Multiplexed *in situ* proximity ligation method for identifying functional tumor-immune interactions

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Background

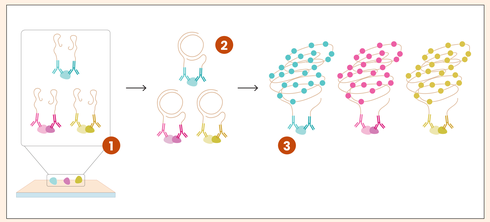
Inhibitory signaling pathways such as PD1/PD-L1 can be hijacked as immune escape routes by cancer cells in the tumor microenvironment (TME). Their blockade is thus an attractive, widely applied immunotherapeutic approach, but its efficacy has been limited, even for patients positive for diagnostic biomarkers.

This underscores the importance of a holistic view of the TME and a functional assessment of key protein interactions rather than expression profiling alone. Here, we introduce a new threeplex *in situ* proximity ligation (*is*PLA) approach based on Naveni® *is*PLA technology. We demonstrate the threeplex approach by co-detecting proteins and functional protein interactions in human cancer and healthy tissues.

Methods

Formalin-fixed, paraffin-embedded (FFPE) tissue sections were deparaffinized and underwent epitope retrieval. Tissues were blocked and incubated with three pairs of oligonucleotide-conjugated antibodies (Navenibodies, see step 1 below) followed by circle formation (step 2) and signal amplification (step 3). Each Navenibody pair results in a distinct amplification product.

The products were detected with oligonucleotides labeled with Atto647N, Atto550 and AlexaFluor488, respectively. Imaging was carried out on the Olympus VS200 scanner. Two different panels of targets were used. One panel included assays to detect the protein interactions PD1/PD-L1 and CD8/MHC-I, and the T-cell marker CD3. The other panel included three interaction assays targeting PD1/PD-L1, PD1/PD-L2 and PD1/SHP-2.



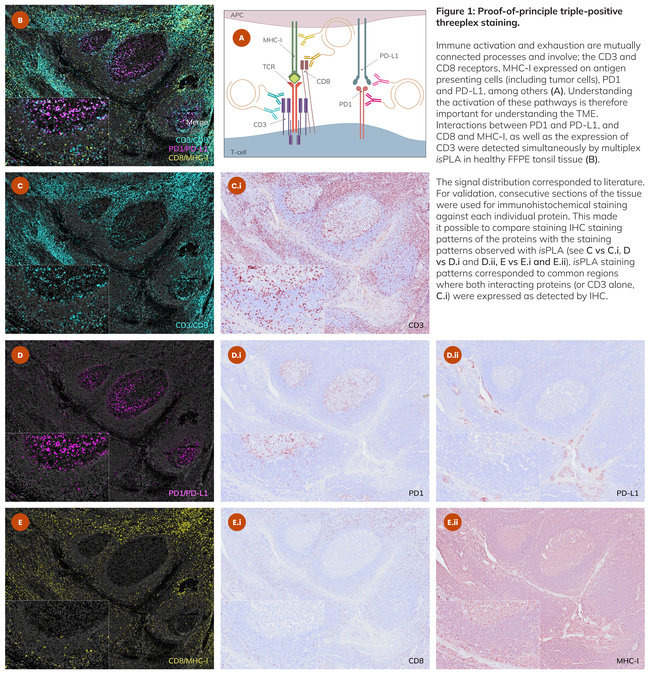


Figure 1: Proof-of-principle triple-positive threeplex staining.

Immune activation and exhaustion are mutually connected processes and involve; the CD3 and CD8 receptors, MHC-I expressed on antigen presenting cells (including tumor cells), PD1 and PD-L1, among others **(A)**. Understanding the activation of these pathways is therefore important for understanding the TME. Interactions between PD1 and PD-L1, and CD8 and MHC-I, as well as the expression of CD3 were detected simultaneously by multiplex *is*PLA in healthy FFPE tonsil tissue **(B)**.

The signal distribution corresponded to literature. For validation, consecutive sections of the tissue were used for immunohistochemical staining against each individual protein. This made it possible to compare staining IHC staining patterns of the proteins with the staining patterns observed with *is*PLA (see **C vs C.i**, **D vs D.i** and **D.ii**, **E vs E.i and E.ii**). *is*PLA staining patterns corresponded to common regions where both interacting proteins (or CD3 alone, **C.i**) were expressed as detected by IHC.

Conclusion

Understanding the dynamics of immune checkpoint pathways within the TME can enhance the development of effective strategies to overcome immune escape mechanisms employed by cancer cells. Functional studies that show protein involvement in key signaling pathways such as PD1/PD-L1, PD1/PD-L2 and CD8/MHC-I offer a more biologically relevant and comprehensive view of the TME than traditional IHC.
The simultaneous visualization of three functional markers and the possibility to analyze and quantify the data contributes to more meaningful spatial profiling. Further research is needed to explore the therapeutic potential of protein interactions as superior biomarkers, advancing cancer immunotherapy, and improving patient outcomes.

Data Analysis

Regions of interest (ROIs) were defined for analysis in QuPath (0.5.1) for tonsil images stained with the threeplex *is*PLA (Figure 3). Zone A encompasses germinal centers, Zone B is an expansion of 100 µm around the germinal centers to approximate the mantle zone, and Zone C is drawn around T-cell rich areas.

