

Integration of Multiplex isPLA with High-Plex Spatial Phenotyping on the PhenoCycler Fusion

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1 INTRODUCTION

Spatial proteomics can define cell phenotypes and tissue architecture at high dimensionality, but conventional antibody-based imaging does not directly report protein–protein interactions. In our previous work, *in situ* proximity ligation assay (isPLA) enabled spatial detection of PD-1/PD-L1 interactions together with multiplex phenotyping in FFPE tissue¹.

Here, we extend this concept to **multiplex isPLA (misPLA)** and integrate **Navinci Diagnostics OMNI panel** for multiple protein–protein interaction readouts with **Quanterix Human IO60 Phenocode® Discovery Panel** for high-plex spatial phenotyping on the PhenoCycler Fusion. Two complementary assay configurations were used. A 9-marker tonsil panel was designed primarily to evaluate multiplex detection performance, reporter elution and potential oligonucleotide cross-reactivity across immune and epithelial phenotyping targets. A second panel was applied to urothelial carcinoma tissue, focusing on biologically relevant protein interactions including **PD-1/PD-L1**, **PD-1/PD-L2**, **CD8/MHC-I** and **E-cadherin/β-Catenin-1**, followed by integration with the IO60 phenotyping panel.

The aim was to establish a single automated workflow and integrated image dataset that combines cell-type-assigned protein interactions with surrounding immune and tissue phenotypes, while also evaluating compatibility between the misPLA chemistry and downstream high-plex PhenoCycler imaging.

