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High-resolution analysis of immune checkpoint activation utilizing a combined PD1/PD-L1 *in situ* proximity ligation assay (isPLA) and multiplex immunofluorescence (mIF) imaging approach

Arne Christians[1], Jannik Boog[1], Charles E. Jackson[2], Matthew H. Ingalls[2], J. Spencer Schwarz[2], Sara Bodbin[3], Agata Zieba Wicher[3], Subham Basu[3], Oliver Braubach[2]
Canopy Biosciences®- A Bruker Company, [1]St. Louis, Missouri, USA; [2]Hannover, Germany; [3]Navinci Diagnostics, Uppsala, Sweden

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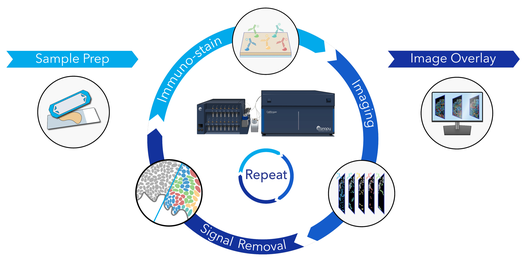
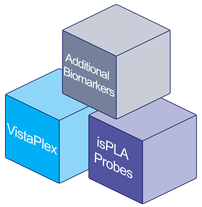
Cellscape™ Precise Spatial Multiplexing



The CellScape platform enables automated cyclic mIF for quantitative spatial phenotyping.

VistaPlex Spatial Immune Profiling Biomarkers		
CD3	CD68	Ki-67
CD4	CD45	PD-L1
CD8	CD45RA	Pan-CK
CD20	CD45RO	Grn B
FoxP3	PD-1	DNA



isPLA probes	
PD-1	PD-L1

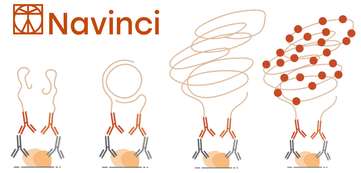
Additional Biomarkers	
CD11c	CD27
Vimentin	

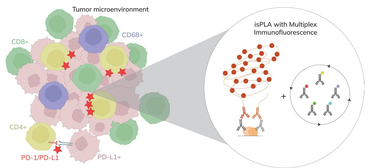
VistaPlex™ Multiplex Assay Kits are ready-to-use validated antibody panels for comprehensive phenotyping of the tumor microenvironment. The kits are modular and can be combined with other commercially available and custom antibodies.

The Navinci *in situ* Proximity Ligation Assay (isPLA) was used to visualize protein-protein interactions between PD-1 and PD-L1 along with the Spatial Immune Profiling kit and three additional antibodies to identify cell populations.

isPLA on the CellScape Platform

Navinci





1. FFPE tissues were prepared for the CellScape workflow and stained with anti- PD1 & PD-L1; primary antibodies were labeled with oligo-modified Navenibody probes
2. Oligos in close proximity formed a circular DNA strand that acted as a template for rolling circle amplification
3. HRP-labeled probes were used to visualize the interaction through fluorescent TSA (AF488-Tyramide)
4. After imaging the isPLA signal, mIF was performed with the standard CellScape workflow

