Multiplexed proximity ligation-based method uncovers immune checkpoint activation in the context of the tumor microenvironment

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Background

Immune checkpoints (ICs), such as the PD1/PD-L1 axis, are inhibitory signaling pathways that down-regulate the immune responses of T cells and play a crucial role in maintaining immune self-tolerance in peripheral tissues. IC pathways can be manipulated by cancer cells to evade immune surveillance, thus making them excellent immunotherapy targets. However, the success of these therapies has been hampered by poorly defined patient groups. By combining the power of the PD1/PD-L1 interaction with the concomitant visualization of relevant biomarkers (such as CD20, CD3d, and cytokeratin), it is possible to create an informative immune profile, improving the likelihood of successful immunotherapy treatment. Using Navinci's highly sensitive and specific proximity ligation technology combined with additional biomarker staining, it was possible to uncover the tumor microenvironments (TME) in Hodgkin's lymphoma and non-small-cell lung cancer (NSCLC) patient samples.

Technology and Methods

An optimized *in situ* proximity ligation method for the detection of PD1/PD-L1 protein-protein interaction with fluorescent readout was developed. To visualize the target interaction, FFPE tissues of Hodgkin's lymphoma and NSCLC (Biomax) were incubated with monoclonal antibodies specific to PD-1 and PD-L1, followed by incubation with Navenibodies (affinity reagents conjugated to proprietary oligo arms). Strong and distinct signal is generated only if the Navenibodies are in close enough proximity to generate a rolling circle amplification reaction. To observe relevant biomarkers such as pan-cytokeratin, CD8a, CD20, and CD3d, fluorescently labelled antibodies were added during the detection step, and the signal was assessed by epifluorescence microscopy.

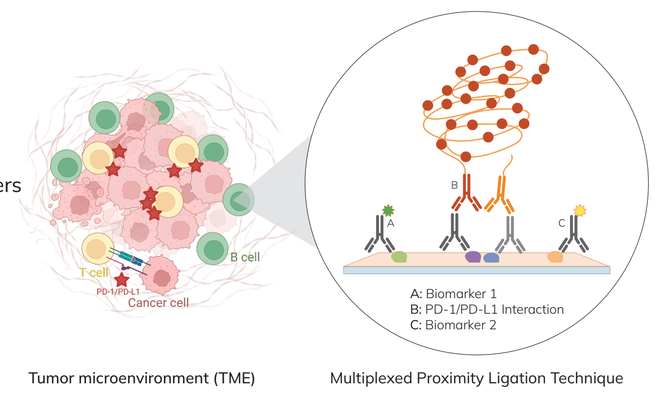


Figure 1: Using a multiplexed approach to the proximity ligation technique, it is possible to detect IC interactions and additional biomarkers at the same time. This provides greater insight into the TME and ultimately may aid the selection of appropriate immunotherapy.

Results

To assess whether the multiplexed proximity ligation approach would be effective in identifying TME, the assay was performed on cancer tissues. Hodgkin's lymphoma is characterized by the presence of Reed-Sternberg cells, which are large, abnormal cells originating from B-cells (CD20). Meanwhile NSCLC is known to have a complex TME, the composition of which is critical to the progression and prognosis of the disease.

Hodgkin's lymphoma tissue was stained for PD1/PD-L1 interaction and the additional biomarkers CD20 and CD3d **(Fig 2)**, revealing a complex TME. The staining pattern of the PD1/PD-L1 interaction is indicative of cancer cell clusters and the co-localisation of CD3d positive T cells around these sites is visible. The B cells are characterized by CD20 staining in green.

To identify the TME within NSCLC tissues, alongside the PD-1/PD-L1 interaction, additional markers cytokeratin and CD8a were assessed **(Fig 3).** This allows the visualization of the tumor area marked by cytokeratin, surrounded by PD1/PD-L1 interactions. Also present in the TME are CD8a-positive cytotoxic T cells, which play a crucial role in the anti-tumor response.