A

Experimental design: 4-plex isPLA + IO60 workflow

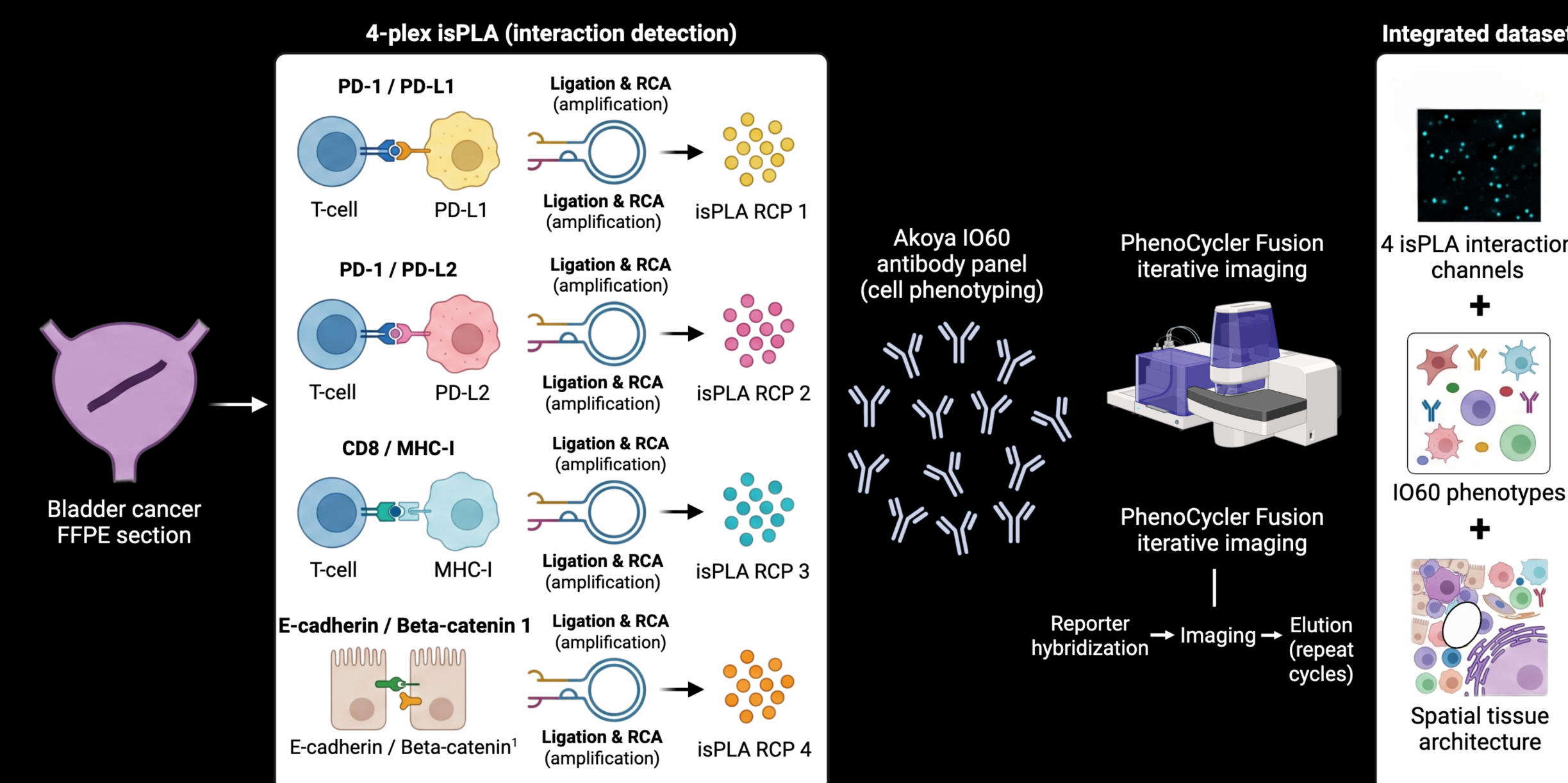


Fig. A) Integrated misPLA–PhenoCycler Fusion workflow. Multiple protein–protein interactions are detected by misPLA and visualized through sequential reporter hybridization and imaging, followed by high-plex phenotyping with the IO60 panel on the PhenoCycler Fusion. The workflow generates interaction and phenotypic information within a single spatial image dataset.

2 METHOD

FFPE tissue processing and preparation for PhenoCycler Fusion imaging were performed at the SciLifeLab Spatial Proteomics Unit according to the applicable Akoya/IO60 workflow, while multiplex isPLA (misPLA) was performed at Navinci Diagnostics in Uppsala.

Initial optimization and cross-reactivity testing were performed on FFPE tonsil using a 9-plex detection panel (CD3, MHC-1, CD19, B7H3, EGFR, CD79b, CD8, LAT, VCAM-1) workflow across AF488, Atto550 and Cy5 channels, followed by blank imaging after each elution cycle.

For the final integrated experiment, a 4-plex misPLA panel (PD-1/PD-L1, PD-1/PD-L2, CD8/MHC-1 and E-cadherin and β-catenin-1) was combined with the IO60 antibody panel in FFPE urothelial carcinoma tissue. In this configuration, the AF488 detection channel used during tonsil optimization was replaced by AF750, while Atto550 and Cy5 were retained. The workflow enabled acquisition of misPLA interaction signals and high-plex phenotyping markers within the same spatial image dataset. Assay compatibility was assessed by reporter-elution efficiency, bidirectional oligonucleotide cross-reactivity testing, and exploratory comparison of downstream IO60 staining.

3 RESULTS

MULTIPLEX DETECTION & ASSAY COMPATIBILITY

Multiplex isPLA was integrated with high-plex phenotyping on the PhenoCycler Fusion using sequential detection and elution cycles. A 9-plex detection workflow was first evaluated in FFPE tonsil using phenotype-oriented markers distributed across the AF488, Atto550 and Cy5 detection channels. Marker-specific fluorescence was readily detected during reporter cycles and strongly reduced in the corresponding post-elution blank cycles, supporting sequential detection with minimal residual fluorescence.

Two complementary experiments were used to assess potential oligonucleotide cross-reactivity between the multiplex isPLA and PhenoCycler chemistries. No detectable signal was observed when PhenoCycler reporter sequences were applied against multiplex isPLA rolling-circle amplification products, or when multiplex isPLA detection oligonucleotides were applied against the IO60 antibody-barcode panel.

