Exploring functional interactions *in situ* using a combined multiomic approach

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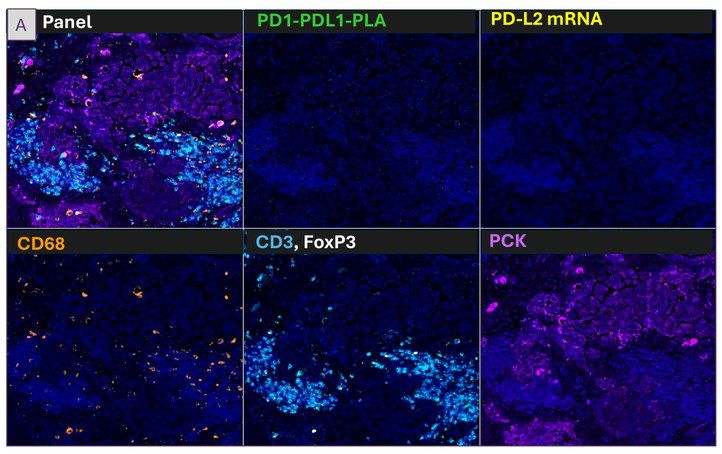
Background and purpose

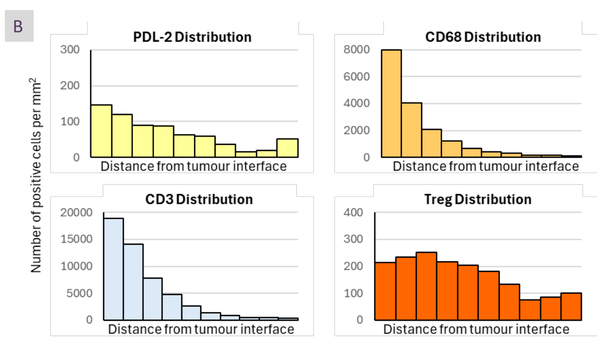
Conventional multiplex fluorescence techniques, such as *in situ* hybridisation (ISH) and immunohistochemistry (IHC), provide valuable insights into analyte expression patterns and spatial co-localisation within tissue. However, these approaches often offer limited information regarding functional molecular interactions. Integrating mRNA and protein detection with proximity-based assays enables the direct interrogation of molecular interactions *in situ*, providing a powerful framework for decoding cellular function and accelerating therapeutic discovery. Our goal was to develop and apply two multiplexed multiomic assays to investigate mechanisms of functional immune evasion and stromal remodeling, enable quantitative and spatially resolved functional characterisation of tumour biology within the native tissue context and facilitate deeper interrogation of complex immune–tumour interactions.

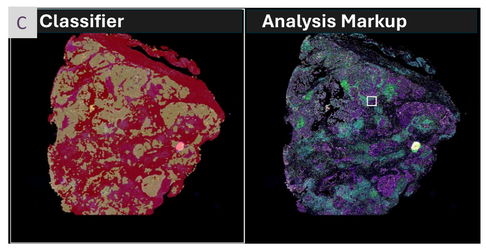
Multiomic assay development and image analysis

We developed two panels integrating RNAscope™ Multiomic technology (Bio-Techne) with Akoya Opal™ (Quanterix) multiplex immunohistochemistry (mIF) on the Leica BOND RX automated platform. Stained slides were scanned using a PhenoImager HT (Quanterix) and image analysis was performed using HALO® (Indica Labs).

Immune-evasion panel : simultaneous detection of PD-1/PD-L1 interaction (ProximityScope™ PLA), PD-L2 mRNA expression and CD3, CD68, FOXP3 and pan-cytokeratin protein expression in ovarian carcinoma. This panel enables high-resolution spatial profiling of immune evasion mechanisms within ovarian cancer tissue, allowing concurrent visualisation of immune checkpoint engagement, T cells, regulatory T cells, macrophages, and epithelial cells. Spatial analysis tools were used to interrogate functional interaction between populations detected in this panel.

