Spatial Proteomic Profiling of TME with a Cross Species Capable, IO Aligned, High Plex Panel on the Akoya PhenoCycler®-Fusion 2.0

Veronique Neumeister MD[1], Danica Wuelfing[1], Hong Cao PhD[1], Rachel Schaefer[1], Caitlin Looney[1], Briana Cooper[1], Adam Dickinson PhD[1], Ritu Mihani PhD[1], Bassem Ben Cheikh[2], Kishore Malyavantham PhD[1], Emily Neil PhD[1]

1. Reagent Research & Development, Quanterix Corporation, Billerica, Massachusetts, USA
2. Tixomics, Los Angeles, CA, USA

Background

Understanding the spatial organization and functional state of the tumor microenvironment (TME) is critical for elucidating mechanisms of immune response, resistance, and therapeutic activity in immuno-oncology (1, 2). High-plex spatial proteomics enables simultaneous characterization of immune populations, activation and exhaustion states, tumor–stromal interactions, and cellular neighborhoods within intact tissue (3). Expanding this framework to include markers of tumor metabolism and key antibody–drug conjugate (ADC) targets further enables characterization of metabolic states, therapeutic targets, and spatial determinants of treatment response (4). However, translating relevant findings from preclinical mouse models to human disease remains challenging, in part because differences in marker selection and profiling approaches can limit direct biological comparison. An expanded IO-aligned, cross-species spatial profiling framework may provide a more consistent approach for connecting strategically selected, preclinical models with human tumor biology and clinical development.

PhenoCycler-Fusion 2.0 Approach: Ultra-High Plex Spatial Proteomics

The PhenoCycler®-Fusion 2.0 (PCF) integrates high-plex barcode-based chemistry with automated imaging and fluidic control to enable subcellular-resolution spatial proteomics across large tissue areas. Cyclic in situ detection permits quantification of tens to hundreds of protein targets within a single intact section while preserving tissue morphology.

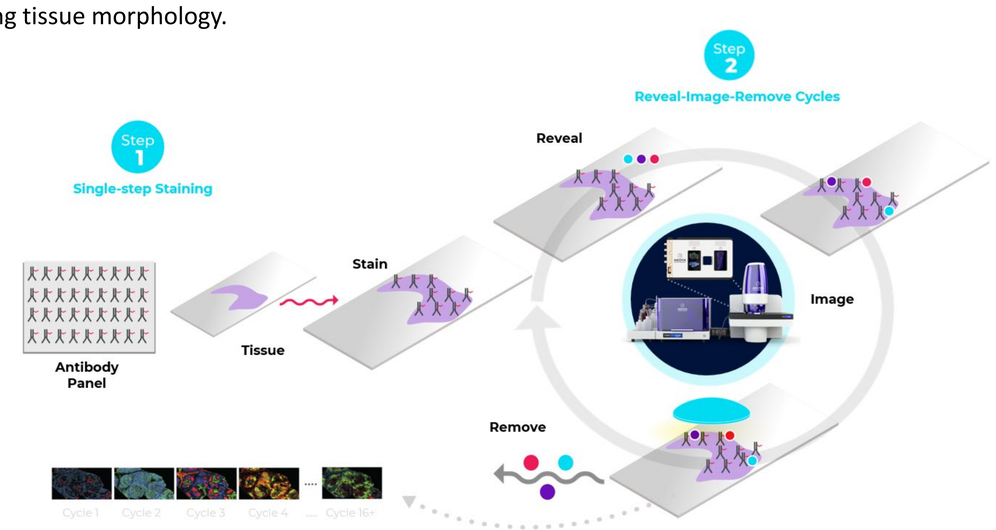


Figure 1. PhenoCycler®-Fusion 2.0 workflow
A gentle, molecular barcoding-based chemistry enables highly multiplexed detection of dozens of protein biomarkers simultaneously.

Methods

FFPE NSCLC, breast cancer, and multi-tissue TMA specimens were processed on the PCF targeting 79 biomarkers. Information on the tissue samples/TMA and on the design of an ultra-high plex PCF biomarker panel are listed in Table 1 and Table 2. Table 2 also summarizes information on cross-species reactivity of selected targets. Images were analyzed using **Celtix™**, an end-to-end, code-free analysis platform integrating image analysis, bioinformatics, and statistical analysis. The workflow included study design, quality control, cell segmentation, phenotyping, and spatial analysis, with custom figures and visualizations generated directly in **Celtix™**.

