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Multiplex *in situ* proximity ligation assay for functional studies of proteins and their interactions in cell and tissue samples

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Introduction

Detecting protein-protein interactions (PPIs) and their localization is essential for the understanding of protein function and enables deeper understanging of cell biology by delineating signal transduction. This allows enhanced identification of biomarkers and can lead to improvements in patient stratification and other aspects of diagnostics. In situ proximity ligation assay (*is*PLA) is a well-established tool for the study of PPIs in cell and tissue samples. It detects proteins located within 40 nm of each other via antibodies conjugated to oligonucleotides that generate amplified fluorescent or chromogenic signal (Fig. 1). The method can be automated and/or combined with multiplexed immunofluorescence (mIF) staining of individual proteins to visualize tissue context e.g., the tumor microenvironment or the immune landscape. However, current *is*PLA technology has been limited to the detection of a single PPI at a time. Hereby, we propose a method to solve this limitation.

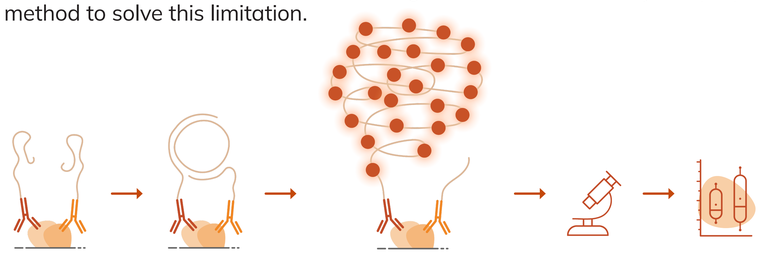
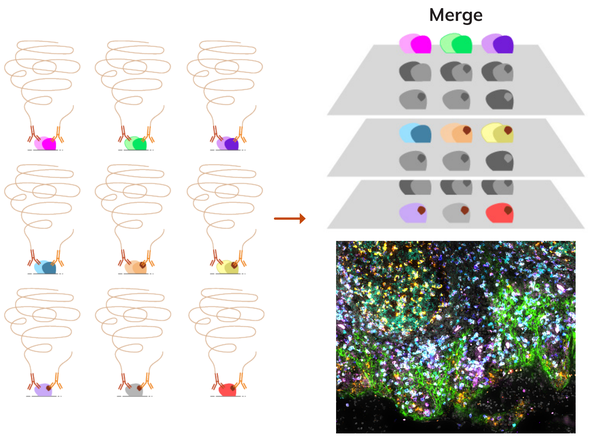


Fig. 1. *is*PLA workflow
A pair of primary antibodies conjugated to proprietary oligonucleotide arms (Navenibodies) are simultaneously incubated with an FFPE tissue or fixed cell sample to detect the interaction between two proteins, or two epitopes on the same protein. Only if the Navenibodies are in close proximity will they generate a rolling circle amplification reaction, leading to a robust and distinct signal detection by fluorophore-labeled detection oligos.

Method

We developed multiplex *is*PLA capability built on the Naveni® technological solution. The approach allows the simultaneous and precise detection of up to nine *is*PLA reactions. Each *is*PLA probe pair results in an amplified signal with a unique DNA barcode. The barcode is detected using complementary, fluorophore-labelled oligonucleotides. The approach provides a way to look beyond protein expression and into functional states of proteins in both cells and tissues. It is suitable to study, for example, signal transduction, responses to drug treatments, or patient stratification.



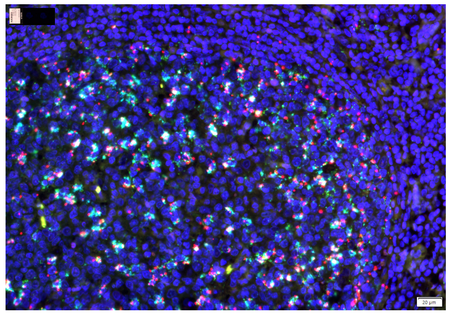


Fig. 3. Three-plex *is*PLA.
Simultaneously detecting the interactions between PD1 and PD-L1 (green), PD1 and PD-L2 (cyan), and PD1 and SHP2 (red) in FFPE tonsil tissue. DAPI in blue. Even though the same protein, PD1, participates in all three of the detected interactions with different partners, detection remains specific and robust. Signal localization correlates with reports in literature and across the three *is*PLA reactions.

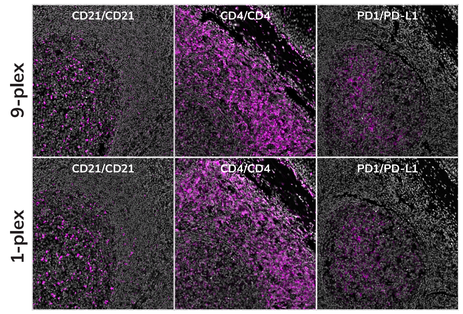


Fig. 4. Regardless of plex level, each *is*PLA reaction remains sensitive, specific and with the same localization.
We compared three *is*PLA assays (for single proteins CD21 and CD4, and the PD1/PD-L1 interaction) as part of a 9-plex panel (top) and as individual assays used for reference (bottom) in FFPE tonsil tissue, and there were no significant difference in expression patterns or localization.

