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New approaches to understanding the tumor microenvironment: *in situ* detection of immune checkpoint interactions in cancer patient tissue samples

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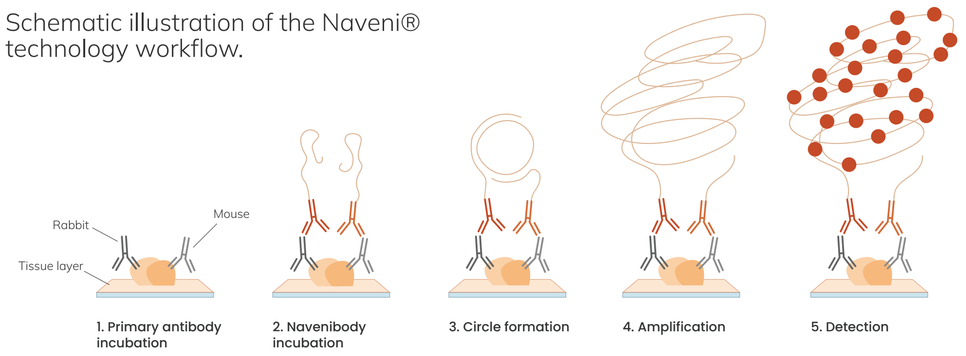
Background

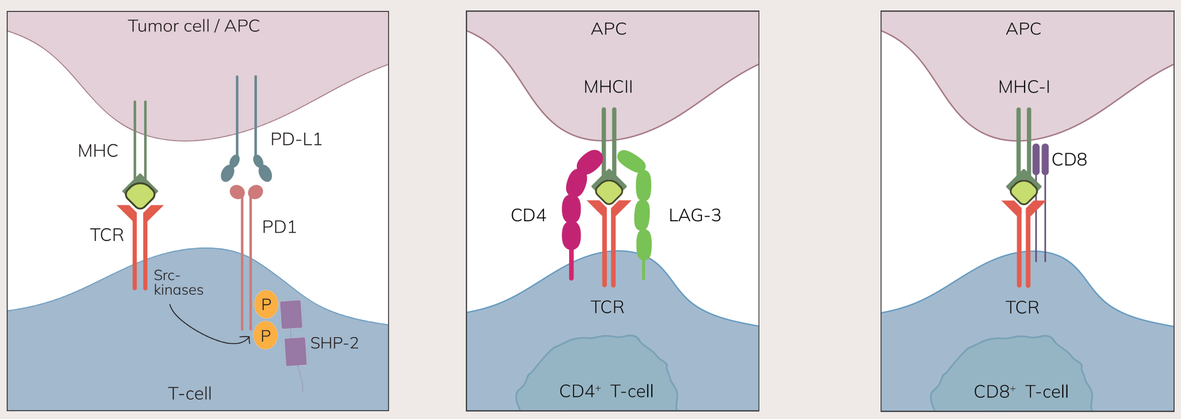
The CD8/MHC I interaction is crucial for cytotoxic T cells to recognize and kill abnormal/tumor cells by binding to the MHC I-presented antigen peptides. Conversely, receptors PD-1 and LAG3, and their respective ligands PD-L1 and MHCII form immune checkpoint (IC) interactions that lead to inhibitory signals in the T cell and promote immune exhaustion to avoid autoimmunity. All abovementioned interactions are interconnected through their roles in immune regulation, antigen presentation, and potential synergistic effects in immune therapy strategies. The complex interplay of these proteins is critical for maintaining immune balance and modulating immune responses. IC pathways, however, can be hijacked as immune escape routes by cancer cells in the tumor microenvironment (TME), hence their blockade is an attractive and widely applied immunotherapeutic approach. However, the efficacy of immune checkpoint inhibitors (ICIs) has been limited. Patient stratification is traditionally based on immunohistochemical stainings against single IC proteins such as PDL-1 which often prove inconclusive. Disease outcomes may be better correlated to high levels of IC interactions, which are indicative of pathway activation, than to single protein expression. In addition, it can be useful to include other relevant biomarkers to visualize and understand the TME, reveal T cell responses in the context of the tumor and/or healthy tissue and understand signaling in cancer. Here, we demonstrate the possibility for deeper understanding of the functional processes in the TME by looking at key interactions and biomarker co-stains with the Naveni® technology.

Technology and Methods

Formalin-fixed, paraffin-embedded tissue (FFPE) sections of tonsil, skeletal muscle and non-small cell lung cancer TMAs were stained for immune interactions with or without immunofluorescent co-stains. To ensure that interactions and not simply an overlap of fluorescent signal was detected, we employed the Naveni® proximity ligation method. This technology detects proteins *in situ*, located within an interaction range (<40 nm) by means of antibodies conjugated to oligonucleotides that create amplified fluorescent or chromogenic signal only if the proximity requirement is fulfilled. The technology is compatible with simultaneous IF co-stains and Opal dye staining protocols.

Schematic illustration of the Naveni® technology workflow.





