/PDL1 interactions are inhibitory, causing T cell dysfunction and exhaustion, while the CD8/MHC-I interaction is essential for initiating an effective anti-tumor response. Here, we used a cutting-edge *in situ* proximity ligation strategy combined with immunofluorescence (IF) to reveal these key interactions and TME biomarkers in hepatocellular carcinoma and Hodgkin lymphoma. Since the method does not compromise the structural integrity of tissues, communication between proteins can be studied in their native milieu.

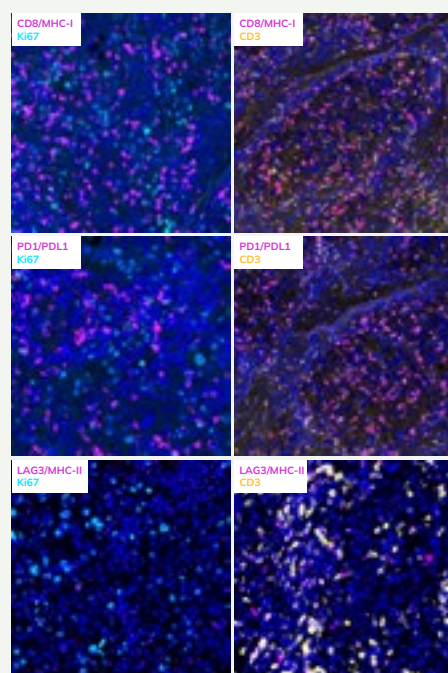


Fig. 2. FFPE sections of hepatocellular carcinoma show T cell priming and activation of inhibitory pathways in the TME. Liver cancer samples were highly positive for primed CD8+ T cells (as evidenced by the abundant CD8/MHC-I interaction) and for the PD1/PDL1 interaction, whereas the interaction between LAG3 and MHC-II was sparser. High proliferation was demonstrated with Ki67 IF co-staining. T cells were visualized with CD3 IF co-staining.

Results

All hepatocellular carcinoma stainings (Fig. 2) originated from the same tissue block. The tumor microenvironment was visualized by either Ki67 (a proliferation marker indicative of the aggressiveness of the tumor) or CD3 (a general T cell marker) as shown by IF. The studied samples were of "hot" tumors with high proliferation activity. The presence of active and functional CD8+ cells was evident by the high number of CD8/MHC-I interactions detected with NaveniFlex™ Tissue. At the same time, inhibitory pathways like PD1/PDL1 and LAG3/MHC-II were also activated. The proximity between the Ki67+ cells, CD3+ cells and the inhibitory interactions suggests crosstalk between proliferating tumor cells and the immune system.

In the case of Hodgkin lymphoma (Fig. 3), cytotoxic T cells (positive for CD8/MHC-I interaction) and cells undergoing immune checkpoint activation (positive for the PD1/PDL1 interaction) were detected in similar areas, whereas in healthy tissue (tonsil, data not shown) the opposite pattern is observed. CD3 co-staining partially overlapped with interaction signals, indicating the presence of immune cells in the TME and the possible crosstalk between them and cancer cells.

Technology and methods

Protein-protein interactions (PPIs) were detected with the NaveniFlex™ Tissue Atto647N kit in human FFPE tissues according to the manufacturer's protocol. This technique can detect proteins located within interaction range (<40 nm) via oligonucleotide-antibody conjugates that create amplified fluorescent signal. Parallel IF co-staining for Ki67 and CD3 was performed during the Naveni® detection step 5 in Fig. 1A. Slides were mounted and imaged on the Olympus VS200 automated scanner.

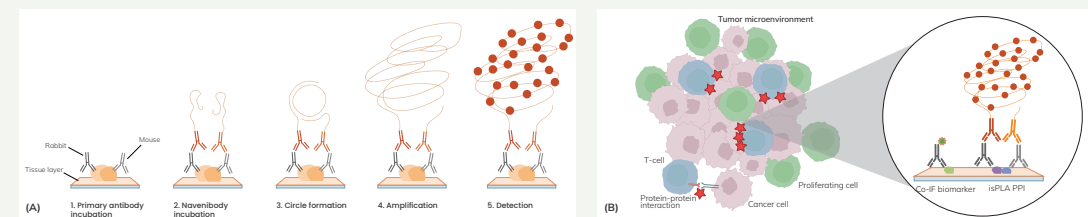


Fig. 1. Schematic depiction of the Naveni® technology for PPI detection and the integration of IF for co-staining of single biomarkers.