Table 1. Tissue Samples

Stained Sections							
Multi-Tissue TMA	Tonsil 1	Tonsil 2	Tonsil 3	Tonsil 4	Normal Breast 1	Normal Breast 2	Normal Breast 3	Normal Breast 4
	Breast Cancer 1	Breast Cancer 2	Breast Cancer 3	Breast Cancer 4	Breast Cancer 5	Breast Cancer 6	Breast Cancer 7	Breast Cancer 8
	Breast Cancer 9	Breast Cancer10	Normal Lung 1	Normal Lung 2	Normal Lung 3	Normal Lung 4	NSCLC 1	NSCLC 2
	NSCLC 3	NSCLC 4	NSCLC 5	NSCLC 6	NSCLC 7	NSCLC 8	NSCLC 9	NSCLC 10
	Normal Skin 1	Normal Skin 2	Normal Skin 3	Normal Skin 4	Melanoma 1	Melanoma 2	Melanoma 3	Melanoma 4
	Melanoma 5	Melanoma 6	Melanoma 7	Melanoma 8	Normal Liver 1	Normal Liver 2	Normal Liver 3	Normal Liver 4
	NASH 1	NASH 2	NASH 3	NASH 4	NASH 5	NASH 6	NASH 7	NASH 8
Breast Cancer	WSS; Infiltrating ductal carcinoma of the breast; G2, T2N0M0; ER+ (75%), PR+(75%), Her2: 1+							
NSCLC	WSS; NSCLC; adenocarcinoma, G3, T3N1M0							

Table 2. Human IO60 PhenoCode® Discovery Panel, the Metabolism Spike-In Module, and additional Spike-In Targets and Cross Species Compatibility

IO60 Panel

Immune Cell Typing	Lymphocyte Profiling	Immune Activation	Myeloid Profiling	Proliferation	Tissue Architecture	Tumor-specific Markers
CD11c	CD107	ICOS	CD11b	Histone H3 pSer28	β-Catenin1	BCL-2
CD14	CD21	IDO-1	CD163	Ki67	Beta-Actin	EpCAM
CD20	CD38	IFNG	CD206	PCNA	Caveolin-1	ER
CD3e	CD39	LAG-3	CD209		CD31	GP100
CD4	CD57	PD-1	CD66		CD34	Keratin14
CD44	CD79A	PD-L1	iNOS		Collagen IV	Keratin 5
CD45	FOXP3	VISTA	MPO		E-Cadherin	Keratin 8/18
CD45RO	Granzyme B				Podoplanin	Pan-CK
CD56	TCF-1				SMA	SOX2
CD68	TOX				Vimentin	TP63
CD8						
HLA-A						
HLA-DR						

Spike-in Module

IO Spike-In	ADC Targets Spike-In	Metabolism Spike-In
Arginase-1	EGFR	ASCT2
CTLA-4	B7H3	ATP5A
p53	Mesothelin	CS
CD15	FBP	G6PD
CD19		GLUT-1
OX40		HK1
		IDH2
		LDHA
		SDHA

Legend
Conjugated antibody compatible and validated on human and mouse tissue samples
Conjugated antibody compatible with human tissue only
Antibody cross species reactive for human and mouse tissue but validation data for conjugated antibody not available at this time

References:
1. Aung TN et al. Spatial signatures for predicting immunotherapy outcomes using multi-omics in non-small cell lung cancer. Precision Oncology. 2025. doi: 10.1038/s41588-025-02351-7. PMID: 41073787.
2. Tam CW et al. The development of a high-plex spatial proteomic methodology for the characterisation of the head and neck tumour microenvironment. Precision Oncology. 2025. doi: 10.1038/s41592-021-01203-6. PMID: 40533476
3. Liu Y, et al. Conserved spatial subtypes and cellular neighborhoods of cancer-associated fibroblasts revealed by single-cell spatial multi-omics. Cancer Cell. 2025. doi: 10.1016/j.ccell.2025.03.004. PMID: 40154487.
4. Nicolò E, et al. Combining antibody-drug conjugates with immunotherapy in solid tumors: current landscape and future perspectives. Cancer Treatment Reviews. 2022;108:102395. PMID: 35468539.