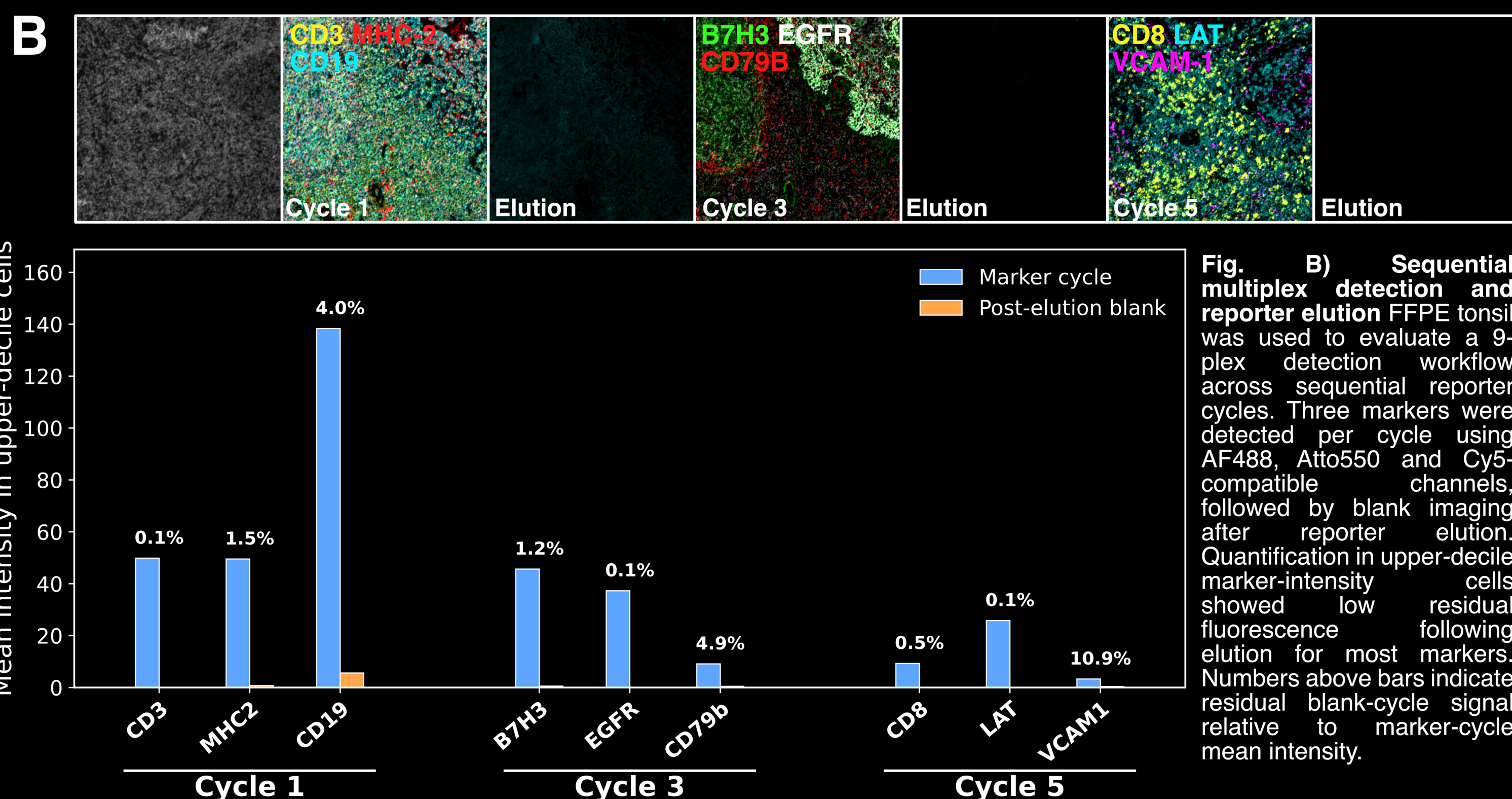
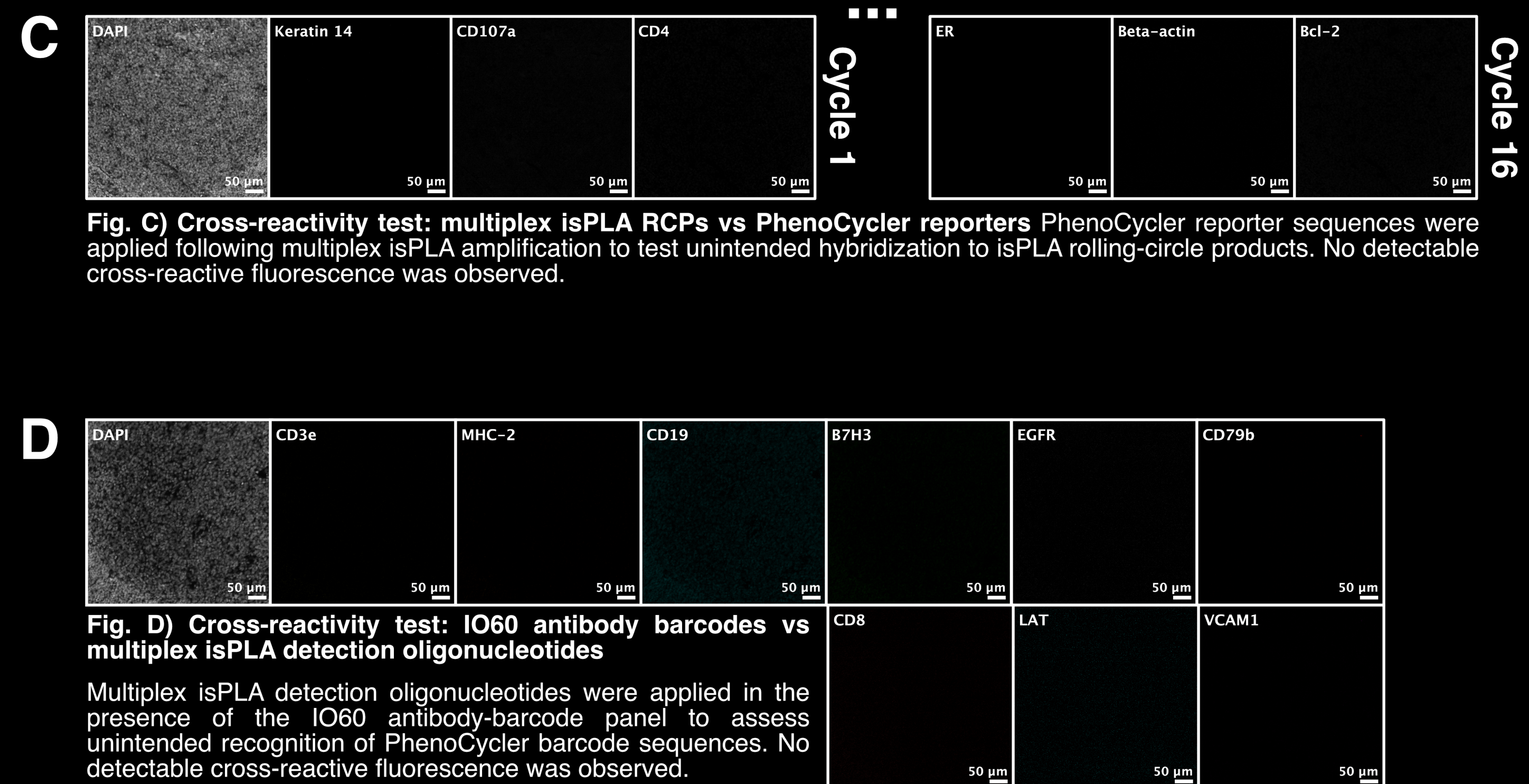


