Development of an Ultrahigh-plex Antibody Panel for Spatial Phenotyping of Murine Cancer Models

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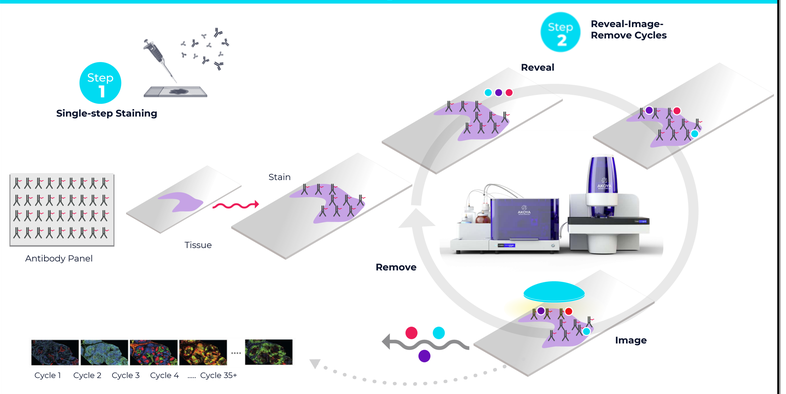
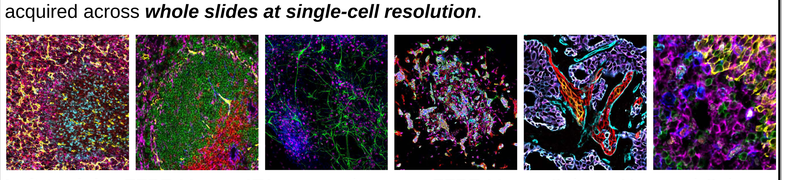
1. Introduction

Single cell spatial phenotyping of protein biomarkers is an established tool for cancer researchers aiming to understand the biology of the tumor microenvironment (TME). The technology has been developed around human tissues. Mouse models are widely used for immuno-oncology (IO) research, thus justifying the development of spatial phenotyping applications for use in mice. In collaboration with Cell Signaling Technology Inc. (CST), we developed a 32-plex murine IO antibody panel that covers immune cell lineages, immune checkpoints and cell state markers, as well as proliferation and structural markers. The antibody panel that was developed as part of this study can be readily deployed for discovery and translational research and will engender future comparative mouse and human spatial phenotyping studies that further our understanding of cancer biology.

2. The PhenoCycler Workflow



The **PhenoCycler®-Fusion** is an automated spatial biology platform for ultra-high plex imaging. The workflow consists of iterative cycles of labeling, imaging, and removing fluorescent reporters. During each imaging cycle, three fluorescent reporters are assayed to their corresponding barcode-conjugated antibodies and imaged via epifluorescence optics. Thereafter, the three reporters are removed, and a new cycle begins during which additional reporters are imaged. The technology is fully automated and widely compatible with formalin-fixed paraffin embedded (FFPE) or fresh frozen samples. All data are acquired across ***whole slides at single-cell resolution***.



Left to right: FFPE Mouse 4T1 syngeneic tumor; FFPE Mouse Spleen; Neuronal Culture; FFPE Human breast organoid; FFPE Ductal Adenocarcinoma; FFPE EBV+ Tonsil.

3. Design and Development of FFPE Mouse Panel

3.1 Panel Design. This 32-plex antibody panel was designed using commercially available Akoya antibodies together with CST antibodies conjugated to PhenoCycler barcodes.

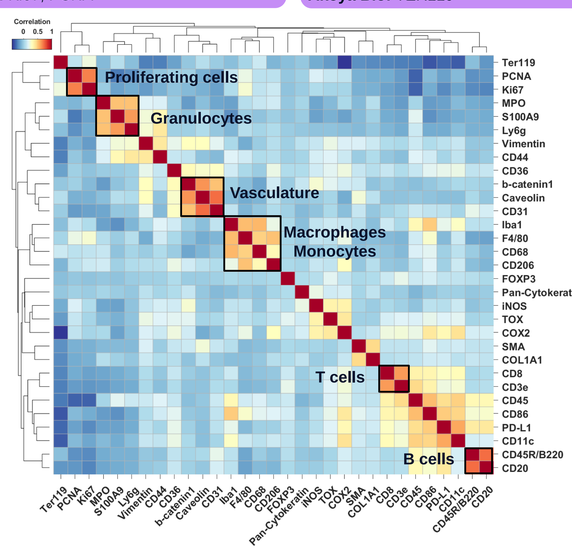
Immuno-Phenotyping Markers:
CST: CD45, CD44, CD3ε, CD20, CD68, CD11c, CD31, CD56
Akoya Bio: CD8, CD45R/B220

Myeloid Markers:
CST: F4/80, Ly6g, Iba1/AIF1, CD206, iNOS, S100A9, MPO

Structural Markers:
CST: Vimentin, COL1A1, Caveolin-1
Akoya Bio: αSMA, β-Catenin-1, Pan-Cytokeratin

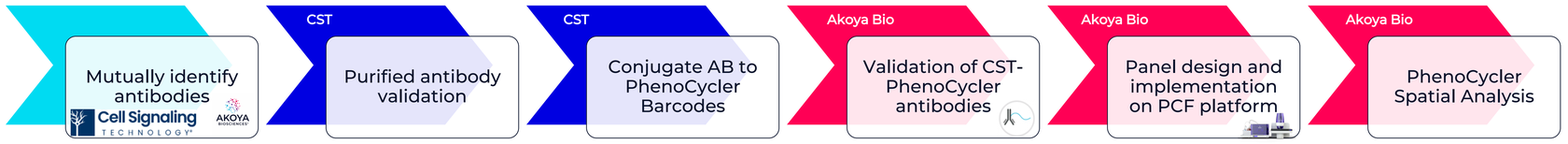
Proliferation and Transcription Markers:
CST: FoxP3, TOX2
Akoya Bio: Ki67, PCNA