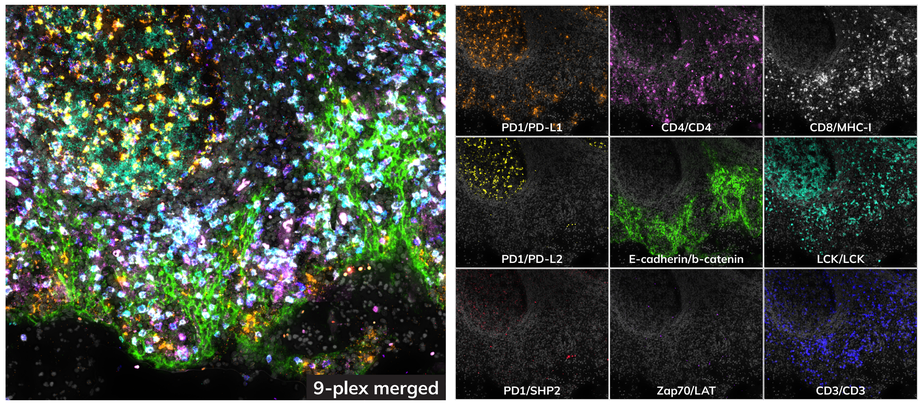


Fig. 5. Nine-plex *is*PLA in FFPE tonsil tissue.
Six PPIs and three individual proteins were detected simultaneously. There was high correlation but not complete overlap between interactions such as PD1/PD-L1 and PD1/PD-L2, likely indicating local immune suppression, confirmed also by downstream signaling activation (via PD1/SHP2). In regions not fully suppressed by PD1 signaling, early signs of T cell activation are observed as shown by the expression of CD3, LCK and some limited Zap70/LAT. Helper (CD4+) and cytotoxic (CD8+) T cell populations can be differentiated.

Conclusion

In summary, our multiplex *is*PLA approach offers a powerful and flexible tool for the simultaneous detection of up to nine protein-protein interactions or single proteins in tissue and cell samples. This method enhances the spatial understanding of molecular interactions, increases confidence about expression of individual markers and supports applications in translational research such as patient stratification and biomarker discovery.

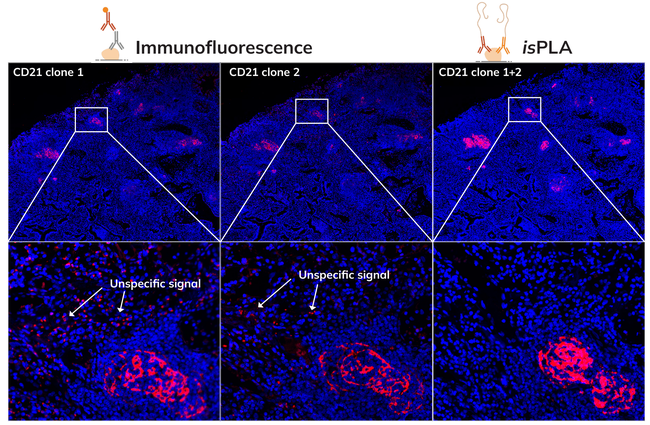


Fig. 6. *is*PLA can be used for highly specific and sensitive detection of proteins that are challenging to detect.
Here, we compared the detection of CD21 using two different antibody clones with immunofluorescence (IF) versus combining the same two clones in *is*PLA in consecutive lung cancer FFPE sections. The zoom-ins clearly demonstrate the presence of unspecific signal using each clone individually in IF. In contrast, in *is*PLA only specific signal is detected. This data demonstrates the value of including single protein detection in *is*PLA multiplex panels.

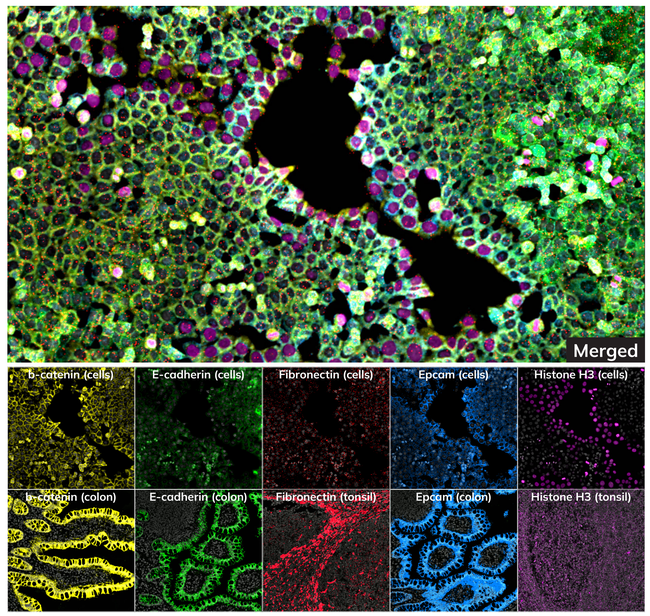


Fig. 7. Five-plex *is*PLA for detection of individual proteins in cells and tissues.
Five individual proteins' expression was compared using the same antibodies in cultured HCT116 cells and in two different FFPE tissues (colon and tonsil). We observed that our multiplex *is*PLA protocol performs equally well in these two model systems.