(A) Outline of the NaveniFlex™ Tissue workflow, demonstrating the binding of target-specific primary antibodies, Navenibodies, circle formation when the Navenibodies are bound within interaction range, amplification and detection of the fluorescent signal in Atto647N. (B) Illustration of the TME, showing interactions detected by the Naveni® *in situ* proximity ligation assay (isPLA) and individual markers detected by co-IF.

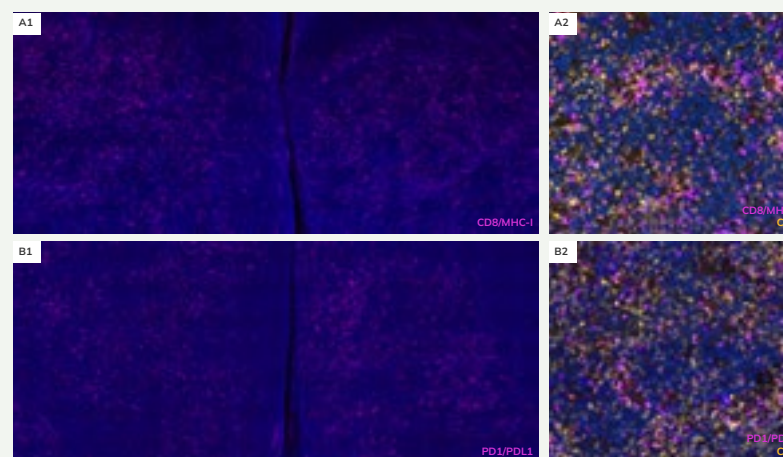


Fig. 3. FFPE sections of Hodgkin lymphoma show activation of the PD1/PDL1 axis along with potent cytotoxic T cell activation.

Images A1 and B1 show overview (20x magnification) of the tissue staining for the CD8/MHC-I and the PD1/PDL1 interactions, respectively (magenta). A2 is a zoom-in of A1, including anti-CD3 IF co-staining (yellow). T cell activation is demonstrated by the abundant interaction staining. As expected, CD8/MHC-I partially coincides with CD3 staining as it represents a sub-population of T cells. B2 is a zoom-in of B1, including anti-CD3 IF co-staining (yellow). The powerful activation of the PD1/PDL1 axis is evident by the strong interaction stain (magenta) and is adjacent to T cells, indicating a "hot" tumor with strong immune system involvement.

Conclusion

The interactomics approach, employing *in situ* proximity ligation for observing protein function as opposed to mere expression, allows the detection of key PPIs in the TME. Understanding the intricate crosstalk between them is crucial in developing effective immunotherapies. Targeting these pathways simultaneously or in combination, such as using immune checkpoint inhibitors against PD1/PDL1 while exploring strategies to modulate LAG3/MHC-II and CD8/MHC-I interactions, holds promise in reinvigorating T cell responses against cancer and improving the efficacy of immunotherapies within the complex landscape of the TME.

Have more questions?
contact@navinci.se

Download the poster here:



Visit
www.navinci.se

Exploring the role of the MSLN/MUC16 interaction in cancer progression via the *in situ* proximity ligation technique

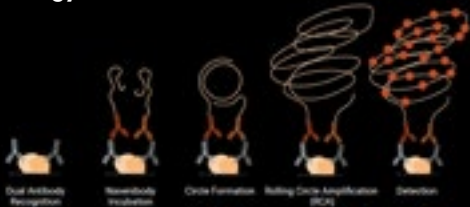
Authors
Sara Bodbin, [Doroteya Raykova](#), Agata Zieba-Wicher
Navinci Diagnostics, Research and Development, Uppsala, Sweden. contact@navinci.se



Introduction

Mesothelin (MSLN) is a glycoprotein present on the surface of mesothelial cells, sometimes used as a tumor marker. Its overexpression in various cancers, including mesothelioma, ovarian, pancreatic, and lung, is linked to increased tumor aggressiveness, evasion of apoptosis, metastasis, and resistance to treatments. Mucin16 (MUC16) belongs to a family of proteins that play a crucial role in cell adhesion, signaling, and immune responses. In cancer, alterations in mucin expression and structure may contribute to tumor progression and metastasis by enhancing cell survival and immune evasion. Mesothelin is known to interact with mucins, influencing tumor cell behavior and metastatic potential. More specifically, the MSLN/MUC16 interaction facilitates tumor cell invasion and peritoneal spread in ovarian cancer. However, reliable molecular tools to study protein interactions have been scarce. Here, we applied the Naveni® *in situ* proximity ligation technology to identify MSLN/MUC16 in various cancer samples. The method detects proteins located within interaction range (<40 nm) by means of antibodies conjugated to oligonucleotides that create amplified fluorescent signal. Since the technique does not compromise the structural integrity of tissues, communication between proteins can be studied in their native environment.