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Results

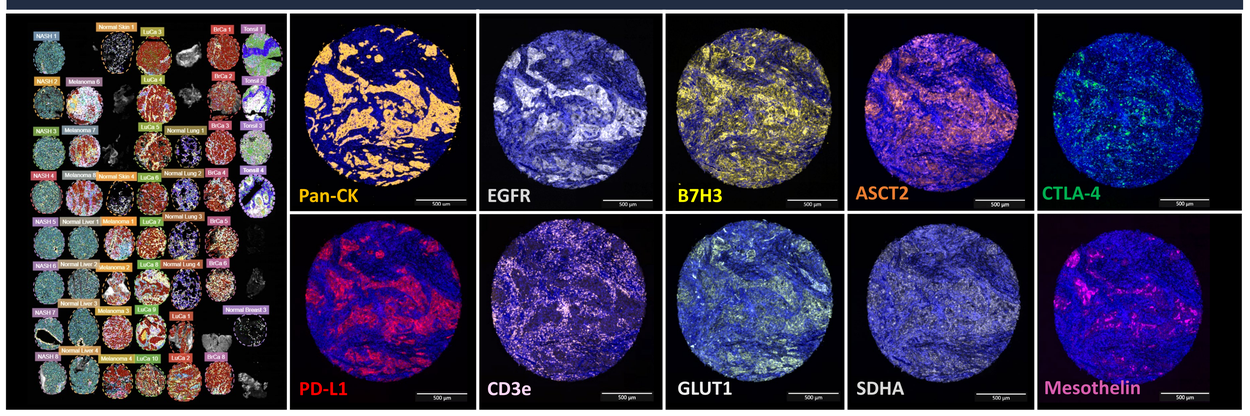


Figure 2. High-dimensional multiplexing of IO-60 and spike-in targets on a multi-tissue TMA.
Shown are the TMA and its tissue cores stained for 79 protein targets. Single-layer images of core NSCLC-08 demonstrate staining performance of selected IO60 spike-in targets.

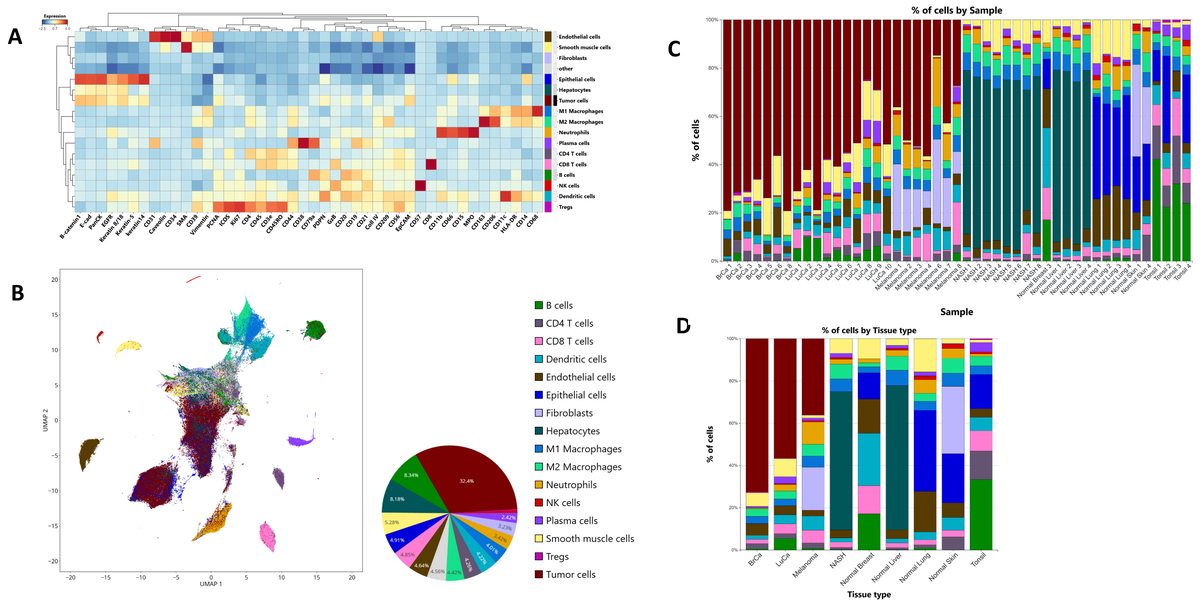


Figure 3. Tumor, immune, stromal, and clinically actionable phenotypes are reliably resolved across diverse human tissues.
A) Heatmap showing the average protein expression across 17 cell phenotypes identified by unsupervised clustering. **B)** UMAP visualization of all cells colored by phenotype, with a pie chart showing overall phenotype proportions. **C)** Stacked bar chart showing the relative cellular composition of each individual TMA core/sample. **D)** Stacked bar chart summarizing the relative abundance of each cellular phenotype across tissue types.

