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Ultra-High-Plex Immunofluorescence Analysis of the Tumor Immune Microenvironment 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 assay for comprehensive analysis of the tumor microenvironment using the CellScape platform.

Method

Formalin-fixed, paraffin-embedded tissue sections were subjected to standard histological processing before automated, iterative staining on the CellScape 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.

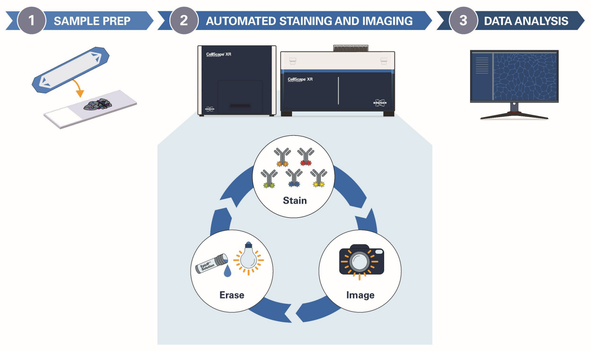


Figure 1. CellScape workflow for high-plex mIF.
1, Sample prep: Application of CellScape Whole-Slide Imaging Chamber using standard histopathology slides.
2, Automated Staining and Imaging: Cycles of staining, imaging, and gentle signal removal using EpicIF technology streamline high-plex data generation.
3, Data Analysis: Integrated image viewing and analysis modules for quantitative spatial phenotyping at single-cell resolution.

A panel to label tumor cells, immune cells, tissue architecture, and key signaling pathways was built using 241 fluorophore-conjugated candidate antibodies against 230 targets, sourced from 3rd party vendors. All antibodies were tested in a low- to mid-plex setting to determine optimal dilutions, before staining with the final **208-plex** panel on a mixed tissue microarray (TMA) section.

