Integrating in situ Proximity Ligation Assay for PD-1/PD-L1 interaction with Multiplexed Immunofluorescence Imaging in Urothelial Carcinoma

Abstract # 4673

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1. Introduction

The PD-1/PD-L1 axis plays a crucial role in immune checkpoint regulation, and blocking this interaction is the target for many immune checkpoint inhibition (ICI) therapies used to treat a broad range of solid tumors. Despite the potential, therapeutic interventions targeting this pathway have exhibited limited efficacy, with approximately 20-30% response rates in advanced urothelial carcinoma patients (1). Today, patients with metastatic disease are eligible for PD-1/PD-L1 immunotherapy treatment, and first line treatment with ICI is based on the PD-L1 status alone, neglecting the complex spatial heterogeneity in patient samples. We hypothesize that the interaction between immune cells and tumor cells via the PD-1/PD-L1 axis is a better prognostic marker for response to ICI therapy.

In situ Proximity Ligation Assay (isPLA), commercialized by Navinci Diagnostics, is a method that allows for detection of protein interactions *in situ*, making use of two antibodies for the respective protein targets. If close in proximity (less than 40 nm apart) a rolling circle amplification product is formed that can be visualized by fluorescence microscopy and thereby detect interactions between the target proteins (2). Using isPLA to target PD-1/PD-L1 the level of PD-1/PD-L1 interactions between individual cells can be explored. If combined with multiplexed immunofluorescence, the interactions can also be assigned to specific cell types such as the tumor cells and different types of immune cells. In addition, the overall tumor microenvironment can be characterized, including detection of tertiary lymphoid structures that also has shown to be an interesting parameter for better response prediction to therapies.

By integrating isPLA and multiplexed immunofluorescence, we aim to enhance the predictive accuracy of treatments and improve patient stratification, paving the way for more personalized and effective cancer therapies.

Aim 1. **To develop an integrated assay to detect protein interactions between individual cells along with multiplexed immunofluorescence to phenotype different cell types and structures in the tumor microenvironment (TME).** In this study, we have successfully integrated isPLA using Navinci Diagnostics NaveniFlex kit for PD-1/PD-L1, and multiplexed immunofluorescence using the Phenocycler technology from Akoya Biosciences, all in one single experiment. This allows for fully automated data generation in a relatively high-throughput manner, with output data as one integrated dataset for further downstream analysis.

Aim 2. To apply the method developed in Aim 1 on a **retrospective cohort of muscle invasive bladder cancer treated with the ICI therapy Pembrolizumab**, to explore the possible correlation between the degree of PD-1/PD-L1 interactions with clinical endpoints. The PEMBRO-100 cohort consists of >100 patients with well defined clinical data. So far isPLA data for 11 patients has been generated, representing complete responders and non-responders and serving as proof-of-concept data set for exploring the correlation between PD-1/PD-L1 interactions and clinical endpoints.

2. Method and Technology integration

Formalin-fixed Paraffin-Embedded (FFPE) tissue samples were obtained from the retrospective PEMBRO-100 cohort of muscle invasive bladder cancer patients. The tissue sections were heated at 60°C for 40 minutes, then placed into a Leica Bond RX machine along with the **Naveni®** reagent, which is designed for automated processes. Within the Leica Bond device, the tissue samples underwent dewaxing and antigen retrieval using ER2 solution for 40 minutes at 100°C. The system was set up to execute the Naveni® sequence, encompassing steps such as blockage, incubation with both primary antibody and Navenibody, ligation, and rolling circle amplification. Following this, multiplexed immunofluorescence was generated using the Phenocycler technology from Akoya Biosciences. By adding the detection oligos (DO) for the isPLA in the first cycle of the Phenocycler assay, a fully integrated dataset of PD-1/PD-L1 protein interaction and the protein markers from a 15-plex antibody panel was generated on the PhenoCycler Fusion instrument (Fig. 1 A). The image datasets were analyzed using Q-path, focusing primarily on the PD-1/PD-L1 isPLA distribution along with phenotyping of the interacting cell types (Fig. 1B). Biological controls such as tonsil and bone marrow were used as technical validation (positive and negative, respectively) for the **Naveni®** PD-1/PD-L1 interaction assay (Fig. 1C).

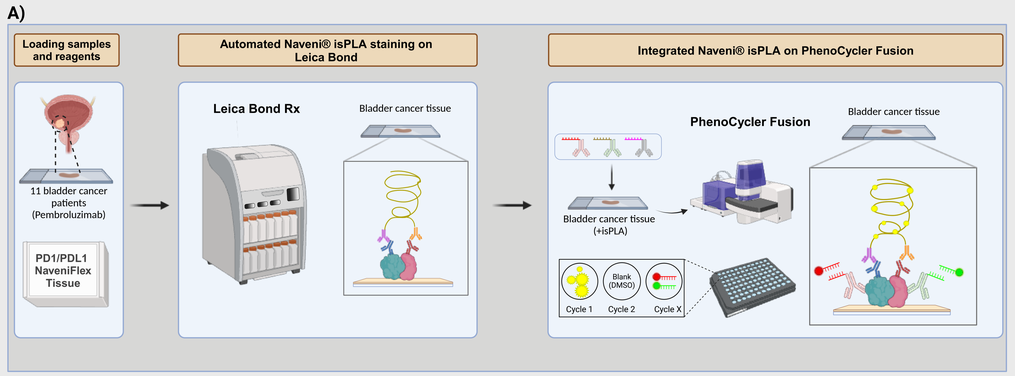


Fig. 1 A) Schematic illustration of automated NaveniFlex (isPLA) on Leica Bond Rx and integration into PhenoCycler Fusion platform.

