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Ultra-High-Plex Immunofluorescence Analysis of the Tumor-Immune Microenvironment with EpicIF™ on the CellScape™ Platform

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Introduction

Spatial biology continues to transform cancer research by revealing the intricate molecular and cellular architecture of tissues. Yet, our understanding of the tumor microenvironment is still incomplete, partly because single-cell *in-situ* proteomic data remain challenging to achieve. Here we present, for the first time, a 208-plex immunofluorescence panel for comprehensive analysis of the tumor microenvironment on the CellScape platform.

Method

Formalin-fixed, paraffin-embedded tissue sections were subjected to standard histological processing before automated, iterative staining on the CellScape XR instrument. Each staining cycle consisted of background imaging, staining of 4 fluorophore-labeled primary antibodies, high dynamic range imaging and rapid signal removal with EpicIF technology (Fig 1.).

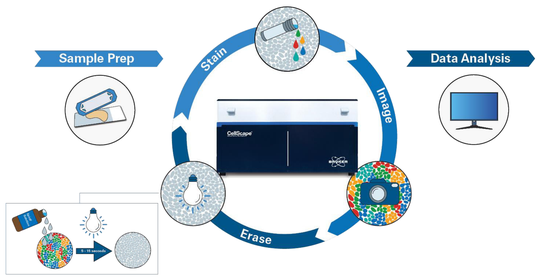

Figure 1. Experimental workflow using CellScape and EpicIF technology. Cycles of staining, imaging, and signal removal detect biomarkers with spatial context at single-cell resolution. Signal removal facilitated by filtered photobleaching and the EpicIF Solution to provide a safe, gentle, and effective fluorophore removal.

First, we assessed antibody signal intensity after 50 EpicIF-enhanced bleach cycles. Four human tonsil sections were exposed to 50 consecutive photobleaching steps on one half while the other half of the tissue was exposed to EpicIF solution without light, followed by staining of 34-plex staining. The stain intensities were quantified and compared (see Fig. 4).

A total of 241 antibodies against 230 biomarker targets were then sourced, entirely from commercially available fluorophore-conjugated antibody catalogs. This panel targets biomarkers in tumor cells, immune cell subsets, the tissue architecture, and key signaling pathways (Table 1). All antibodies were test stained in a low- to mid-plex setting to determine optimal dilutions, before staining full 208-plex panel on a mixed tissue TMA section.

Category	# of antibodies	Example biomarker targets
Tissue Architecture & Differentiation	62	Keratins, Vimentin, CDX2, Napsin A, MelanA, SMA, p63
Immune cell lineage	45	CD45, CD3, CD4, CD8, CD20, CD11b, CD11c, CD68
Mitogenic & Oncogenic signaling	39	EGFR, HER2, phospho-Erk1/2, phospho-Stat1, mTOR
Immune activation & stimulation	20	Granzyme B, ICOS, CD27, GITR, CD69, CD107a, CD40
Immune suppression & escape	16	CD274, CD279, CTLA4, LAG3, IDO, Galectin-9, TIM-3
Cell cyle & Proliferation	15	Cyclin D1, Ki-67, PCNA, CDK1, Rb, p21 Waf1/cip1, TERT
Cell death pathways	11	cleaved PARP, Bcl-2, Bcl-xl, Bcl-6, Survivin, Caspase 3
Homeostasis & Metabolism	10	ATP1A1, GAPDH, GLUT1, G6PD, LDHA, HIF-1A
Inflammation	9	iNOS, Galectin-3, MIF, S100A8, S100A9, Cox2, NFAT1
Angiogenesis	7	VEGF, VEGFR1, VEGFR2, CD31, CD105, CD115, PNAd
Stemness	7	SOX2, Nanog, PRAME, Aiolos, Ikaros, Oct4