Figure 2

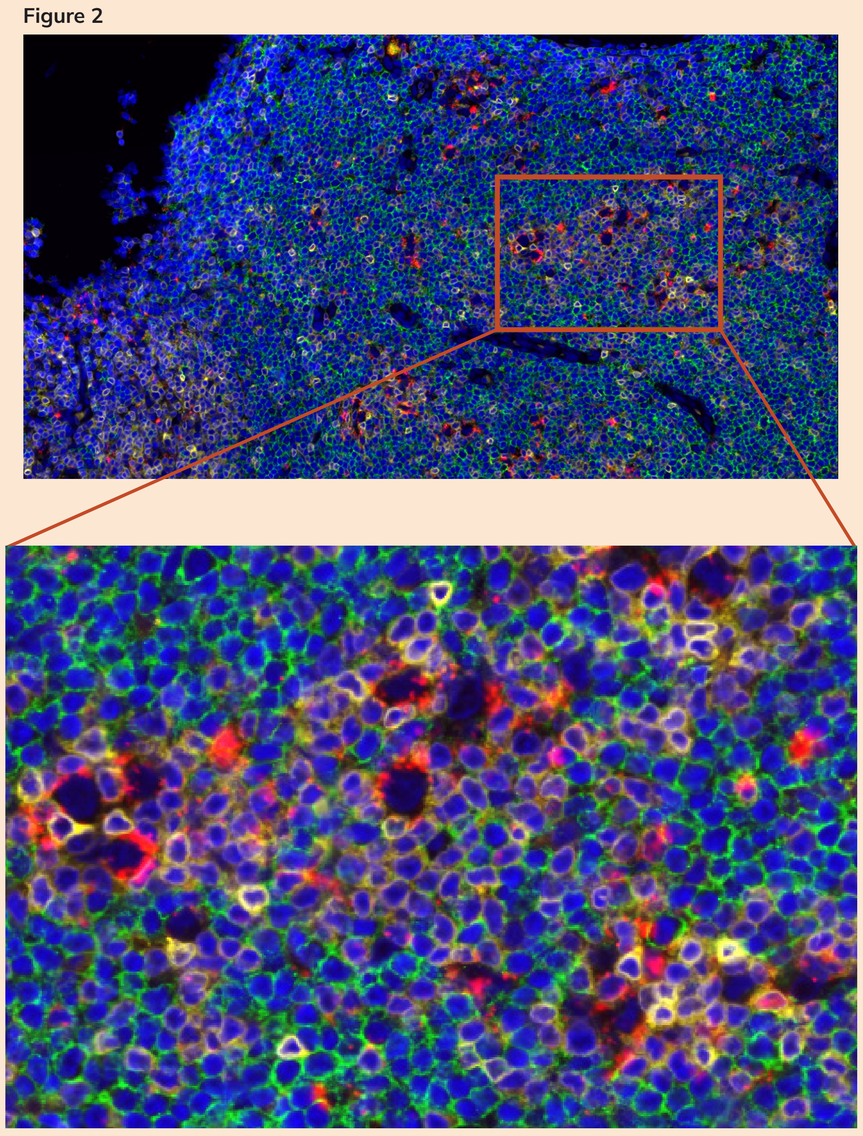
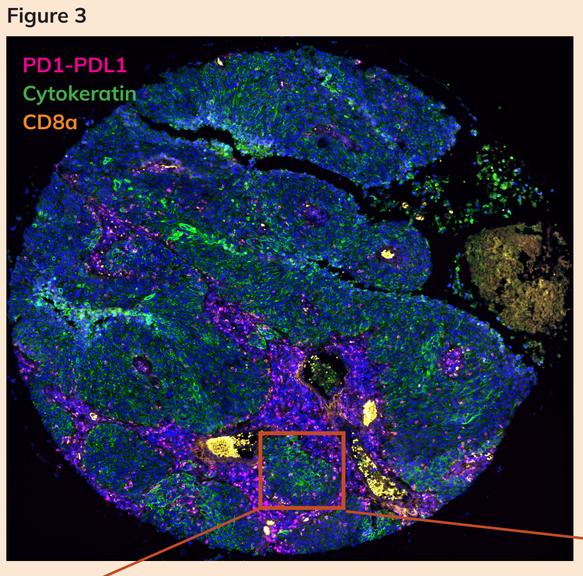


Figure 2: Hodgkin's Lymphoma (10%) cancer tissue, with PD-1/PD-L1 interaction in red, CD20 in green and CD3d in yellow. Clustering of cancer and immune cells visualizes the TME.

Figure 3



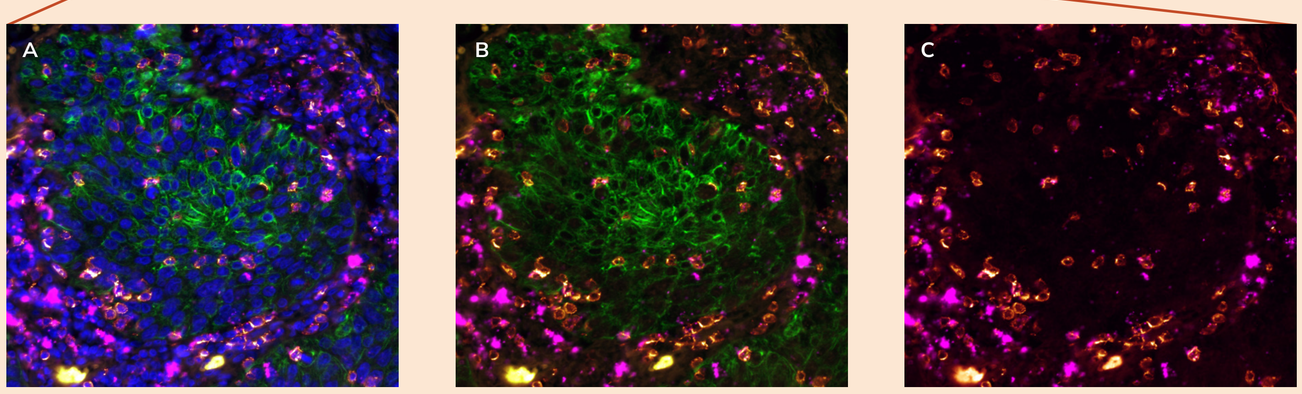


Figure 3: NSCLC tissue showing PD1/PD-L1 interaction in fuchsia, cytokeratin in green and CD8a in brown. The lower panel depicts; **(A)** all three markers plus DAPI (blue), **(B)** all three markers, no nuclear stain, and **(C)** PD-1/PD-L1 interaction and CD8a.

Conclusions

Studies have determined that the expression of PD-L1 on Hodgkin's lymphoma cells is associated with a poor prognosis and resistance to therapy. However, the co-expression of PD-L1 and CD3 in the TME may indicate a more favourable prognosis and a better response to immunotherapy. Similarly, the presence of CD8+ T cells in NSCLC tumors is associated with improved clinical outcomes, likely due to their ability to recognize and kill cancer cells. Using the Naveni technology, it is possible to multiplex the proximity ligation method with biomarker staining for the visualization of complex TME. This may lead to improved patient stratification and ultimately, appropriate therapy.

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