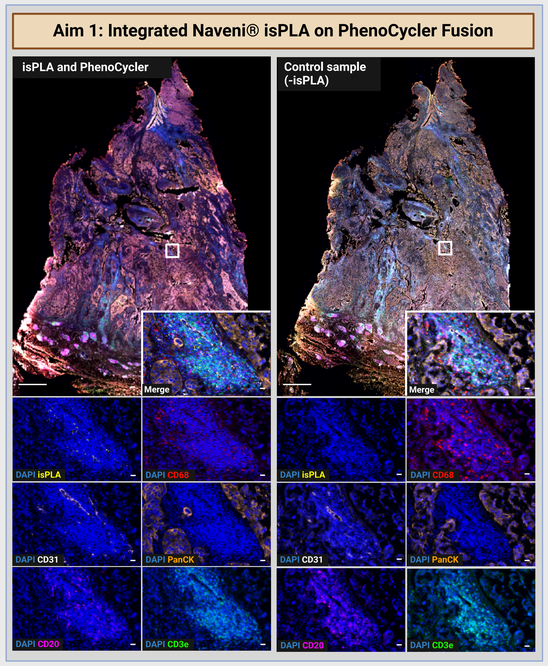
Fig. 1 B) Detection of automated PD-1/PD-L1 interaction in a TLS structure on PhenoCycler Fusion. The PD-1/PD-L1 isPLA was imaged (cycle 1) with no DO present. The DO was then added (cycle 2) and visualized after imaging. An elution step followed after detection of the isPLA (cycle 3), effectively removing the DO. The addition of a 15-plex panel followed, here showing immune and endothelial cells in a TLS structure. Scale bar: 50 um

Fig. 1 C) Biological controls. Staining on consecutive sections. Tonsil, positive control for NaveniFlex (isPLA), PD1-IHC and PD-L1 IHC. Bone marrow (BM), negative control for all assaysthe interaction. Scale bar: 50 um.

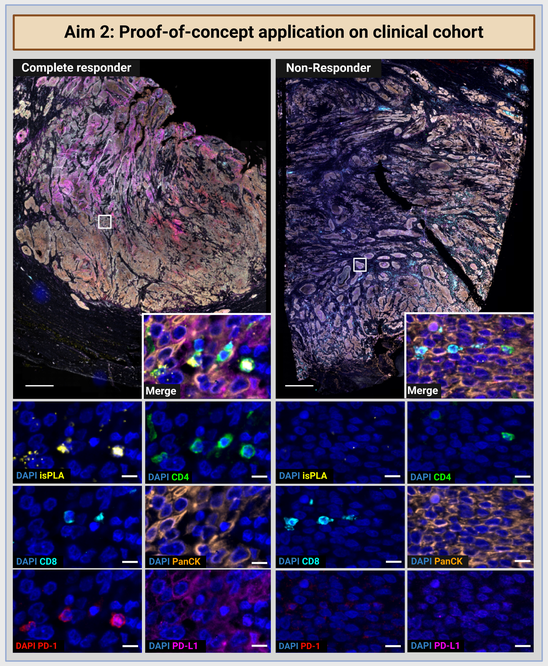
3. Results

The results from our study are summarized and divided according to our two aims. In aim 1, we describe the data we have generated from the **successful PD-1/PD-L1 isPLA method integration into Akoya Bioscience's PhenoCycler Fusion instrument** (Fig. 2). In aim 2, we build upon our technology integration and apply it on a **retrospective bladder cancer cohort.** Here, we share our NaveniFlex isPLA and 15-plex antibody panel results from a **complete responder and a non-responder** patient samples

Aim 1:
Following several internal control runs and optimizations for tonsil and bladder tissue (Fig. 1C), we have successfully integrated the *is*PLA assay for the detection of PD-1/PD-L1 interaction and multiplexed immunofluorescence using the Phenocycler technology. The workflow combines automated *is*PLA assay on Leica Bond, subsequent sample preparation and incubation of Phenocycler antibodies followed by automated data generation and detection of both isPLA signals and the protein markers in one integrated dataset on the Phenocycler instrument (Fig. 2). The use of automation in our integrated assays allows for significant improvements in efficiency and reproducibility. This approach enables the collection of data in a fully automated, high-throughput manner, leading to the creation of a cohesive dataset that's ready for in-depth downstream analysis. More than just increasing our capacity to process samples, automation ensures the data's consistency and reliability which are crucial for detailed investigations of the TME.