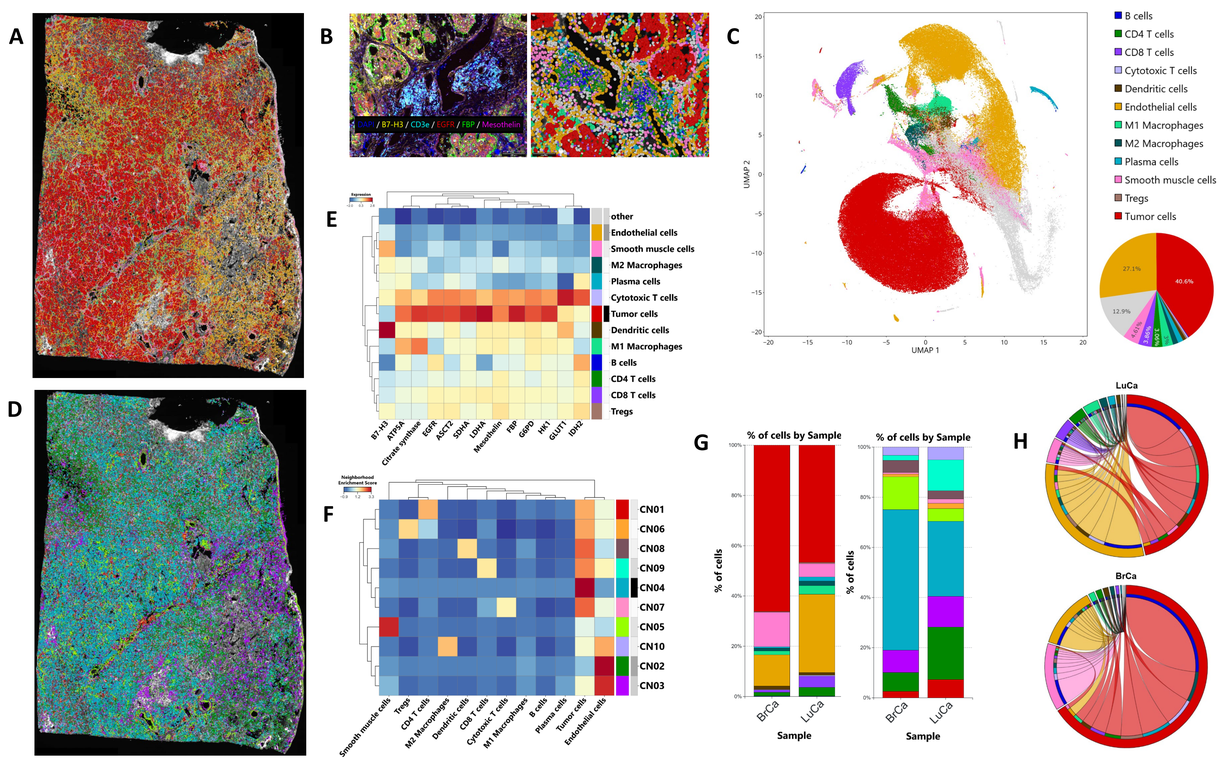


Figure 4. Spatial neighborhood mapping uncovers distinct tumor–immune niches and cell–cell interaction patterns in whole tissue sections.
A) Spatial distribution of 13 cell phenotypes identified by unsupervised clustering. **B)** Representative region showing 6-marker staining and corresponding cell phenotypes. **C)** UMAP of all cells colored by phenotype. **D)** Spatial map of 10 cellular neighborhoods. **E)** Heatmap of average metabolic-marker expression across phenotypes. **F)** Neighborhood enrichment scores. **G)** Relative proportions of cell phenotypes and cellular neighborhoods across tissue sections **H)** Circos plots showing cell–cell interactions based on 5-nearest-neighbor analysis.

Summary and Conclusions

- High-plex spatial proteomic profiling on the PhenoCycler®-Fusion 2.0 enabled simultaneous characterization of immune, tumor, stromal, metabolic, and ADC-associated biomarkers within intact human tissue; Unsupervised analysis identified biologically interpretable cellular phenotypes across diverse tissue types.
- Integration of metabolic markers and ADC targets, including GLUT1, ASCT2, SDHA, EGFR, B7-H3, and Mesothelin, with immune and checkpoint markers enables interrogation of tumor–immune–metabolic relationships and therapeutic target expression within their spatial context.
- The inclusion of human/mouse-compatible targets provides a framework for more direct comparison of spatial biology across preclinical models and human disease, supporting translational biomarker development.
- Together, these findings demonstrate the potential of an expanded, IO-aligned, high-plex panel to generate an integrated view of TME composition, functional state, spatial organization, metabolism, and therapeutic targets within a single tissue section.