Fig. B) Sequential multiplex detection and reporter elution. FFPE tonsil was used to evaluate a 9-plex detection workflow across sequential reporter cycles. Three markers were detected per cycle using AF488, Atto550 and Cy5-compatible channels, followed by blank imaging after reporter elution. Quantification in upper-decile cells showed low residual fluorescence following elution for most markers. Numbers above bars indicate residual blank-cycle signal relative to marker-cycle mean intensity.



INTEGRATION WITH HIGH-PLEX IO60 PHENOTYPING

Potential downstream effects of multiplex isPLA on high-plex phenotyping were explored in urothelial carcinoma tissue. Nearby tissue sections were processed either with multiplex isPLA followed by the IO60 panel or with IO60 alone. Within a matched 3000 × 3000 px tumor-region ROI (2307923 μm²), the overall hierarchy of marker intensities was broadly preserved between conditions (Pearson $r = 0.874$; Spearman $\rho = 0.906$; regression slope = 0.888; $R^2 = 0.763$), although individual markers displayed variable increases or decreases in P90 intensity.

Because this comparison was performed on separate nearby sections and a limited ROI, it represents an exploratory assessment of assay compatibility rather than a definitive measurement of isPLA-induced effects.

Finally, multiplex isPLA interaction signals could be visualized together with conventional phenotyping markers in the same spatial dataset. PD-1/PD-L1, PD-1/PD-L2 and CD8/MHC-I interactions were demonstrated within a tertiary lymphoid structure, while E-cadherin/β-catenin interactions were visualized in urothelial carcinoma tissue, enabling simultaneous localization of protein interactions and surrounding cellular phenotypes.