Results

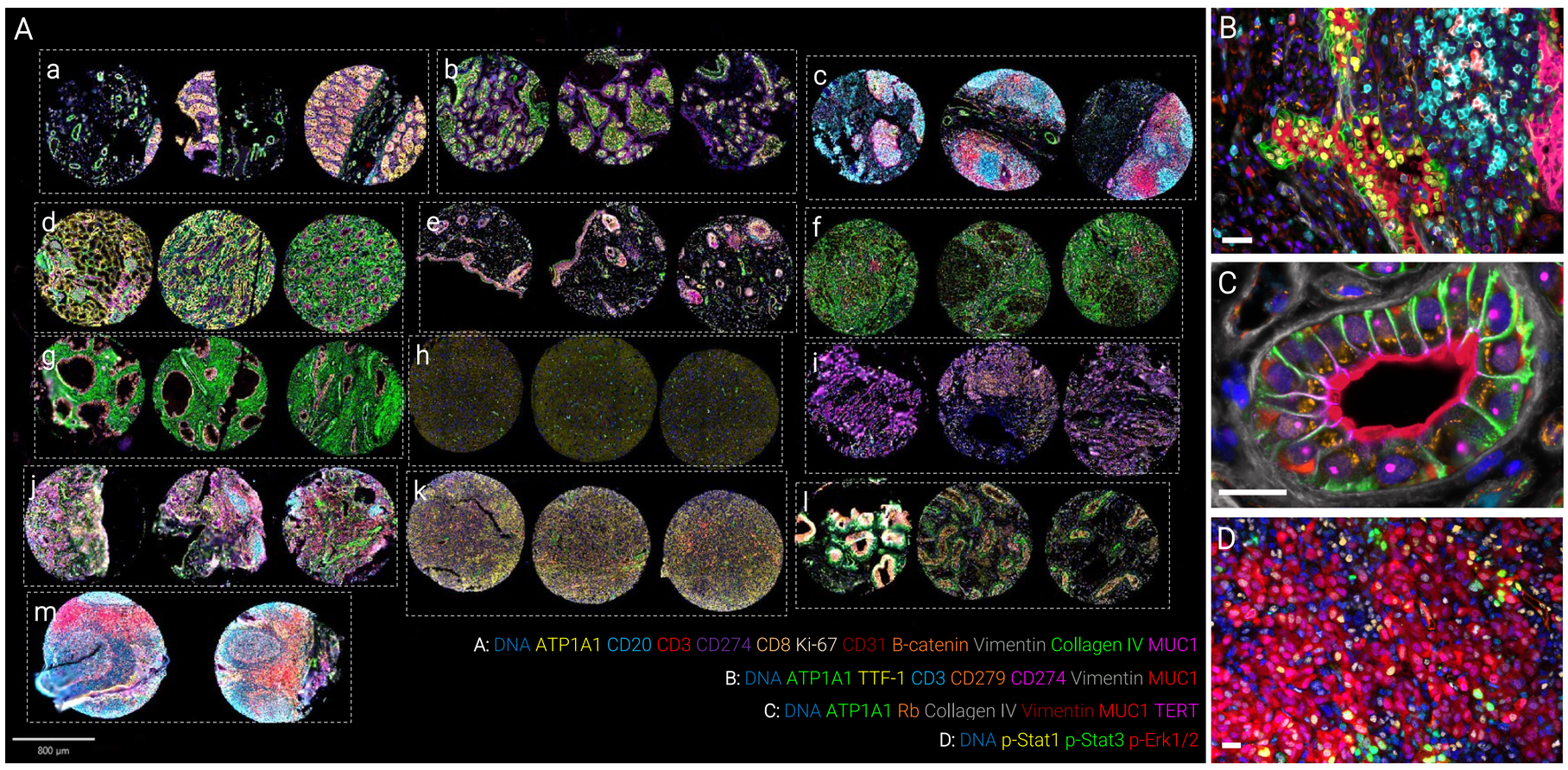


Figure 2. High plex mIF staining of a tissue microarray. A. TMA consisting of 12 neoplastic and non-neoplastic human FFPE tissue samples with colon (a), placenta (b), tonsil (c & m), kidney (d), skin (e), liver (f), prostate cancer (g), brain (h), breast cancer (i), lung cancer (j), melanoma (k), and 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) antibodies allows profiling of signal pathways in the TME. Scalebars: A=800 µm, B/C/D=20 µm.

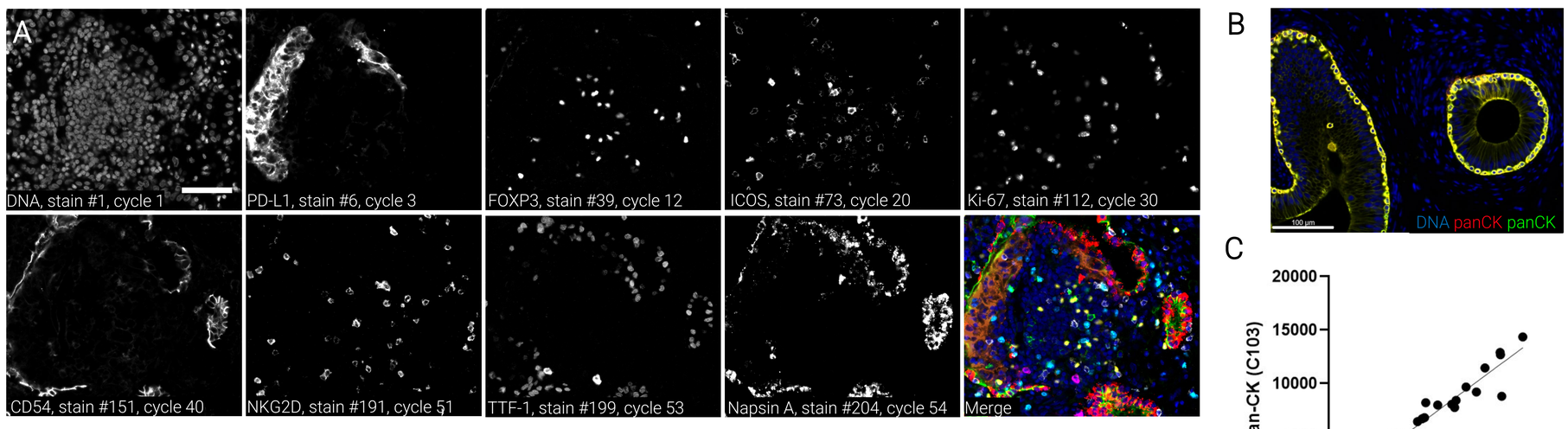


Figure 3. Consistent and specific staining of markers across 54 cycles. A. The panel shows staining of 9 immune regulatory and tissue differentiation markers in an area of the lung cancer TME. Staining is specific for both high and low-expressing biomarker targets across 54 cycles and reveals immune-regulatory 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. Scalebar represents 100 µm. C. Quantification of panCK signal across all cores shows near 100% correlation of signal, indicating that no signal loss, and hence loss of epitope stability, antigenicity or steric hindrance occurred after 22 staining and EpicIF-enhanced photobleaching cycles.