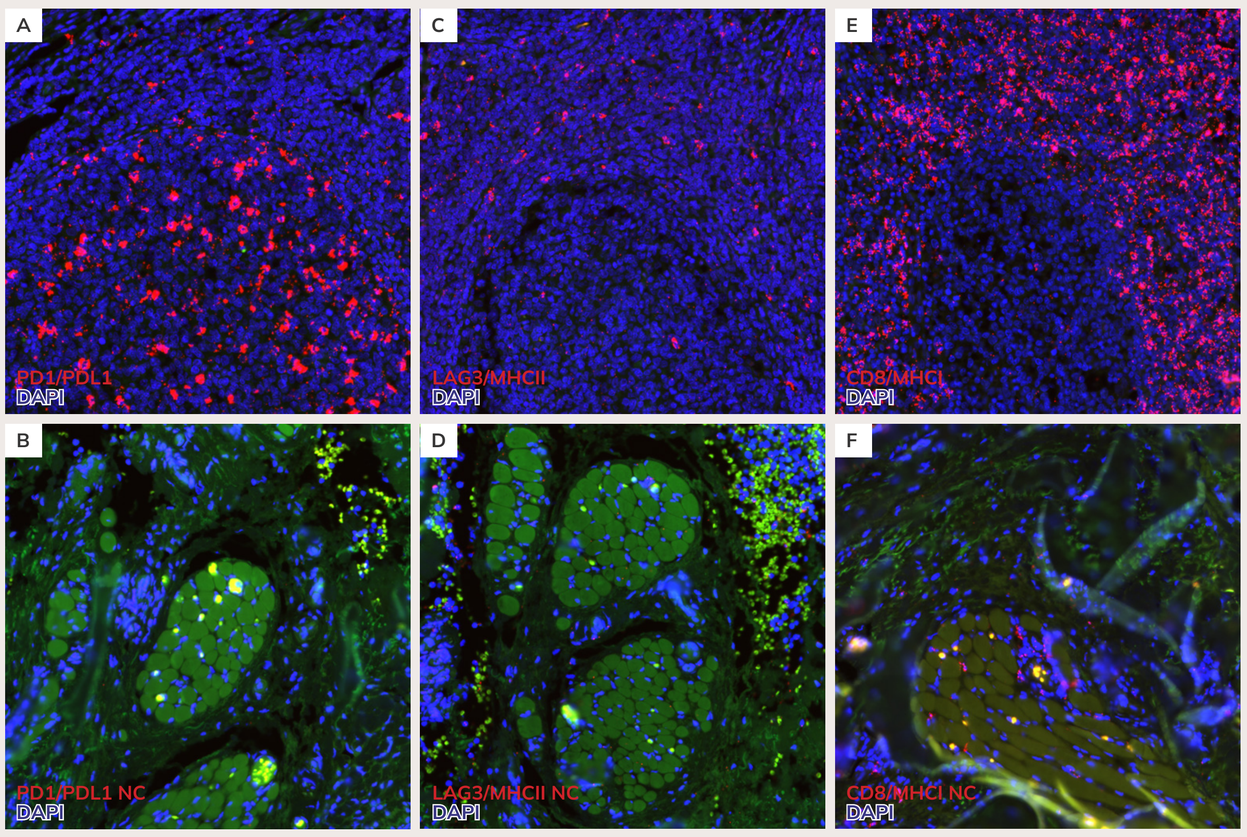


Fig. 1. Various immune interactions detected in tonsil (signal-positive tissue) and in skeletal muscle (biological negative control) with NaveniFlex™ Tissue Atto647N. **(A)** PD1/PDL1 interactions are abundant in the germinal center of tonsil and **(B)** absent in the negative control. **(C)** LAG3/MHCII interactions are seen outside of the germinal center and are **(D)** absent from the negative control. **(E)** Activation of CD8+ T cells is evident thanks to multiple CD8/MHCI interactions outside the germinal center while **(F)** the biological negative control is blank. Cartoons above microscopy images depict the interaction detected below.