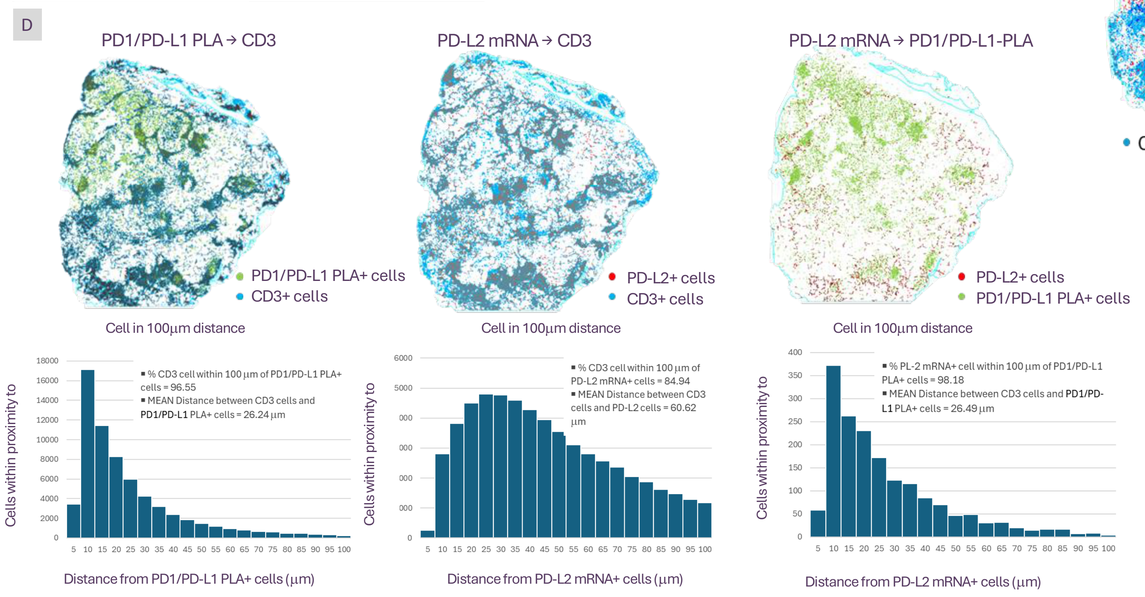


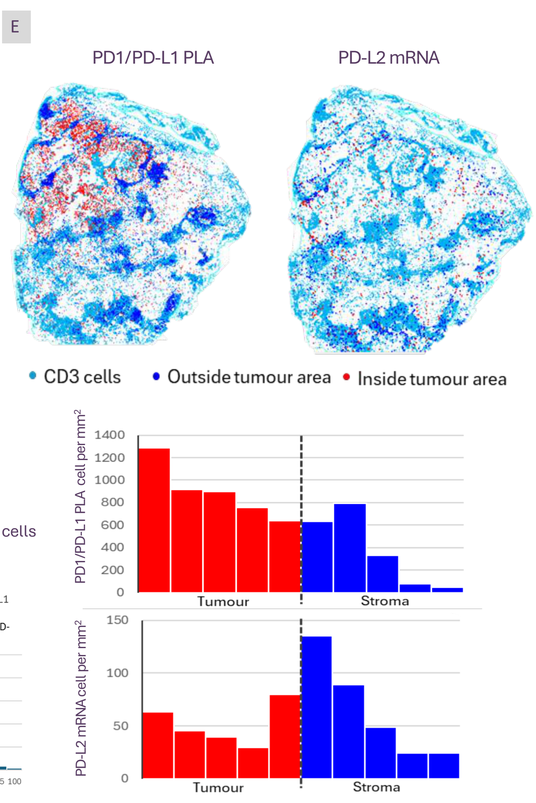




Analysis Output Table

	Tumour	Stroma
PDL2 mRNA+ Cells (% of total)	0.70	1.08
PD1-PDL1 PLA+ Cells (% of total)	7.34	6.74
CD68+ Cells (% of total)	21.06	14.82
CD3+ Cells (% of total)	19.37	46.65
[CD3+ FOXP3+] Cells (% of CD3)	7.82	4.50
[PD1-PDL1 PLA+ PDL2+] Cells (% of total)	0.09	0.10
Area Analysed (mm²)	7.65	10.92