Technology and methods



Human FFPE tissue microarrays (TMA) with ovarian primary carcinoma and matched metastatic cancer (80 cores) and ovarian cancer tissue with normal tissue (100 cores) were deparaffinized and subjected to standard heat-induced antigen retrieval and blocking. Staining against the MSLN/MUC16 interaction was performed with the NaveniFlex™ Tissue MR Atto647N kit (Navinci Diagnostics). Co-staining antibodies against pan-Cytokeratin, Ki67 or MMP1 were included in the detection mix as indicated. Immunofluorescence (IF), using the same MSLN and MUC16 primary antibodies, was completed on an additional slide of each TMA, and then secondary antibodies conjugated to Alexa Fluor™ 488 and 594 were used for detection. Imaging was performed on an Olympus scanner VS200.

Conclusion

In this study, we showed that the interaction is abundant in ovarian cancer, with a distinguishable difference in the signal presence between cancerous and normal adjacent tissue. Furthermore, the additional tumor marker co-staining provide insights to the tumor invasion and growth. When comparing the Naveni® signal of MSLN/MUC16 against IF staining of each, the Naveni® signal shows true interactions, as opposed to individual expression patterns. Understanding the roles of mesothelin and mucin in cancer, both individually and in interaction, is crucial for developing targeted therapies and diagnostic tools. Further research on their interaction and specific molecular pathways triggered by it is essential for devising effective strategies to combat cancer progression.

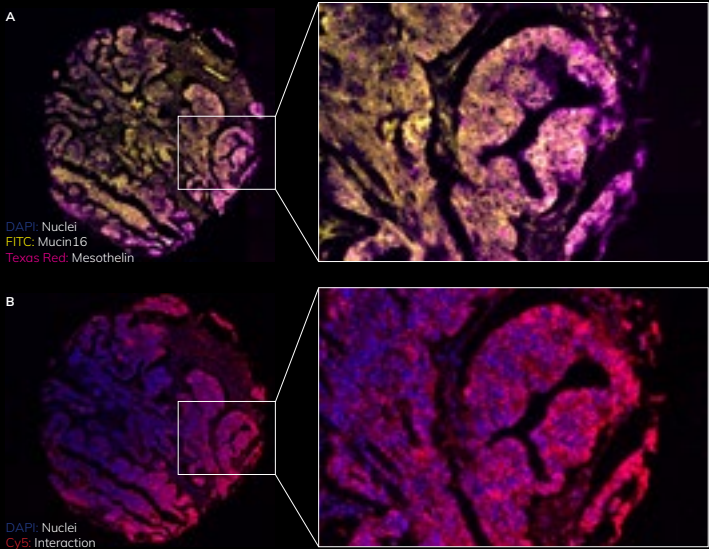


Fig. 1. Comparison between expression of MUC16 and MSLN and their interaction levels in ovarian cancer TMA cores. (A) Immunofluorescent staining against Mucin16 (yellow) and Mesothelin (magenta). The zoomed-in section shows an area of high overlap of fluorescent signals (white) suggesting but not proving interaction between the two proteins. (B) Mucin16/Mesothelin interaction (red) detected with NaveniFlex™ Tissue. Although the individual expression patterns of the two proteins largely overlap with the isPLA staining, IF co-expression is not a synonym for interaction. The zoomed-in area shows the same region as in (A) with a lot of interactions visible.

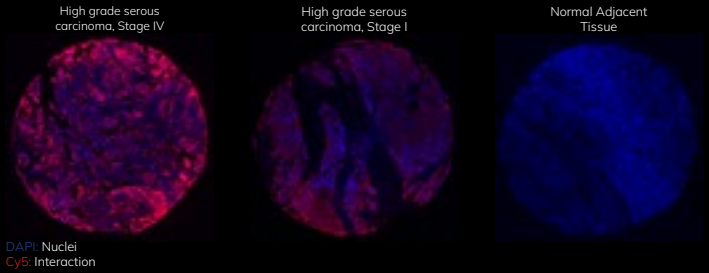


Fig. 2. Comparison of MUC16/MSLN interaction levels in different disease stages compared to normal adjacent tissue. High grade serous ovarian carcinoma cores in different stages and a normal adjacent tissue core were stained for the Mucin16/Mesothelin interaction and variable degrees were observable. Although additional studies are needed in order to make a definitive conclusion, the staining patterns suggest that the severity of disease is positively correlated to the number of interactions detected (core on the far left vs middle core) and thus healthy tissue (core on the far right) is negative for the interaction.