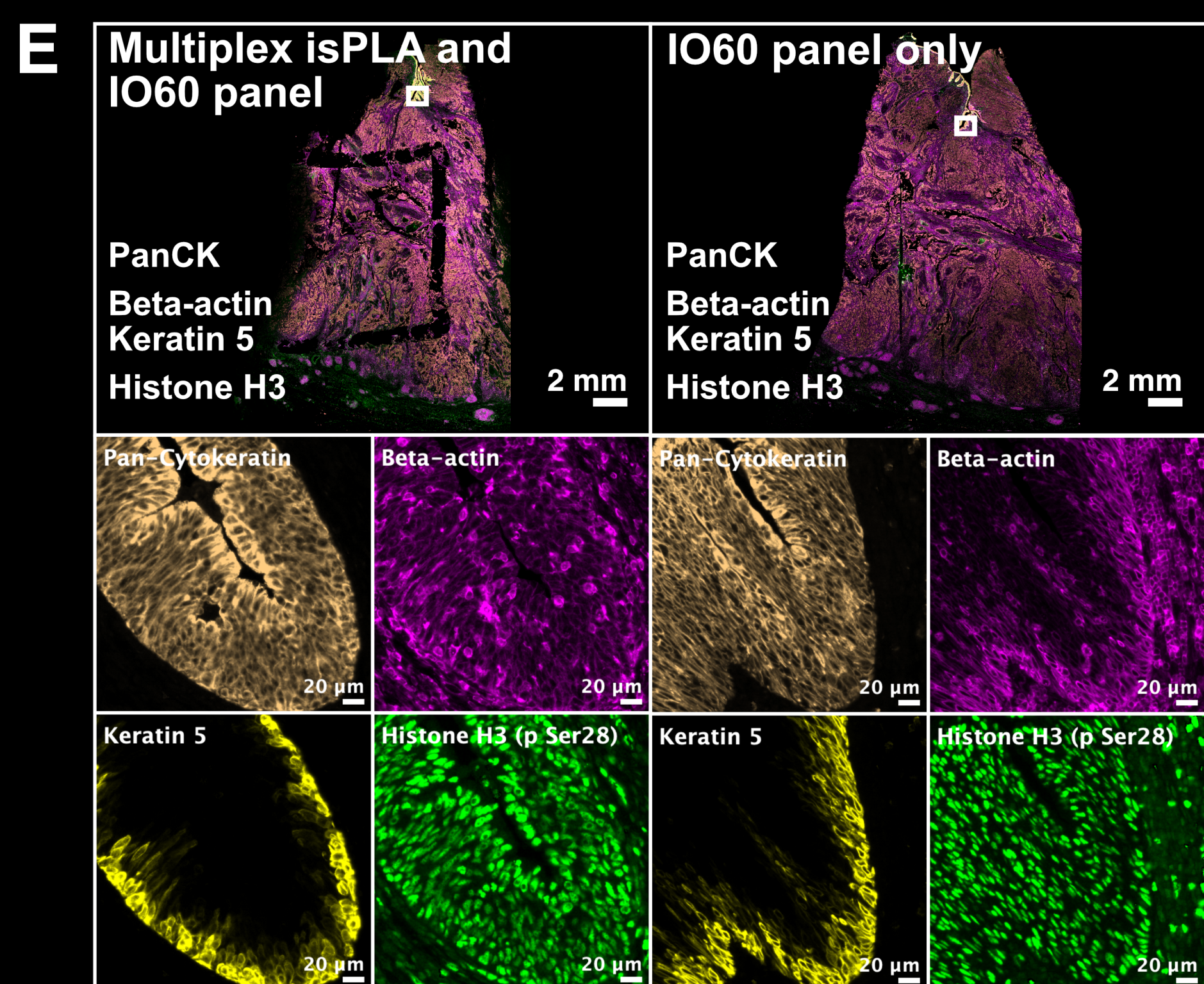


Fig. E) Downstream IO60 staining following multiplex isPLA. Representative urothelial carcinoma regions stained with multiplex isPLA followed by IO60 or with IO60 alone. Selected epithelial, stromal and immune markers are shown to illustrate preservation of downstream phenotyping.