Validation of Combined VistaPlex mIF and isPLA Staining

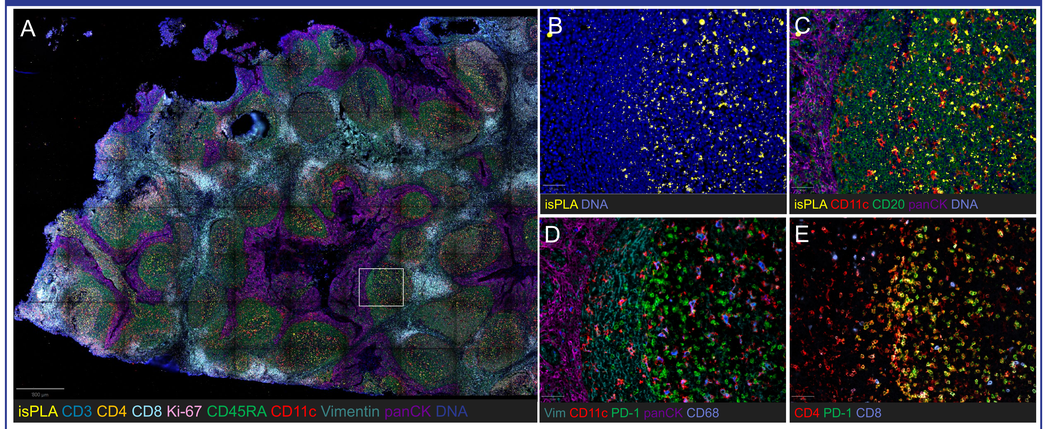


Figure 1: Combined isPLA and VistaPlex staining on FFPE tonsil tissue. (A) Whole slide overview image. The isPLA signal is most prevalent in germinal center regions. The marked germinal center is shown at higher magnification in B-E. **(B - E)** The isPLA signal is strongest in germinal center areas with CD11+CD68+Vim+ follicular dendritic cells and PD1-expressing follicular CD4+ Helper T cells.

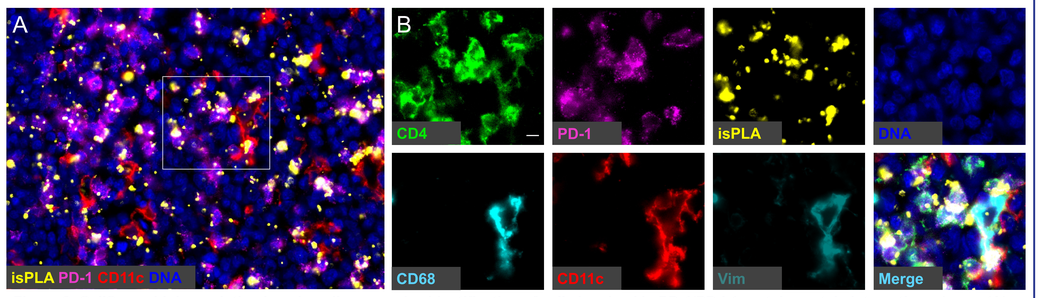


Figure 2: CellScape high resolution imaging allows precise identification of cells involved in PD-1/PD-L1 protein-protein interactions. Image **(A)** shows the same region as Fig. 1 B-E. The marked area is expanded in subcellular magnification in B. **(B)** Expression of CD11c, CD68 & Vimentin is a hallmark of follicular dendritic cells. FDCs interact with follicular helper T & B cells to regulate immune responses and prevent autoimmunity.

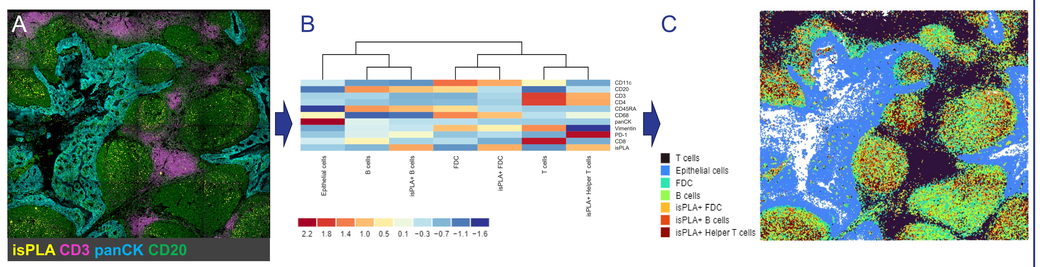


Figure 3: Bioinformatic analysis of isPLA / mIF data. (A) Images are exported as 32-bit OME TIFF files compatible with image analysis platforms, like Enable Medicine that was used here. Cell segmentation was performed with StarDist and followed up with unsupervised clustering analysis for cell phenotyping. **(B)** Cluster analysis shows high correlation of isPLA signal with CD4 & PD-1 and CD11c & CD68. **(C)** Precise spatial mapping of cell populaions of interest with the isPLA signal shown in orange to red.