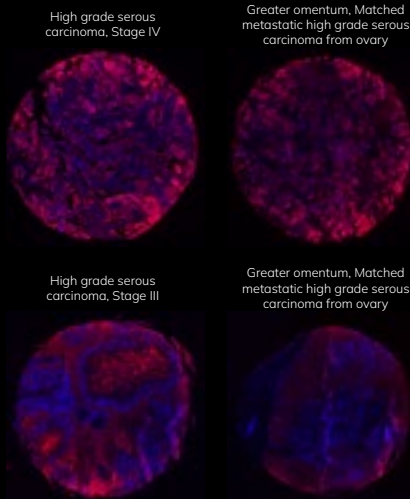


Fig. 3. MUC16/MSLN interaction levels in primary and metastatic high grade serous carcinoma of different stages. Metastasis of ovarian cancer to the peritoneal cavity can occur in an alternative manner compared to classic hematogenous metastasis seen in many solid cancers. Ovarian cancer cells detach from the main tumour and form spheroids that survive and recruit specific cell types to create a specialized TME that they can survive in. They then metastasise predominantly to the peritoneal milieu. We compared stage III (below) and stage IV (above) high grade serous ovarian carcinoma to a matched metastatic carcinoma in the greater omentum and observed similar levels of interaction between the primary and secondary site tumors.

Have more questions?
contact@navinci.se

Download the poster here
Visit www.navinci.se

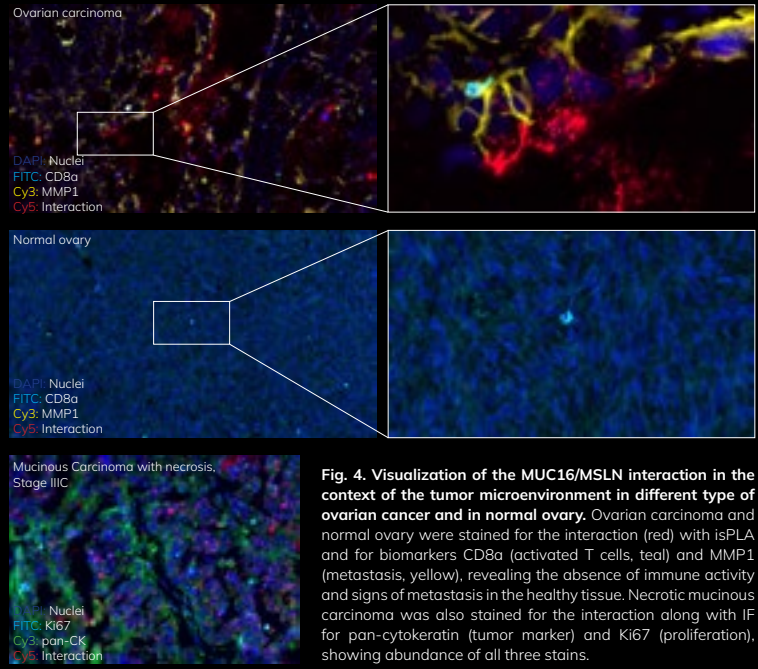


Fig. 4. Visualization of the MUC16/MSLN interaction in the context of the tumor microenvironment in different type of ovarian cancer and in normal ovary. Ovarian carcinoma and normal ovary were stained for the interaction (red) with isPLA and for biomarkers CD8a (activated T cells, teal) and MMP1 (metastasis, yellow), revealing the absence of immune activity and signs of metastasis in the healthy tissue. Necrotic mucinous carcinoma was also stained for the interaction along with IF for pan-cytokeratin (tumor marker) and Ki67 (proliferation), showing abundance of all three stains.

Integrating in situ Proximity Ligation Assay for PD-1/PD-L1 interaction with Multiplexed Immunofluorescence Imaging in Urothelial Carcinoma

Abstract # 4673

Tony Ullman¹, David Krantz², Hampus Elofsson³, Maria Lung², Nima Rafati⁴, Carolina Oses Sepulveda¹, Agata Zieba-Wicher³, Päivi Östling³, Anders Ullén³, Subham Basu¹, Charlotte Stadler¹

1. KTH - SciLifeLab, Dept of Protein Science, Stockholm, Sweden 2 Karolinska Institutet, Dept of Oncology-Pathology, Stockholm, Sweden. 3 Navinci Diagnostics, Uppsala, Sweden 4. Uppsala University, NBIS



Scan for a digital poster!