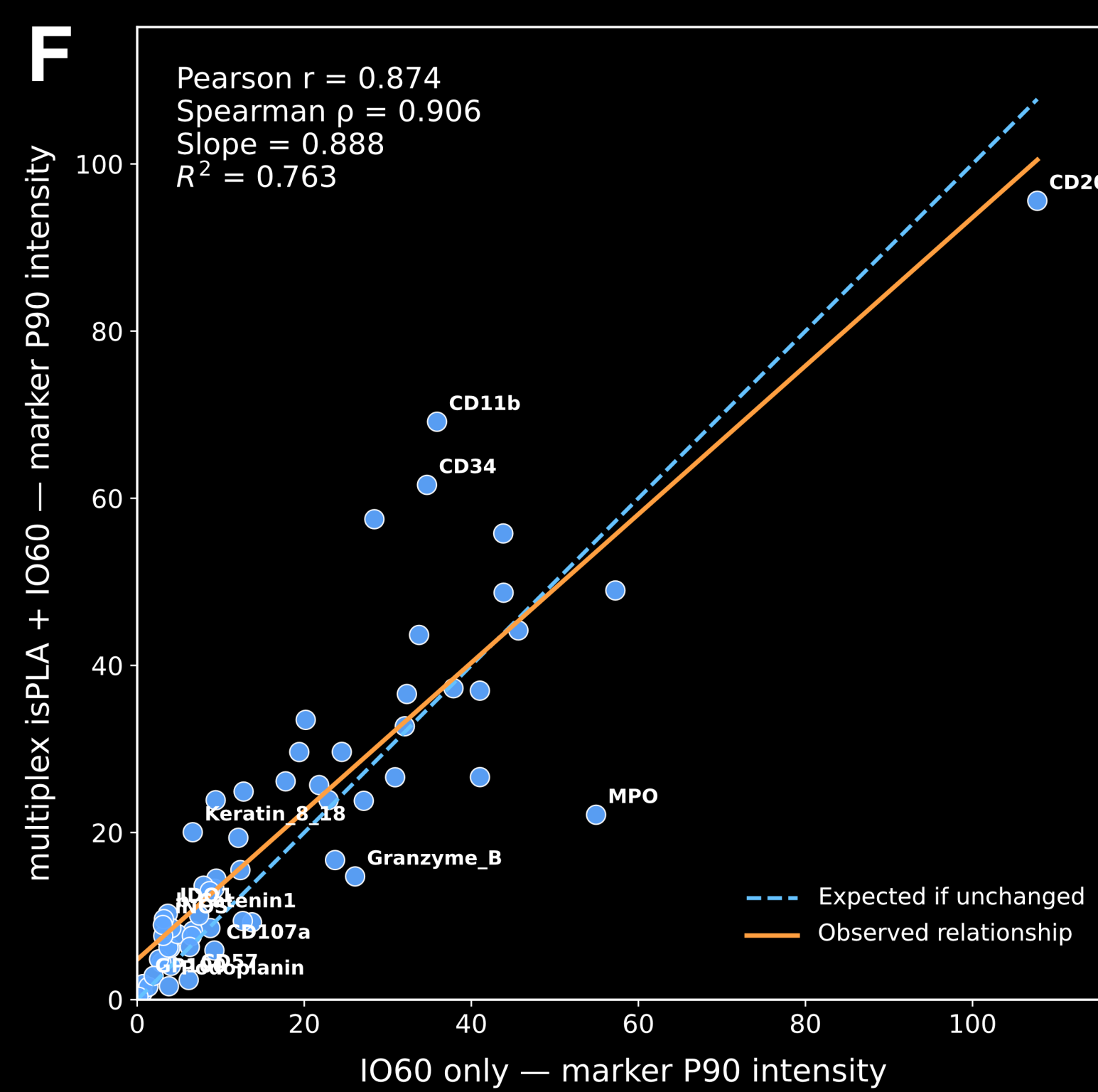


Fig. F) Exploratory quantitative comparison of downstream IO60 staining. P90 cellular marker intensities were compared between a 3000 × 3000 px tumor-region ROI (2307923 μm²) from nearby urothelial carcinoma sections processed with multiplex isPLA + IO60 or IO60 alone. Marker intensities showed strong overall association (Pearson $r = 0.874$; Spearman $\rho = 0.906$), while individual marker differences remained evident.