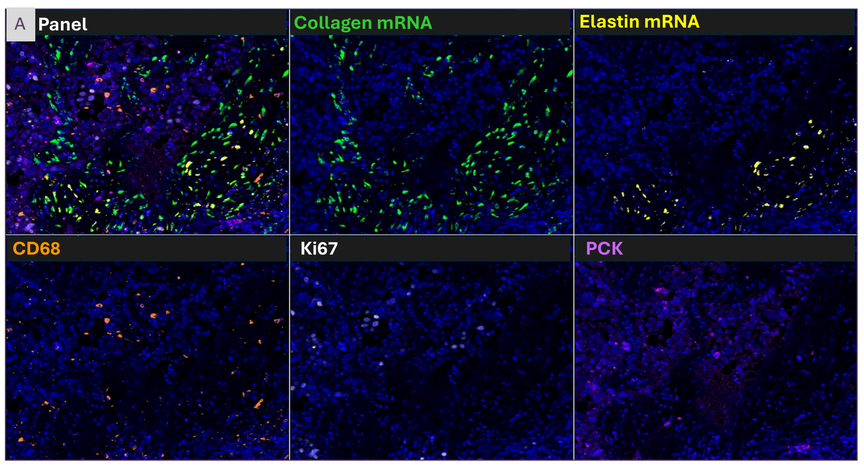


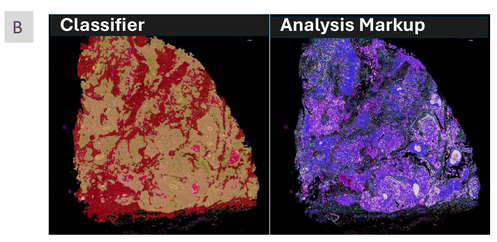


Immune evasion panel

A) Representative image of a region of interest (ROI) from an ovarian carcinoma multiomic-stained section. Individual channels are shown **B)** Spatial distribution of PD-L2, CD3, CD68, and Treg within a 220µm distance from the tumour–stroma interface showing T cells and macrophages localise close to the tumour border, whereas Treg and PD-L2 expressing cells are mainly distributed throughout the stroma. **C)** Tissue was classified into tumour and stroma areas for spatial analysis (Classifier). Quantitative analysis of the distribution of the panel populations in stromal and tumour regions (Analysis Markup and Table), showing T cell and macrophage infiltration of the tumour areas. **D)** Spatial relationship (proximity analysis) between PD1/PD-L1 PLA+, PD-L2 mRNA+ and CD3+ cells. Histogram depicting the percentage of cells within a 100µm radius for PD1/PD-L1 PLA proximity to CD3 and PD-L2, and CD3 proximity to PD-L2. PD1/PD-L1 PLA+ cells are observed in closer proximity to CD3+ cell than PD-L2 mRNA cells. Additionally, inhibited T cells (PD1/PD-1 PLA+) are detected close PD-L2 mRNA+ cells. **E)** Spatial distributions of PD1/PD-L1 PLA+ cells and PD-L2 mRNA+ cells across a 250µm within and outside the stromal–tumour margin (dotted line on the histogram). Immune checkpoint engagement (PD1/PD-L1 PLA) was higher in the tumour areas, whilst PD-L2 mRNA distribution was more clustered in the immediate vicinity of the margin.