1. Introduction

The PD-1/PD-L1 axis plays a crucial role in immune checkpoint regulation, and blocking this interaction is the target for many immune checkpoint inhibition (ICI) therapies used to treat a broad range of solid tumors. Despite the potential, therapeutic interventions targeting this pathway have exhibited limited efficacy, with approximately 20-30% response rates in advanced urothelial carcinoma patients (1). Today, patients with metastatic disease are eligible for PD-1/PD-L1 immunotherapy treatment, and first line treatment with ICI is based on the PD-L1 status alone, neglecting the complex spatial heterogeneity in patient samples. We hypothesize that the interaction between immune cells and tumor cells via the PD-1/PD-L1 axis is a better prognostic marker for response to ICI therapy. *In situ* Proximity Ligation Assay (isPLA), commercialized by Navinci Diagnostics, is a method that allows for detection of protein interactions *in situ*, making use of two antibodies for the respective protein targets. If close in proximity (less than 40 nm apart), a rolling circle amplification product is formed that can be visualized by fluorescence microscopy and thereby detect interactions between the target proteins (2). Using isPLA to target PD-1/PD-L1 the level of PD-1/PD-L1 interactions between individual cells can be explored. If combined with multiplexed immunofluorescence, the interactions can also be assigned to specific cell types such as the tumor cells and different types of immune cells. In addition, the overall tumor microenvironment can be characterized, including detection of tertiary lymphoid structures that also has shown to be an interesting parameter for better response prediction to therapies.

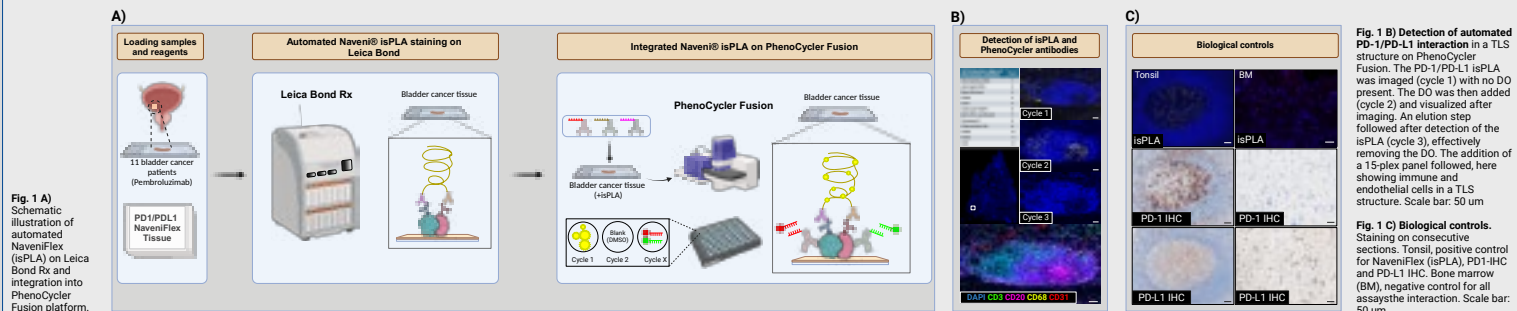
By integrating isPLA and multiplexed immunofluorescence, we aim to enhance the predictive accuracy of treatments and improve patient stratification, paving the way for more personalized and effective cancer therapies.

Aim 1. To develop an integrated assay to detect protein interactions between individual cells along with multiplexed immunofluorescence to phenotype different cell types and structures in the tumor microenvironment (TME). In this study, we have successfully integrated isPLA using Navinci Diagnostics NaveniFlex kit for PD-1/PD-L1, and multiplexed immunofluorescence using the PhenoCycler technology from Akoya Biosciences, all in one single experiment. This allows for fully automated data generation in a relatively high-throughput manner, with output data as one integrated dataset for further downstream analysis.

Aim 2. To apply the method developed in Aim 1 on a retrospective cohort of muscle invasive bladder cancer treated with the ICI therapy Pembrolizumab, to explore the possible correlation between the degree of PD-1/PD-L1 interactions with clinical endpoints. The PEMBRO-100 cohort consists of >100 patients with well defined clinical data. So far isPLA data for 11 patients has been generated, representing complete responders and non-responders and serving as proof-of-concept data set for exploring the correlation between PD-1/PD-L1 interactions and clinical endpoints.