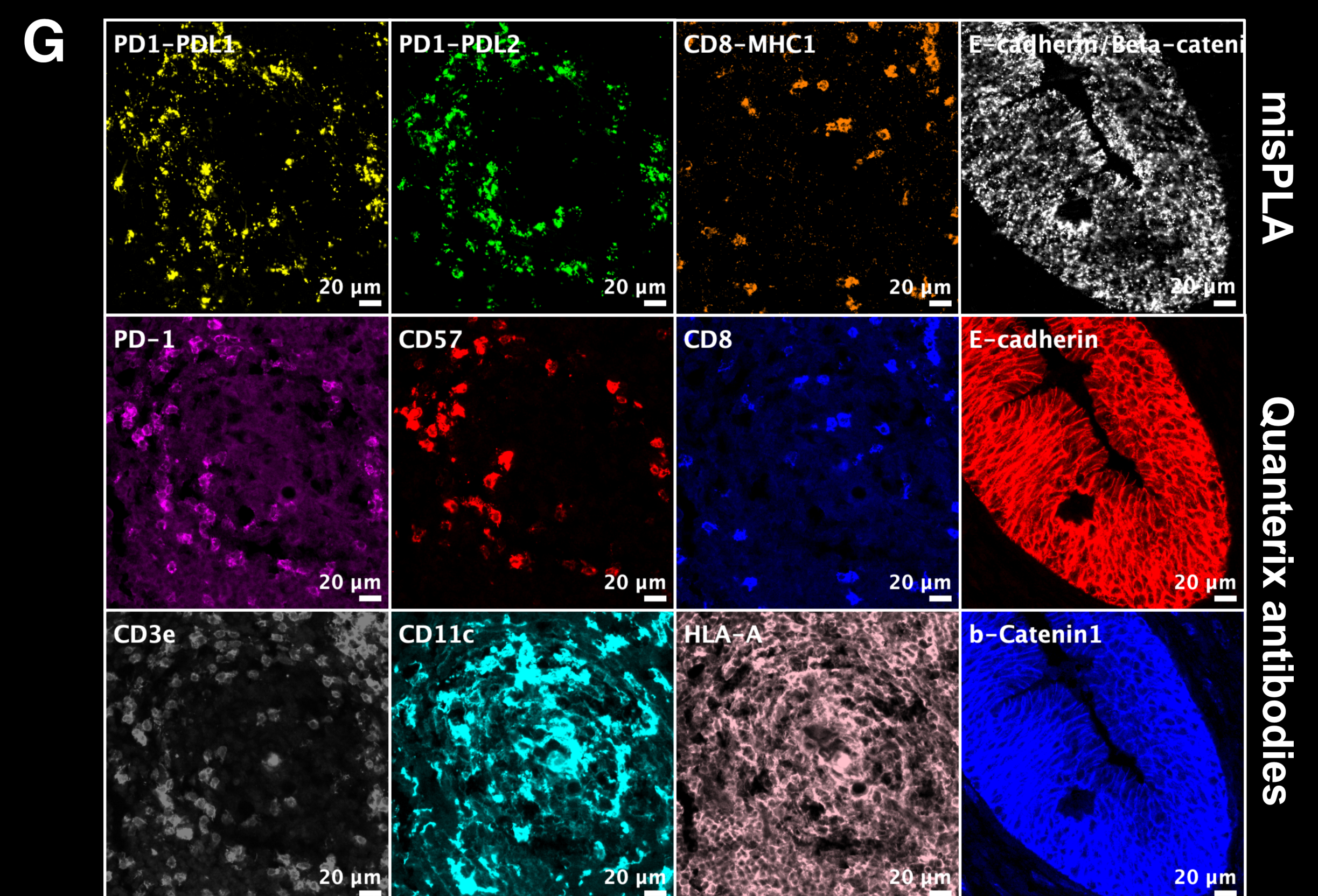


Fig. G) Integrated spatial detection of protein interactions and cellular phenotypes. Representative multiplex isPLA interactions visualized together with PhenoCycler phenotyping markers. PD-1/PD-L1, PD-1/PD-L2 and CD8/MHC-I interactions are shown within a tertiary lymphoid structure, while E-cadherin/β-catenin interaction is shown in urothelial carcinoma tissue.

4 CONCLUSION

- Multiplex isPLA enables simultaneous detection of multiple protein–protein interactions on the PhenoCycler Fusion.
- Sequential reporter detection showed minimal residual fluorescence and no detectable oligonucleotide cross-reactivity in the evaluated experiments.
- Integration with IO60 preserved the overall high-plex marker-intensity pattern and enabled cell-contextualized interaction mapping in FFPE tissue.

