THOROUGH ANTIBODY VALIDATION FOR ROBUST SPATIAL BIOLOGY DATA

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Background and purpose

Understanding immune-checkpoint ligand distribution within the tumour microenvironment is essential for robust biomarker analysis in triple-negative breast cancer (TNBC), where PD-L1 and PD-L2 expression is highly heterogeneous, and in some cases very low. Reliable detection of these heterogeneous markers underpins actionable immunohistochemistry (IHC) and/or multiplex immunofluorescence (mIF) staining and the downstream quantitative image analysis. However, accurate detection of some of these immune-checkpoint ligands, such as PD-L2, can be technically challenging due to variable antibody specificity and contradictory staining patters, evidenced by comparing multiple antibodies against the same target.

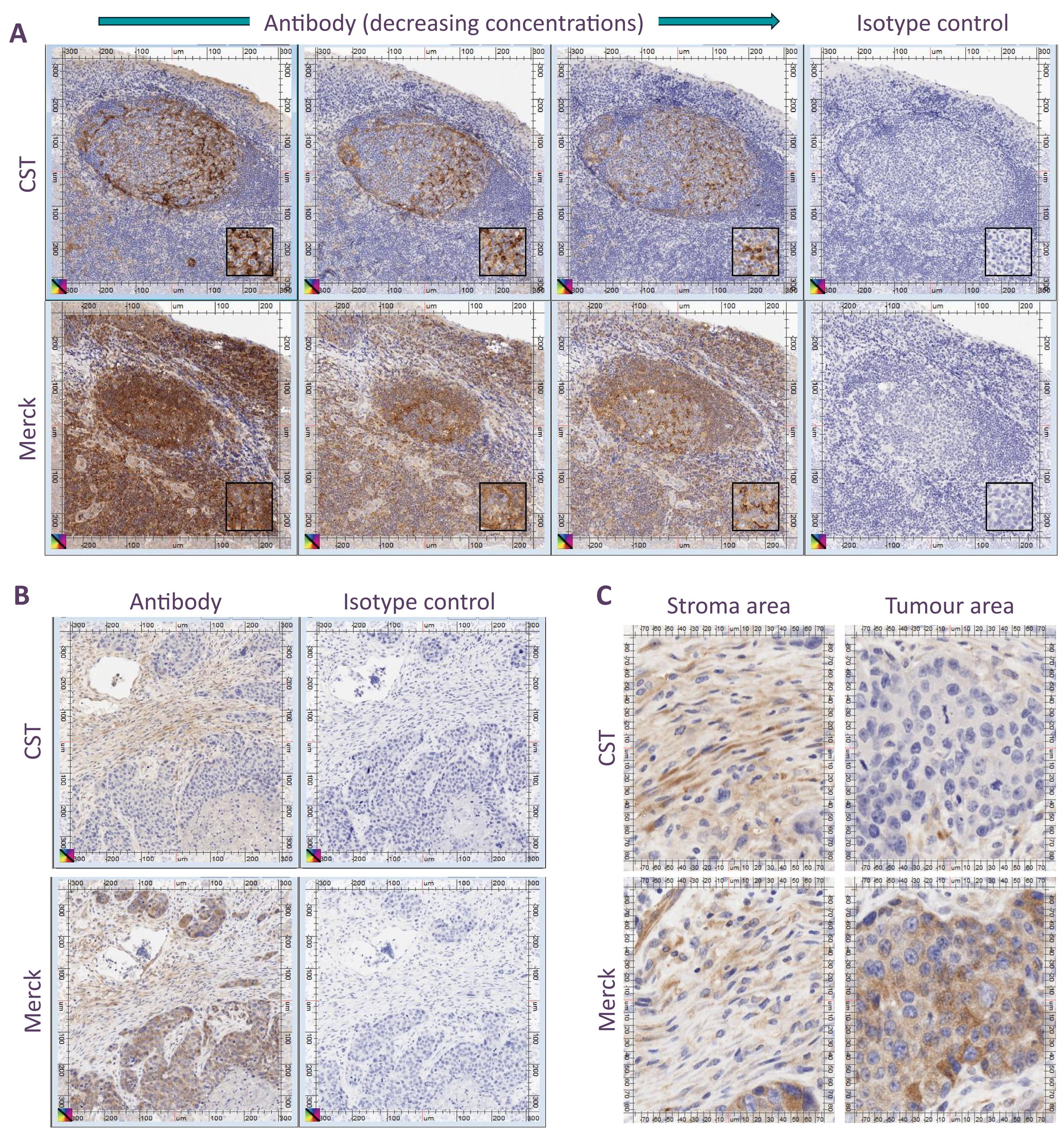
To overcome this challenge, we designed and conducted a structured, optimisation programme to evaluate commercial PD-L2 antibodies using singleplex IHC, orthogonal validation with RNAscope™ *in situ* hybridization (ISH) and *in situ* proximity-ligation assay (*is*PLA), to enable design and optimisation of a mIF panel to robustly localise PD-L1 and PD-L2 populations in TNBC. This approach enabled the development of a mIF panel capable of delivering reliable, spatially resolved PD-L2 measurements, alongside other key tumour microenvironment markers in a cohort of TNBC samples.