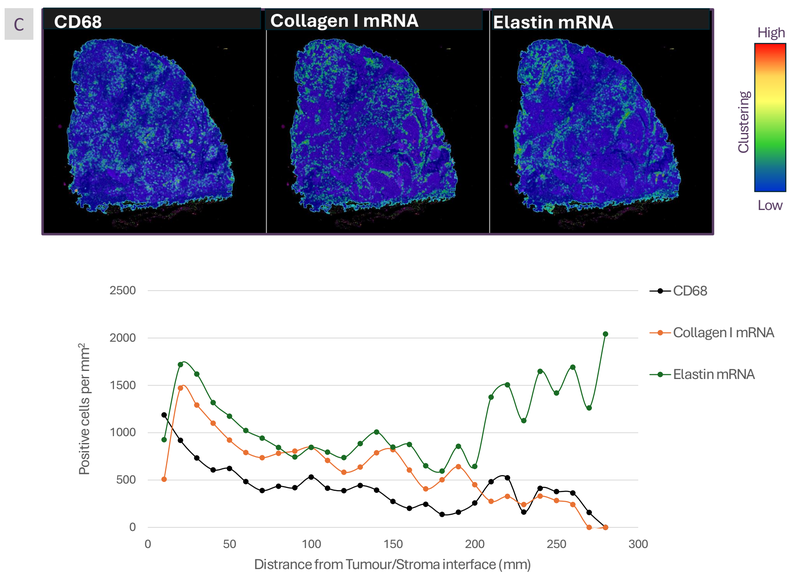
Stroma remodeling panel : multiomic detection of collagen I and elastin mRNA expression and CD68, Ki67 and pan-cytokeratin protein expression in ovarian carcinoma. This panel enables spatially resolved characterisation of macrophage-driven stromal remodelling in ovarian cancer tissue.





Analysis Output Table

	Tumour	Stroma
PCK+ Cells (% of total)	65.48	21.3
[PCK+ Ki67+] Cells (% of PCK+)	19.82	0.9
Col I mRNA+ Cells (% of total)	1.05	12.09
Elastin mRNA+ Cells (% of total)	2.32	14.28
CD68+ Cells (% of total)	10.08	9
Area Analysed (mm²)	10.87	8.1



Macrophages relation to stroma remodeling Panel

A) Representative image of a region of interest (ROI) from an ovarian carcinoma multiomic-stained section. Individual channels are shown. **B)** Tissue was classified into tumour and stroma areas for spatial analysis (Classifier). Quantitative analysis of the distribution of the panel populations in stromal and tumour regions (Analysis Markup and Table). **C)** Spatial distribution analysis of CD68+ cells, collagen I mRNA+, elastin mRNA+ cells. Distribution of populations represented as Heatmap (upper panel) showing clustering patters for each population analysed. Although populations localise in the stroma, clustering patterns do not obviously overlap. However, distribution across the stroma from the tumour-stroma border (lower graph) better depicts the inter-relationship between CD68+ cells and the expression of collagen and elastin mRNA in cells with fibroblast morphology. This observation agrees with the literature where it is described that elastin rich areas promote M2-macrophage switch, that will induce the expression of collagen I by activated fibroblasts.[1,2]

Method

- Proximity and multiplexed mRNA detection were performed using the RNAscope Multiomic LS assay. PD-1/PD-L1 staining was carried out using a combination of rabbit and mouse primary antibodies.
- Multiplex immunofluorescence (mIF) staining was carried out using Akoya Opal-based classic reagents.
- Whole-slide imaging was performed using the PhenoImager HT system.
- HALO® software was used for spatial image analysis using Highplex FL v5.2.2 and spatial analysis tools (cell infiltration, proximity analysis, and density heatmap analysis)

Conclusion

We have established two multiplexed multiomic assays for the interrogation of functional immune evasion and stromal remodeling. Integration with HALO spatial analysis enables quantitative evaluation of immune evasion and characterisation of macrophage-driven extracellular matrix remodeling. Collectively, this approach provides new opportunities for spatially resolved, *in situ* functional profiling, advancing comprehensive assessment of tumour biology and complex immune–tumour interactions.

References

1 Sig Transduct Target Ther 10, 93 (2025). Doi: 10.1038/s41392-025-02124-y
2 Front. Oncol. 11:596798 (2021). Doi: 10.3389/fonc.2021.596798

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