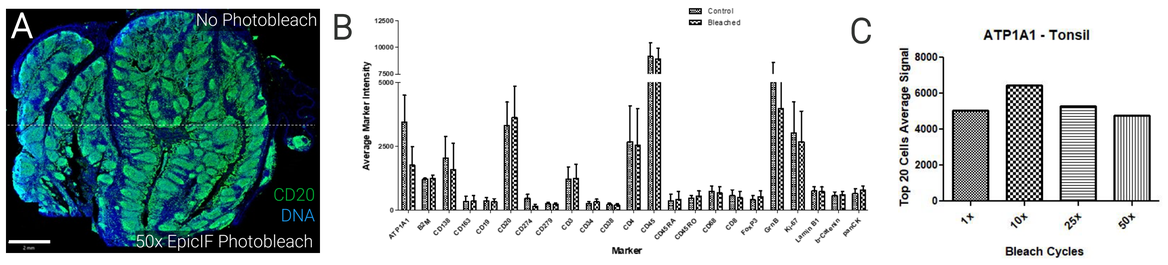


Figure 4. Epitope stability after 50 EpicIF-enhanced photobleaching cycles. A. Four tonsil sections were exposed to repeated EpicIF-enhanced photobleaching cycles on one half of the tissue while the other half was not exposed to photobleaching. This was followed by 34-plex antibody staining with VistaPlex Multiplex Assay panels. Scalebar represents 2 mm. B. 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. C. After a second experiment with 25 cycles of EpicIF-enhanced photobleaching instead of 50 cycles, *no signal reduction was observed for the same antibodies*. These results suggest that unusually photosensitive epitopes will still be accurately captured in earlier cycles of a high-plex assay, which can be addressed by assay design. Further, 85% of epitopes were not affected at all by high-plex cycling with EpicIF technology and affected epitopes experienced only a modest decrease in sensitivity. Last, 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 that is compatible with high-plex staining.

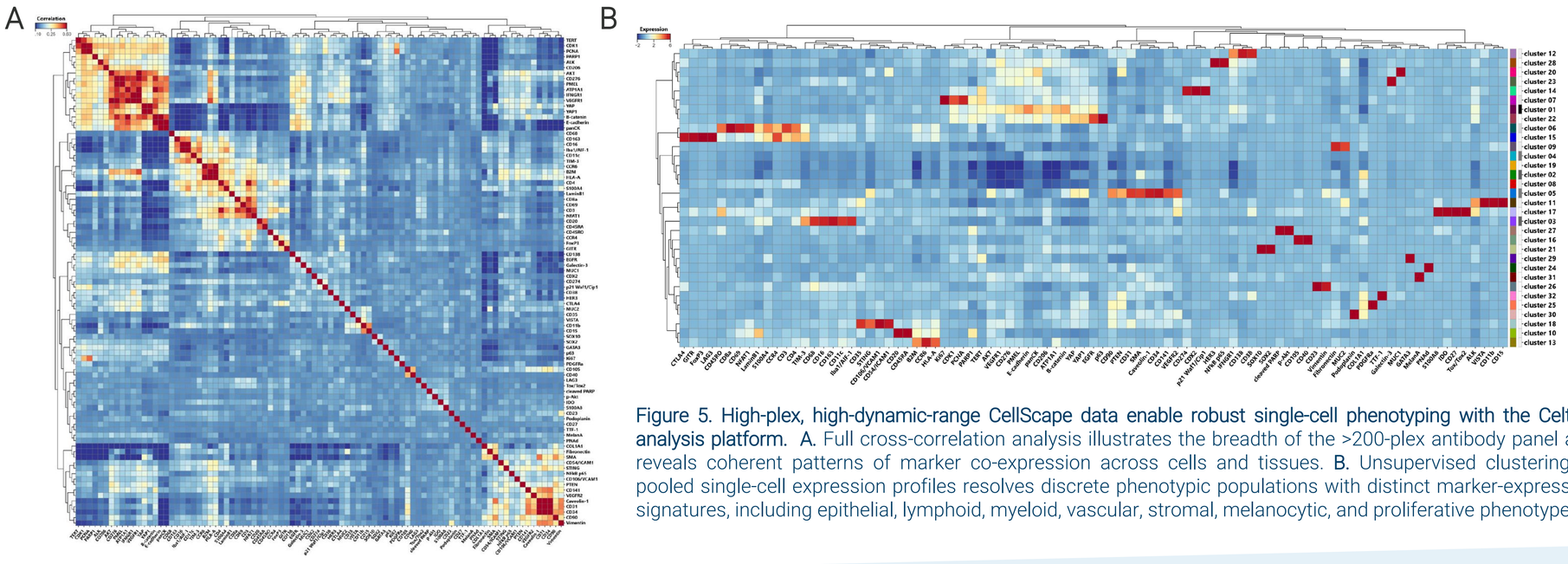


Figure 5. High-plex, high-dynamic-range CellScape data enable robust single-cell phenotyping with the Celtix™ analysis platform. A. Full cross-correlation analysis illustrates the breadth of the >200-plex antibody panel and reveals coherent patterns of marker co-expression across cells and tissues. B. Unsupervised clustering of pooled single-cell expression profiles resolves discrete phenotypic populations with distinct marker-expression signatures, including epithelial, lymphoid, myeloid, vascular, stromal, melanocytic, and proliferative phenotypes.

Conclusions

- EpicIF photobleaching technology is non-destructive and enables ultra high-plex spatial proteomic assay design.
- The CellScape platform supports high-plex, single-cell resolution imaging of **208 antibodies** on a single tissue section.
- High-plex mIF on tissue microarray samples enables quick & efficient proteomic screening of large sample cohorts.

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