Study design overview

Results

Optimisation and comparison of PD-L2 antibodies

IHC assay optimisation was performed using a known positive tissue (tonsil) and a cohort of indication specific (TNBC) samples. The immunostaining showed concentration-dependent binding of each antibody to tonsil sections. However, discrepances between the patterns of immunostaining were evident for the different antibodies in both tonsil and TNBC samples.



Representative images of IHC immunostained sections showing:

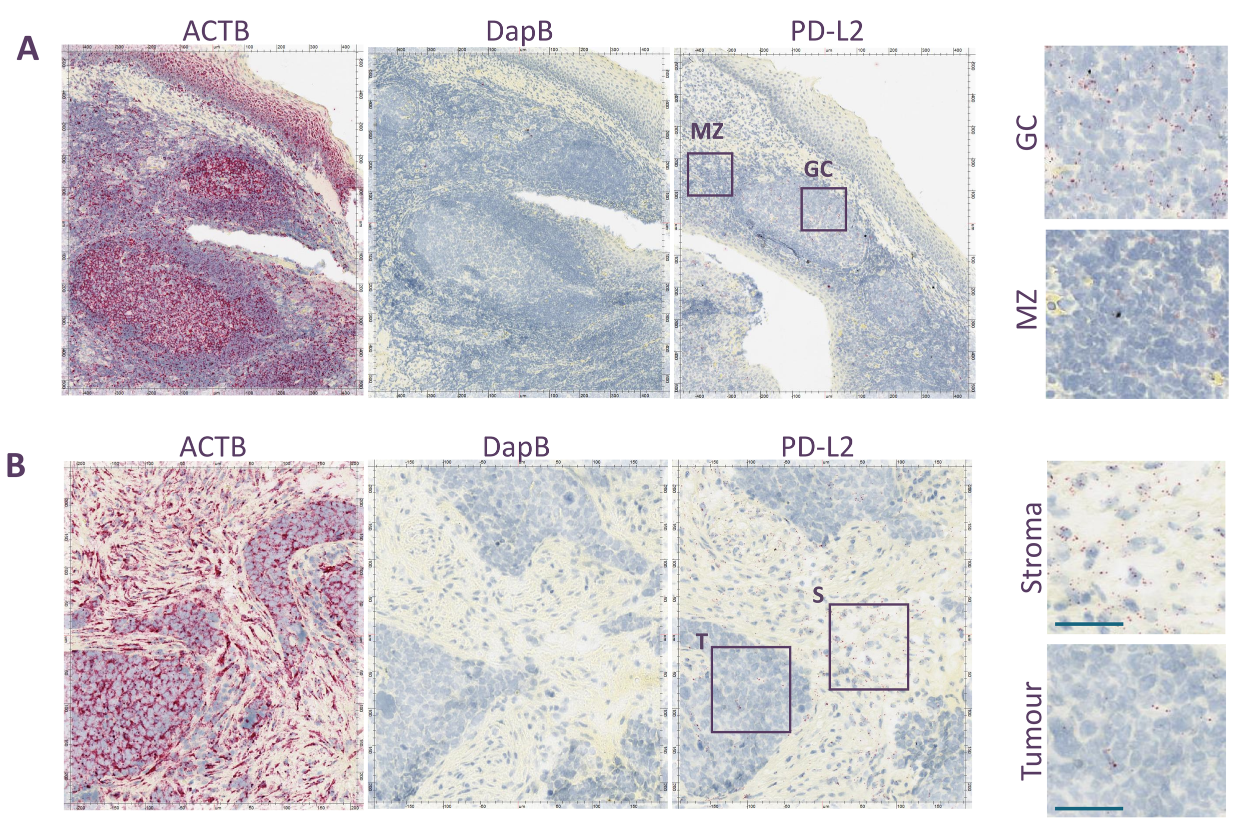
(A) concentration dependent staining with CST and Merck anti-PD-L2 antibodies in human tonsil

(B) stromal-predominant staining in TNBC with CST antibody versus broader tumoural & stromal staining in TNBC with Merck anti-PD-L2 antibody

(C) high magnification images of the stroma and tumour areas

RNA-protein concordance

RNAscope *in situ* hybridisation reveals stromal-predominant PD-L2 RNA expression in TNBC, mirroring PD-L2 IHC patterns with the CST antibody and validating antibody specificity.