Table 1. Antibody target biomarker categories and examples

Results

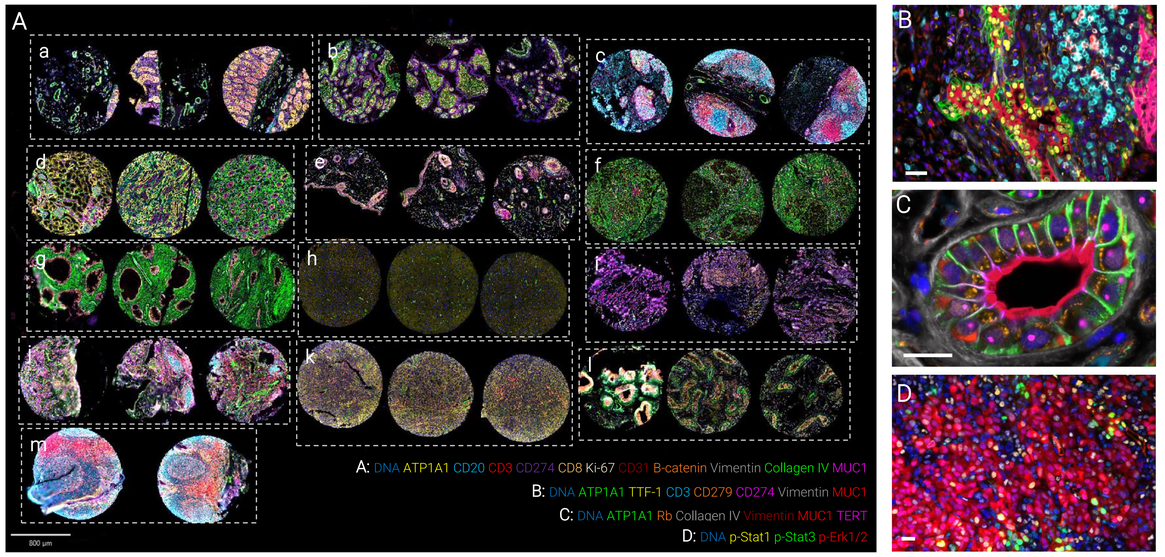

Figure 2. Highplex mIF staining of mixed tissue microarray. A. TMA consisting of 12 neoplastic and non-neoplastic human FFPE tissue samples from different organs of origin, with colon (a), placenta (b), tonsil (c & m), kidney (d), skin (e), liver (f), prostate cancer (g), brain (h), breast cancer (i), lung cancer (j), melanoma (k), testis (l), was stained with 208 antibodies plus DNA stain. **B.** Lung cancer sample stained with immune, structural and tumor differentiation markers showing immune checkpoint activation on T-cells in the TME. **C.** Kidney renal tubule. High resolution imaging reveals fine structural details of tissue architecture. **D.** Melanoma stained with phospho-Erk1/2 (stain #21, cycle 7), phospho-Stat1 (stain #195, cycle 52) and phospho-Stat3 (stain #156, cycle 41) specific antibodies allows profiling of signal pathway activation in the TME. Scalebars: A=800 µm, B/C/D=20 µm.

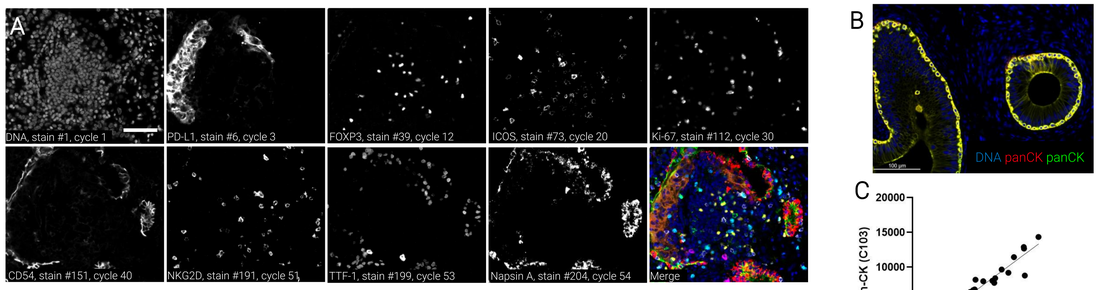

Figure 3. Consistent and specific staining of markers across 54 cycles . A. The panel shows staining of 9 immune regulatory and tissue differentiation markers in an area of the lung cancer TME. Staining is specific for both high and low-expressing biomarker targets across 54 cycles and reveals immune-regulatory processes in the TME. Scale bar represents 50 µm and applies to all images. **B.** Representative image of Testis tissue stained with the same panCK antibody clone in cycle 8 and cycle 30 showing perfect signal overlap. **C.** Quantification of panCK signal across all cores shows near 100% correlation of signal, indicating that no loss of epitope stability and antigenicity or steric hindrance after 22 staining and removal cycles.

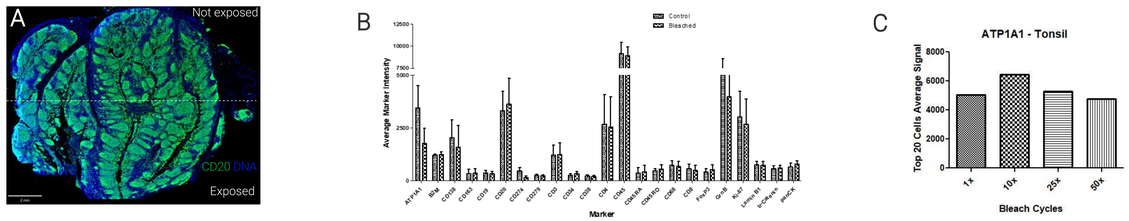

Figure 4. Epitope stability after 50 EpicIF-enhanced photobleaching cycles. A. Four tonsil sections were exposed to repeated EpicIF-enhanced photobleaching cycles on one half of the tissue while the other half was not exposed to photobleaching. This was followed by 34-plex antibody staining with our Vistaplex panels. **B.** Subsequent quantification and comparison of signal intensity enabled identification of sensitive antibodies. After 50 cycles, significant ($p<0.05$) signal reduction could be observed for 5/34 (15%) antibodies with an average of 37% reduction (see histogram). **C.** Notably, no signal reduction could be observed after 25 cycles of EpicIF-enhanced photobleaching for the same antibodies in a second experiment on tonsil. These results inform subsequent assay design decisions by placing antibodies with photosensitive epitopes in early cycles of the assay. Furthermore, the signal reduction only affects certain monoclonal antibodies and thus seems to be epitope-specific. No indication for broad tissue or epitope degradation was found after 50 EpicIF bleaching cycles, highlighting that EpicIF-enhanced photobleaching is a comparably gentle signal removal method, ultimately allowing for high-plex staining without broad loss of antigenicity.

Figure 5. Highplex staining marker expression profiles and UMAP embedding of cells from 10 organs. Heatmap clustering of a subset of 120 markers displays striking cluster diversity, with discrete marker signatures separating immune, stromal, and epithelial lineages across organs. Combining highplex staining with TMA-based sample presentation allows time- and cost-efficient deep profiling of large tumor patient sample cohorts.

Conclusion

- CellScape Platform with EpicIF technology supports ultra-high-plex mIF single-cell resolution imaging of 208 antibodies on a single tissue section
- Highplex mIF in combination with Tissue Microarray samples enables quick and efficient proteomic screening of large sample cohorts
- CellScape enables large scale antibody screening and deep phenotyping of large patient cohorts

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