Combined VistaPlex mIF and isPLA Staining on Tumor Tissue

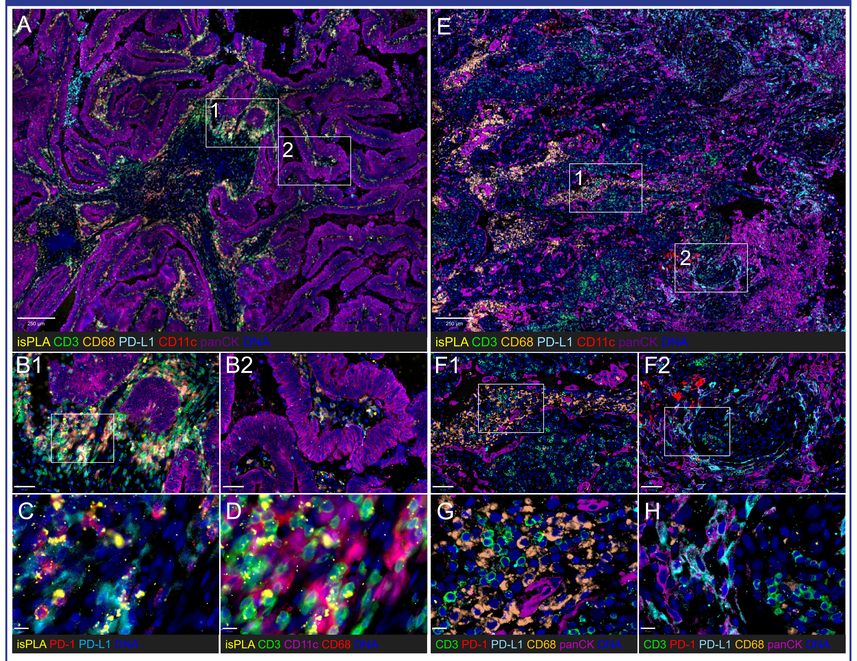


Figure 4: Differential isPLA signal in cancer tissues. (A) mIF immune profiling combined with isPLA on colorectal carcinoma. **(B1-B2)** PD-L1 staining is not present on tumor cells, but on certain immune cell populations in the stroma. These regions also show isPLA signal, suggesting PD-1/PD-L1 interaction and checkpoint activation. **(C)** Marked region in B1 shows an immune cell cluster with staining of PD-1 and PD-L1, correlating with an isPLA signal. **(D)** PD-1+ cells are mostly CD3+, while PD-L1+ cells show staining for CD11c and CD68. This suggests that a potential T-cell anti-tumoral response is suppressed by tumor-associated macrophages. **(E)** mIF/isPLA dual staining on a PD-L1+ lung cancer. **(F1-F2)** Approximately 40% of tumor cells are PD-L1+ and CD68+ cells were abundant. **(G-H)** No PD-1 staining was observed in different tumor regions and, consistently, no isPLA signal. This suggests lacking PD-1/PD-L1 interactions and no checkpoint activation, despite tumor PD-L1 positivity.

Conclusions

- High resolution imaging of immune checkpoint activation utilizing an *in situ* Proximity Ligation Assay and Precise Spatial Multiplexing on the CellScape platform uncovers differential tumor-immune cell regulation in cancer tissues.
- The isPLA signal was consistent with both fluorescent antibody-mediated biomarker localization and known immune cell interactions.
- isPLA enables an additional “interactome” readout for CellScape that provides comprehensive insight into specific interactions of biomarkers and cell populations in both tumor and non-tumor contexts.

References

1. Heesters, B. A., et. al. (2021). Characterization of human FDCs reveals regulation of T cells and antigen presentation to B cells. *The Journal of experimental medicine*, *218*(10).
2. Sage, P. T., et. al. (2018). Dendritic Cell PD-L1 Limits Autoimmunity and Follicular T Cell Differentiation and Function. *Journal of immunology (Baltimore, Md. : 1950)*, *200*(8), 2592–2602.

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