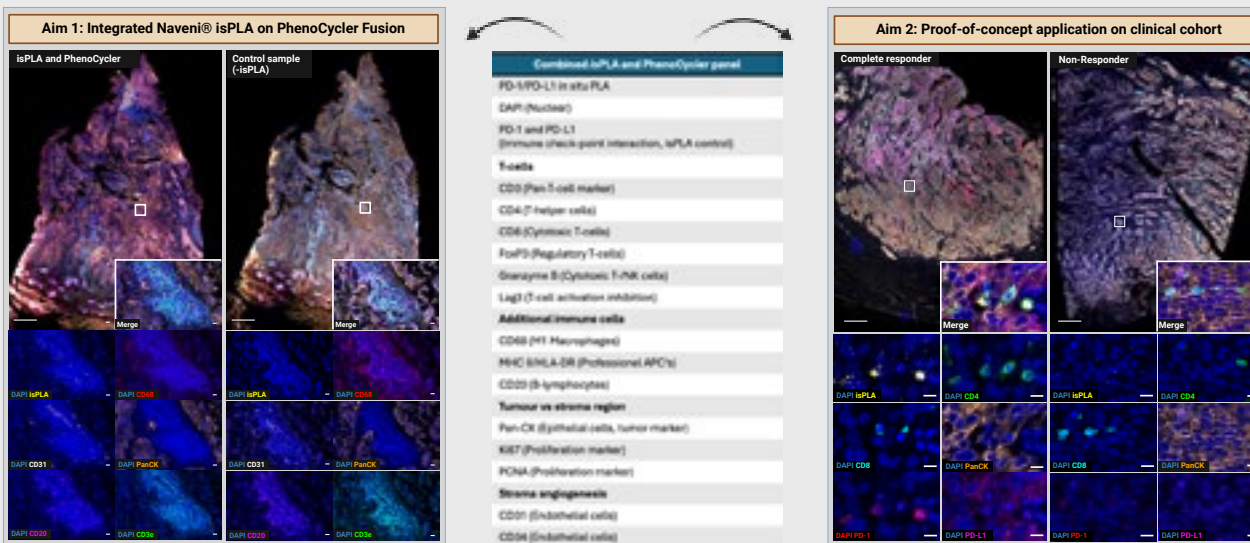
2. Method and Technology integration

Formalin-fixed Paraffin-Embedded (FFPE) tissue samples were obtained from the retrospective PEMBRO-100 cohort of muscle invasive bladder cancer patients. The tissue sections were heated at 60°C for 40 minutes, then placed into a Leica Bond RX machine along with the Naveni[®] reagent, which is designed for automated processes. Within the Leica Bond device, the tissue samples underwent dewaxing and antigen retrieval using ER2 solution for 40 minutes at 100°C. The system was set up to execute the Naveni[®] sequence, encompassing steps such as blockage, incubation with both primary antibody and Navenibody, ligation, and rolling circle amplification. Following this, multiplexed immunofluorescence was generated using the PhenoCycler technology from Akoya Biosciences. By adding the detection oligos (DO) for the isPLA in the first cycle of the PhenoCycler assay, a fully integrated dataset of PD-1/PD-L1 protein interaction and the protein markers from a 15-plex antibody panel was generated on the PhenoCycler Fusion instrument (Fig. 1 A). The image datasets were analyzed using Q-path, focusing primarily on the PD-1/PD-L1 isPLA distribution along with phenotyping of the interacting cell types (Fig. 1B). Biological controls such as tonsil and bone marrow were used as technical validation (positive and negative, respectively) for the Naveni[®] PD-1/PD-L1 interaction assay (Fig. 1C).



3. Results

The results from our study are summarized and divided according to our two aims. In aim 1, we describe the data we have generated from the **successful PD-1/PD-L1 isPLA method integration into Akoya Bioscience's PhenoCycler Fusion instrument** (Fig. 2). In aim 2, we build upon our technology integration and apply it on a **retrospective bladder cancer cohort**. Here, we share our NaveniFlex isPLA and 15-plex antibody panel results from a **complete responder and a non-responder** patient samples



4. Conclusion

- We have successfully developed a method for simultaneous detection of protein interactions between PD-1/PD-L1 and multiplexed immunofluorescence
- The method is fully integrated on the Leica Bond and PhenoCycler platform, with a single output dataset
- The method was applied on 11 samples from a retrospective cohort of muscle invasive bladder cancer patients - PEMBRO-100.
- The results demonstrate reproducible technical performance for all protein markers and isPLA signal intensities across samples.
- Differences in number of isPLA clusters and immune cell distribution indicate correlation to ICI response.

5. Prospects

- Our plan is to apply our method on a large patient cohort of 100 patients (Pembro-100), in order to confirm or deny previously established results.
- Further spatial analysis of PD-1/PD-L1 in concert with multiplex immunofluorescence is needed to better predict treatment outcomes in patients with any type of cancer diagnosis.