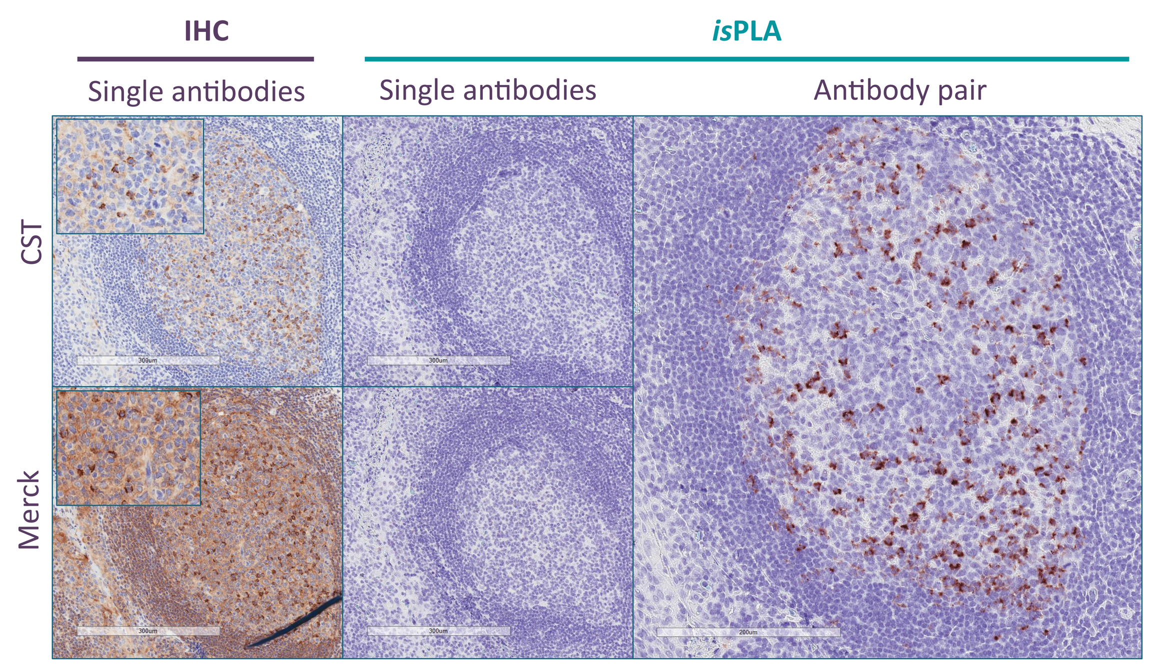


Representative images of RNAScope ISH immunostained sections of Tonsil (A) and TNBC (B) showing ACTB (Actin B), DapB (negative control probe) and PD-L2 mRNA expression.

High magnification images of PD-L2 RNA expression in Germinal Centre (GC) and Mantle Zone (MZ) areas in tonsil and stroma (S) and tumour (T) areas in TNBC (box inserts)