Signals were detected by an ImageJ Macrorunner consisting of Top-Hat filtering with a filter size of 4, segmenting signal regions by Li's autothresholding method and finding local maxima inside the segmented areas with a prominence of 200. Cells were detected in the DAPI channel using the StarDist extension with image normalization of percentiles 0.2, 99.8 using the image normalization builder, a threshold of 0.5 and a pixel size of 0.2.

All cells were then filtered out by a minimum nucleus mean intensity of 500 and a minimum nucleus area of 2 µm^2. Signals were assigned as child objects to cells if they were 100% overlapping within the cell boundary. Lastly, cells were classified as positive for each category by a threshold of 8 *is*PLA signals for CD3, 3 *is*PLA signals for CD8/MHC-I, and 2 *is*PLA signals for PD1/PD-L1.

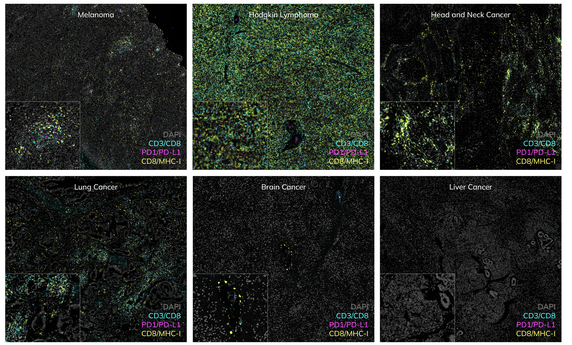


Figure 2: Various levels of pathway activation and CD3 expression in different cancer types.

The threeplex *is*PLA method was used for the simultaneous detection of CD3, CD8/MHC-I and PD1/PD-L1 in FFPE tissue samples from patients with melanoma, Hodgkin lymphoma, head and neck cancer, lung, liver and brain cancer. Various degrees of positivity were observed. In the melanoma sample, there were areas with disperse T-cell activation, as well as a zone enriched in PD1/PD-L1 interactions in the vicinity of CD3+ CD8/MHC-I-activated T cells. In Hodgkin lymphoma, we observed significant overlap between CD3 and CD8/MHC-I positivity, and some PD1/PD-L1 interactions.

In the head and neck and the lung cancer samples, there was T-cell infiltration, but little activation of PD1/PD-L1, whereas liver and brain cancer were negative for staining, with the exception of a few immune cells in the brain. This demonstrates the ability of the threeplex *is*PLA method to capture disparate TME profiles, which can help a more precise patient profiling.

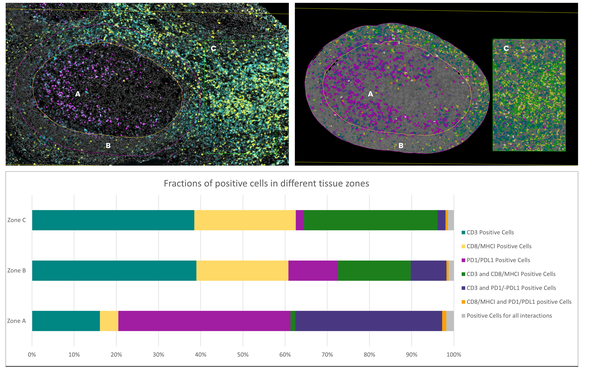


Figure 3: Image analysis and quantification of positive cell fractions in regions of interest (ROIs).

Three ROIs corresponding to the germinal center (zone A), the mantle zone (zone B), and a T-rich area (zone C) were outlined in the multiplex tonsil image and quantified using QuPath as described. Cell segmentations and classifications made after the analysis are shown as an overlay (upper right corner). Teal corresponds to CD3 positive cells, yellow to CD8/MHC-I positive cells, magenta to PD1/PD-L1 positive cells, green to CD3 and MHCI-CD8 double-positive cells, purple to CD3 and PD1/PD-L1 double-positive cells, orange to CD8/MHC-I and PD1/PD-L1 double-positive cells, and grey to cells positive for all three targets.

Image analysis showcases how T-cells infiltrate the B-cell rich zone A, resulting in higher fractions of PD1/PD-L1 signal while zones B and C contain reduced amounts of PD1/PD-L1 interaction and a greater number of CD8/MHC-I positive cells.

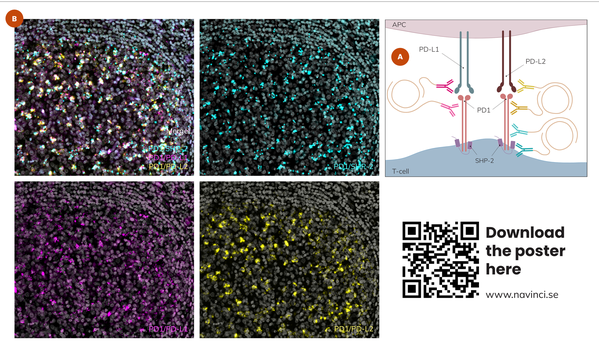


Figure 4: Threeplex *is*PLA for detection of three protein interactions involving PD1.

The detected interactions were PD1/PD-L1, PD1/SHP-2 and PD1/PD-L2. PD-L1 and PD-L2 are ligands to the PD1 receptor, whereas SHP-2 is recruited to the ligand-bound and phosphorylated PD1 **(A)**. We detected high levels of interaction between both PD1/PD-L1 and PD1/PD-L2, along with high recruitment of SHP-2 to the PD1 receptor in the germinal centers, indicating activation of the immune checkpoint pathways. **(B).** All three interactions were localized in clusters together, as expected, confirming the precision and robustness of the threeplex *is*PLA technology for the detection of various interactions with key functions related to responses to immune checkpoint inhibition.

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