Introduction

Head and neck cancer encompasses a group of malignancies that arise in the oral cavity, pharynx, larynx, sinuses, and salivary glands, often linked to tobacco use, alcohol consumption, and human papillomavirus (HPV) infection. Immune checkpoint inhibitors have shown benefit in both HPV positive and negative head and neck cancers, yet biomarkers predictive of benefit are currently needed. While PD-L1 is a commonly used biomarker, it does not reflect a dynamically changing tumour microenvironment. To address this challenge, we have here applied an in situ proximity ligation detection assay (isPLA) of PD-1/PD-L1 interactions (Navinci Technology) to visualise these receptor-ligand activity on spatially mapped head and neck cancer tissues.

METHODS

This study has Human Research Ethics Approval (HREC) from the Royal Brisbane & Women's Hospital and Princess Alexandra Hospital. The study was approved by the University of Queensland. Formalin-Fixed Paraffin Embedded (FFPE) sections from 18 Head and Neck squamous cancer carcinoma (HNSCC) tissue samples were subjected to staining using a 60 plex Phenocycler Fusion (Akoya Biosciences) panel followed by PD-1/PD-L1 isPLA on serial or same sections. Tissues were quantified for isPLA signal, cell types and cellular neighborhoods.

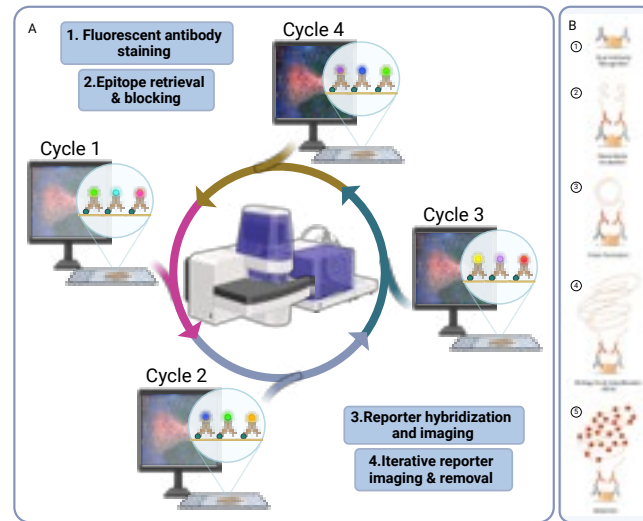


Figure 1. A) Spatial phenotyping of the tumor section using Phenocycler Fusion system. B) Navinci isPLA workflow

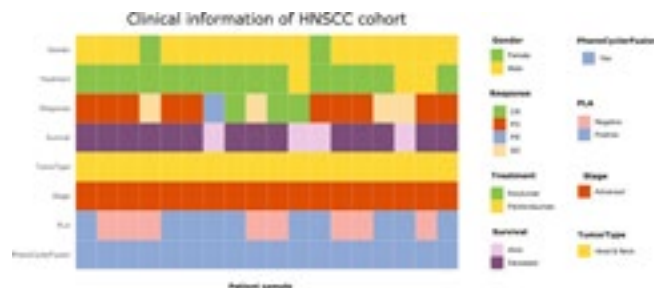


Figure 2. The clinicopathological information for the HNSCC cohort of 18 patients including gender, response, treatment, stage, and survival.