Combined *is*PLA and PhenoCycler panel
PD-1/PD-L1 in situ PLA
DAPI (Nuclear)
PD-1 and PD-L1 (Immune check-point interaction, isPLA control)
T-cells
CD3 (Pan-T-cell marker)
CD4 (T-helper cells)
CD8 (Cytotoxic T-cells)
FoxP3 (Regulatory T-cells)
Granzyme B (Cytotoxic T-/NK cells)
Lag3 (T-cell activation inhibition)
Additional immune cells
CD68 (M1 Macrophages)
MHC II/HLA-DR (Professionel APC's)
CD20 (B-lymphocytes)
Tumour vs stroma region
Pan-CK (Epithelial cells, tumor marker)
Ki67 (Proliferation marker)
PCNA (Proliferation marker)
Stroma angiogenesis
CD31 (Endothelial cells)
CD34 (Endothelial cells)



Aim 2:
Using our developed method from Aim 1, we have so far generated integrated isPLA and multiplexed immunofluorescence data for 11 patients from the retrospective PEMBRO-100 cohort of muscle invasive bladder cancer. Technically, the data has demonstrated robust staining performance (cellular staining pattern and intensity) for the individual protein marker from the Phenocycler antibody panel, and isPLA signal intensity across the patient samples. In contrast, the fraction and distribution of different immune cells and the number of isPLA clusters vary between the patients, demonstrating biological differences between the patients. Preliminary data of these few samples indicate a correlation between the number of isPLA clusters and response to the ICI treatment. The 15 plex antibody panel used in this assay, allows for the assignment of the PD-1/PD-L1 signals to specific cells, the phenotyping of tumor cells (Dapi size and panCK positive), immune cells (CD3, CD4, CD8, CD68, HLA-DR, CD20), and specific cellular states (ki67, PCNA, FoxP3, LAG3, Granzyme B). In addition, the PD-1 and PD-L1 markers serve as technical controls for the isPLA signal and to further distinguish the cell types involved in the interaction (Fig. 3).

Fig. 2 Figure showing the simultaneous detection of PD-1/PD-L1 isPLA with 5 PhenoCycler marker antibodies (CD3e, CD20, CD31, CD68, pan-CK) from a 15-plex mIF panel. A paired bladder cancer sample (consecutive section) with no PD-1/PD-L1 isPLA was used as a control. Protein markers in the PD-1/PD-L1 isPLA stained sample and control displayed similar staining pattern and intensity. However, minor variations was observed (Pan-CK, overview) and can possibly be explained by the two different tissue processing (Dewaxing and ER on Leica Bond and manual) approaches that were used. Overview, scale bar: 2 mm. Protein markers, scale bar: 20 um

Fig. 3 Figure showing isPLA and 15-plex antibody panel in a responder and non-responder from PEMBRO-100 bladder cancer cohort. High-resolution images show interaction between (CD3e +, CD4+, PD1-positive) T-helper cells and (PD-L1-positive) tumor cells in a complete-responder patient sample. Present is also non-interacting (CD3e+, CD8+, PD-1-negative) cytotoxic T-cells. In a non-responder patient sample we see (CD3e+, CD4+ and PD-1-negative) T-helper and (CD3e+, CD8+ and PD-1-negative) Cytotoxic cells having no interaction with surrounding (PD-L1-negative) tumor cells. A Overview, scale bar: 2 mm. Protein markers, scale bar: 20 um

4. Conclusion

- We have successfully developed a method for simultaneous detection of protein interactions between PD-1/PD-L1 and multiplexed immunofluorescence
- The method is fully integrated on the Leica Bond and Phenocycler platform, with a single output dataset
- The method was applied on 11 samples from a retrospective cohort of muscle invasive bladder cancer patients - PEMBRO-100.
- The results demonstrate reproducible technical performance for all protein markers and isPLA signal intensities across samples.
- Differences in number of isPLA clusters and immune cell distribution indicate correlation to ICI response.

5. Prospects

- Our plan is to apply our method on a large patient cohort of 100 patients (Pembro-100), in order to confirm or deny previously established results.
- Further spatial analysis of PD-1/PD-L1 in concert with multiplex immunofluorescence is needed to better predict treatment outcomes in patients with any type of cancer diagnosis.

1) Balar, Arjun V et al. "First-line pembrolizumab in cisplatin-ineligible patients with locally advanced and unresectable or metastatic urothelial cancer (KEYNOTE-052): a multicentre, single-arm, phase 2 study." *The Lancet. Oncology* vol. 18,11 (2017): 1483-1492. doi:10.1016/S1470-2045(17)30616-2
2) Elofsson H, Arngården L, Hahne O, *et al*2009 Fully automated *in situ* detection of protein-protein interactions in tissue.*Journal for ImmunoTherapy of Cancer* 2023;**11**:doi: 10.1136/jitc-2023-SITC2023.0209

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