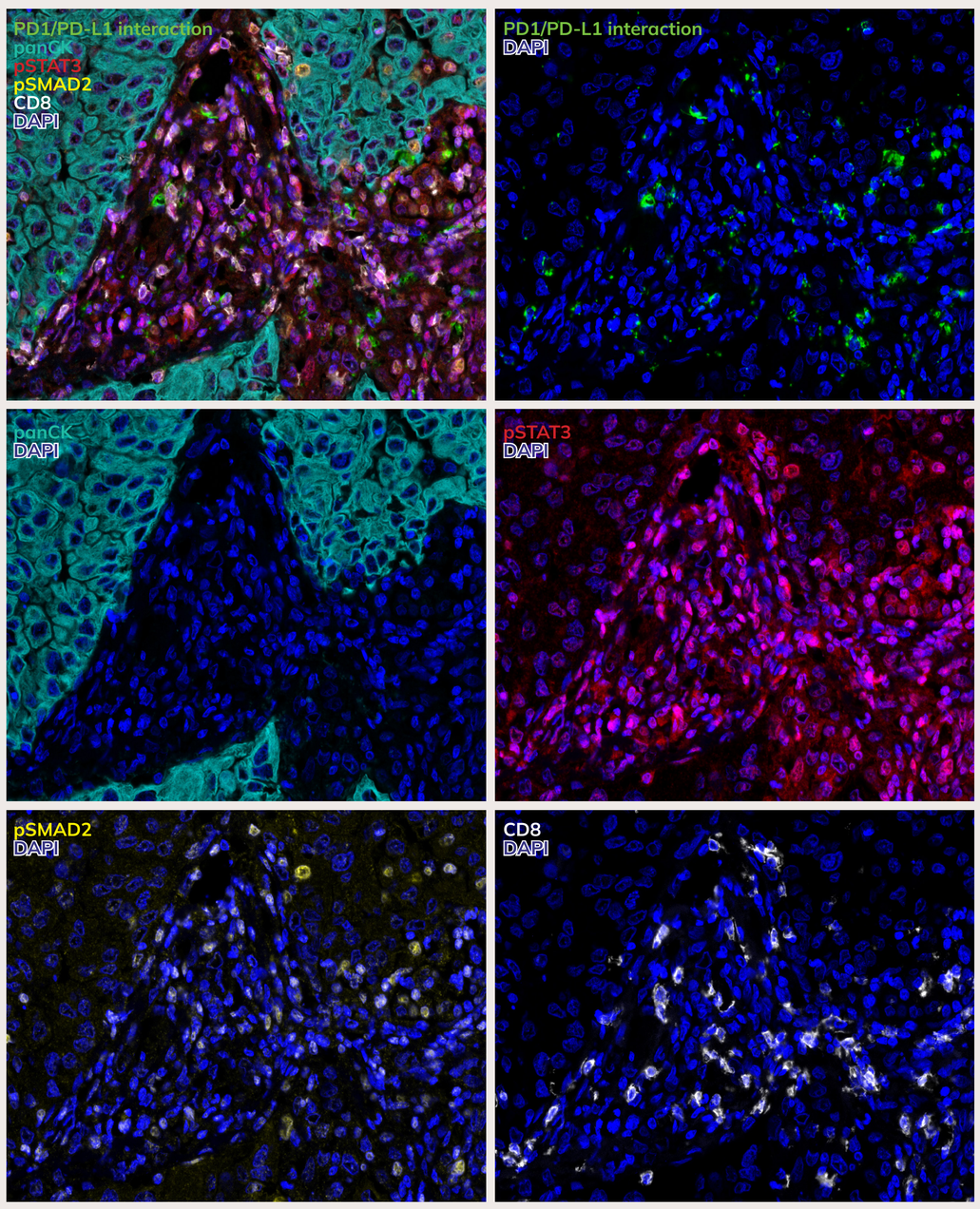


Fig. 2. PD1/PDL1 interaction detection with multiplexed biomarker co-staining in NSCLC. IF multiplexing with Opal dyes was performed on Leica BONd RX first, followed by the IC proximity ligation assay. pSTAT3 and pSMAD2 signaling are potentially involved in the resistance of NSCLC to PD1/PDL1 immune checkpoint inhibitors. Cancer cells, cytotoxic T cells and immune checkpoint activation are simultaneously visualized.

Results

Fig.1 offers proof-of-principle data, demonstrating that key immunological events with significance in cancer, such as the activation of cytotoxic T cells (CD8/MHCI interaction) and of immune checkpoints (PD1/PD-L1 and LAG3/MHCII interactions) can sensitively be detected in situ with the help of the Naveni® proximity ligation technology.

In Fig. 2, the PD1/PD-L1 interaction was put into the context of the TME in a non-small cell lung cancer (NSCLC) sample. The combined multiplex IF and Naveni® technology revealed an immune-excluded tumor where T cells are located mostly in the stroma and not the tumor compartment, and the activation of the PD1/PD-L1 axis is enriched in the stroma. Smad/TGF and STAT signaling also occur in the whole stroma. As pSTAT3 and pSMAD2 signaling are potentially involved in the resistance of NSCLC to PD1/PD-L1 ICIs, expanding this assay to a bigger patient cohort would allow testing for the PD1/PD-L1 interaction along with potential resistance mechanisms on the same tissue section, with the long-term aim to provide better guidance for treatment decisions for patients with primary resistance.

Conclusions

Our study demonstrates the power of the Naveni® technology in unraveling the intricacies of immune checkpoint pathways. Relying solely on PD-L1 expression as a biomarker is insufficient for patient stratification, highlighting the need for a more comprehensive approach that considers various aspects of the immune response within the tumor microenvironment. By visualizing PD-1/PD-L1 and LAG3/MHCI interactions, cytotoxic T cell activation, and co-staining with relevant biomarkers, we have demonstrated the possibility to gain deeper insights into the immune landscape in cancer. This approach not only enhances our understanding of the underlying mechanisms but also provides a promising tool for improving patient selection and treatment outcomes in immunotherapy.

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