Results

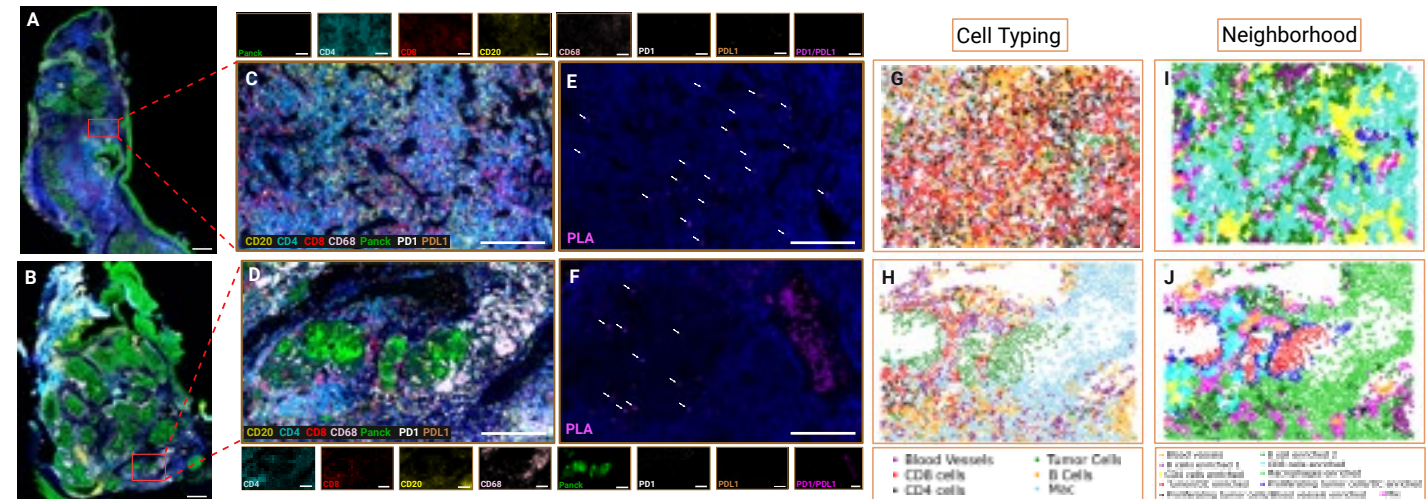


Figure 3. Spatial analysis utilizing Multiplex and isPLA technique. A, B) Comprehensive multiplex images of two distinct head and neck cancer tissues, captured through PhenoCycler-Fusion. C, D) Multiplex images that highlight selected markers, along with their corresponding channels. E, F) isPLA images, pinpointing the regions positive for PD-1/PD-L1 interactions, corresponding to the same areas as depicted in panels C and D, with the PLA channel activated. G, H) Analysis of Cellular Neighborhoods and Cell Typing, employing Cluster-Based Annotation Techniques. This section elucidates the cluster annotation process by which individual cells are categorized into distinct cell types identified within the sample. Each color denotes a unique cell type, facilitating visual differentiation and enumeration. Panels G and H display the predominant cell types within the tissue, represented by varying colors. I, J) Cellular neighborhoods in relation to the clustering algorithm. scale bar = 100 μ m.

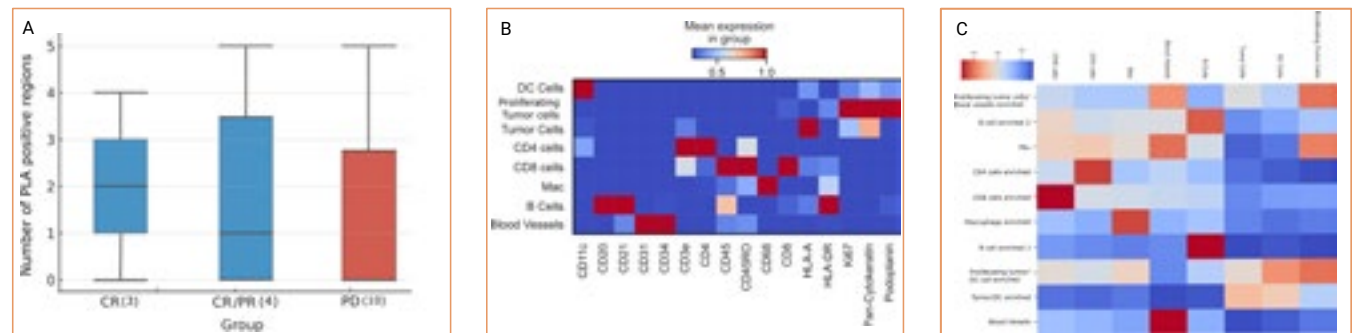


Figure 4. A) Comparative Analysis of Response Categories in Patients with Positive isPLA Results. The figure presents three separate analyses comparing the distribution of 'Number of Positive Regions' between different patient response groups. Positive regions defined as a area with at least 10 isPLA signals for PD-1/PD-L1. Advanced Characterization of Cellular Phenotypes and Spatial neighborhood within Tissues. B) Cell type clustering heatmap constructed based on the expression profiles of a selected panel of cell markers. The heatmap provides a visual representation of the clustering analysis, illustrating the intricate relationships and similarities between different cell types based on their marker expression. C) A neighborhood clustering heatmap derived from the spatial distribution patterns of various cell types. By analyzing the proximity and arrangement of cell types within the tissue matrix, this heatmap offers insights into the cellular microenvironment, revealing the complex interactions and spatial organization that characterize the cancerous tissue.

Conclusion

This study has uncovered that PD-1/PD-L1 interactions predominantly occurs at the interface between the tumor and the tumor microenvironment (TME), with the majority of cell types including CD20 and CD8 cells. Detailed cell typing and cellular neighborhood analyses have elucidated the contributions of diverse immune cell populations to PD-1/PD-L1 ligation events. The employment of cutting-edge spatial phenotyping techniques in conjunction with the proximity ligation assay has been pivotal in underscoring these observations. This combination of technologies has provided a precise mapping of ligation sites and a comprehensive characterization of their cellular composition, thus enhancing our understanding of the immunological landscape within the tumor milieu.

Acknowledgements

The Passe and Williams Foundation and the Princess Alexandra Research Foundation