Antibody staining concordance by *is*PLA

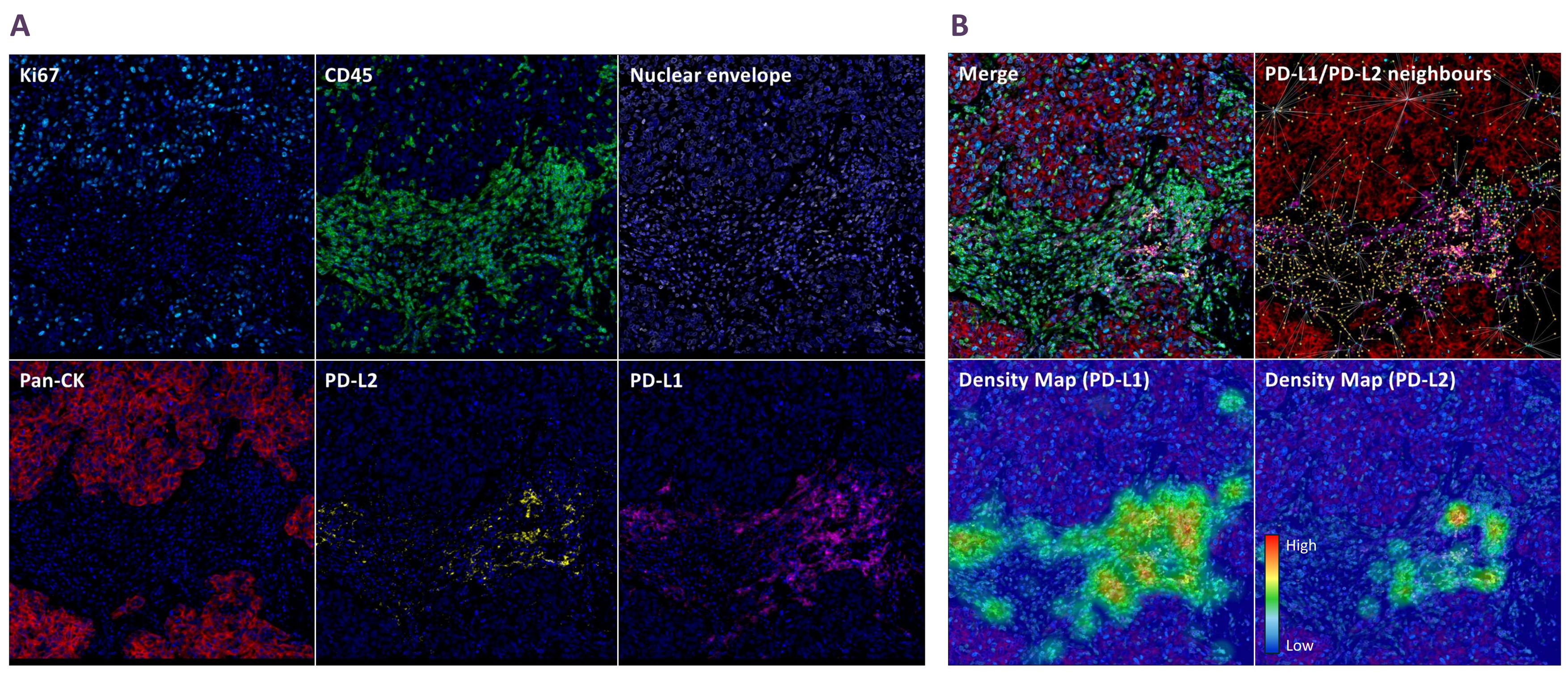
In situ PLA technology was used to build additional confident in PD-L2 protein expression pattern and antibody specificity (see https://navinci.se/technology/)



Representative images of IHC immunostained tonsil sections showing singleplex IHC staining for each individual anti-PD-L2 antibody, *is*PLA with each antibody alone (negative control) and *is*PLA with both antibodies together (where binding of each is exquisitely to the same target and yields an *is*PLA signal)

mIF panel development and spatial image analysis

Iterative optimisation of antibody position, antigen retrieval conditions, and Opal fluorophore assignments resulted in clean multiplex signal without bleed-through (Opal 480, 520, 570, 620, 690, 780). Fluorescent channels display distinct immune (CD45), epithelial (Pan-CK), proliferative (Ki67), nuclear envelop staining and immune-checkpoint (PD-L1/PD-L2) compartments (Panel A). Panel B shows the spatial distribution, cell density and nearest neighbour analysis for PD-L1 and PD-L2 positive cells in TNBC.



Methods

All immunostaining was performed on a Leica Bond RX Autostainer. For each technique, the optimal assay conditions were determined in-house.

- Two commercial anti-PD-L2 antibodies (CST and Merck)
- Singleplex IHC (DAB endpoint) immunostaining using Leica Bond Polymer Refine kit
- *is*PLA (DAB endpoint) using the NaveniBright BOND RX HRP kit
- RNAscope *in situ* hybridisation and Leica BOND Red detection kit
- Selection of optimal anti-PD-L2 antibody for development of multiplex immunofluorescence panel using Akoya classic reagents, including MOTIF opal fluorophores and Akoya HRP polymer
- Spatial image analysis using HALO® Image Analysis software. HighPlex FL algorithm and rainbow forest tissue classifier were used for cell segmentation, marker detection and tissue classification

Conclusion

✓ The development approach successfully identified the **optimal PD-L2 antibody** for Triple-Negative Breast Cancer samples

✓ Multi-modal testing confirmed **high specificity, low background,** and **true biological signal**

✓ Robust multiplex immunofluorescence development workflow and control generated a final **validated, reproducible** and **fit-for purpose** mIF panel for spatial analyses of TNBC

✓ HALO phenotype mapping provides **high-resolution spatial insight**, supporting clinical translation

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