Advanced Markers:
CST: PDL1, CD36, CD86, COX2
Akoya Bio: TER119

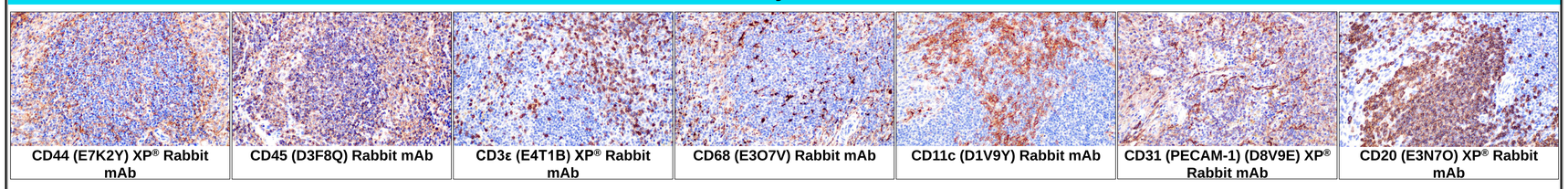


3.2 Cross-correlation matrix of 32 markers showing distinct immune cell populations, their cell states and vascular structures occupying distinct matrix domains.

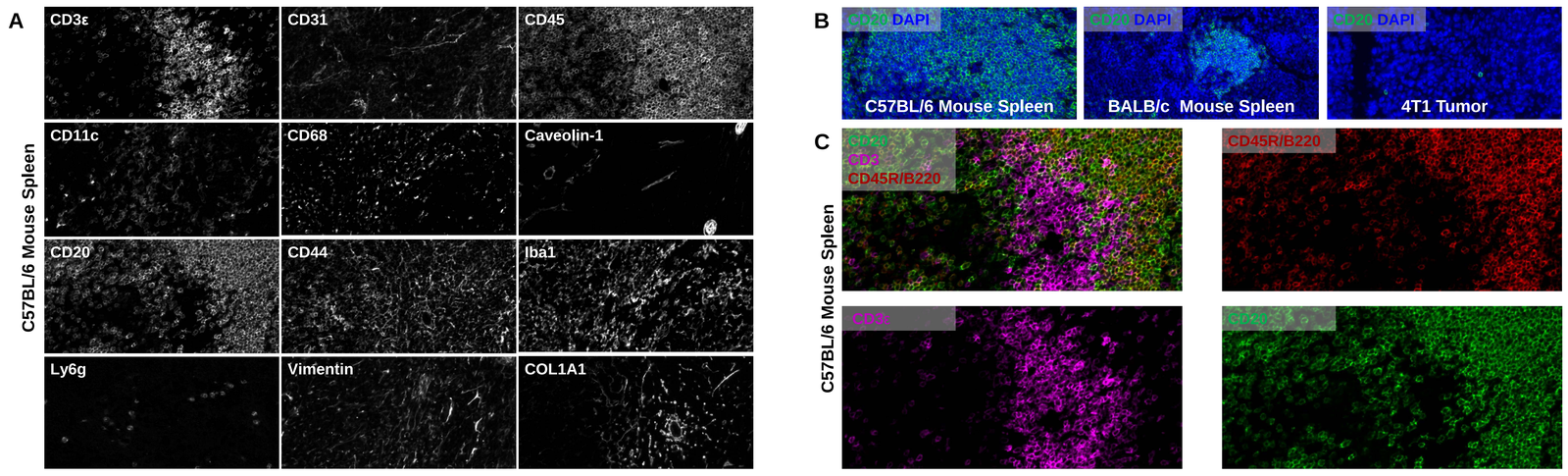
4. Outline of Panel Development Workflow



5. Antibody Validation

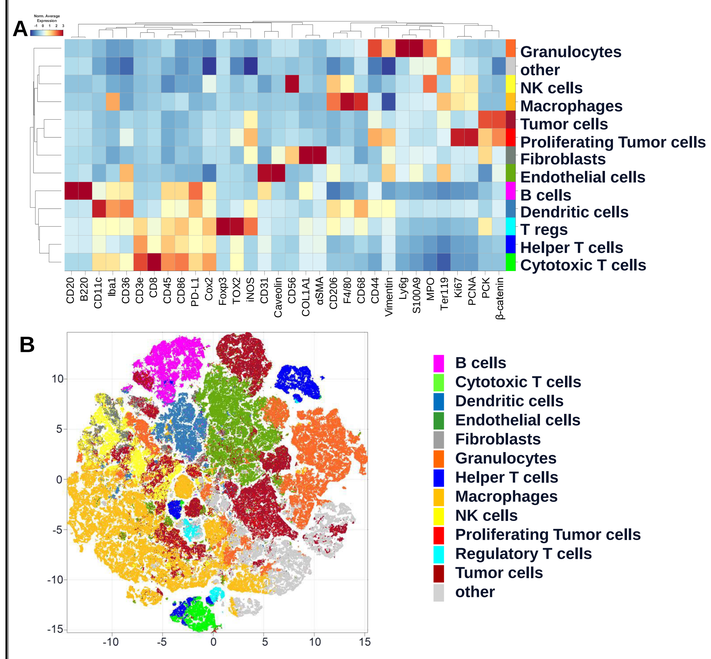


5.1 Validation of purified antibodies conducted by CST on FFPE Mouse Spleen. Immunohistochemical analysis shown in the images above represents antibodies specificity.

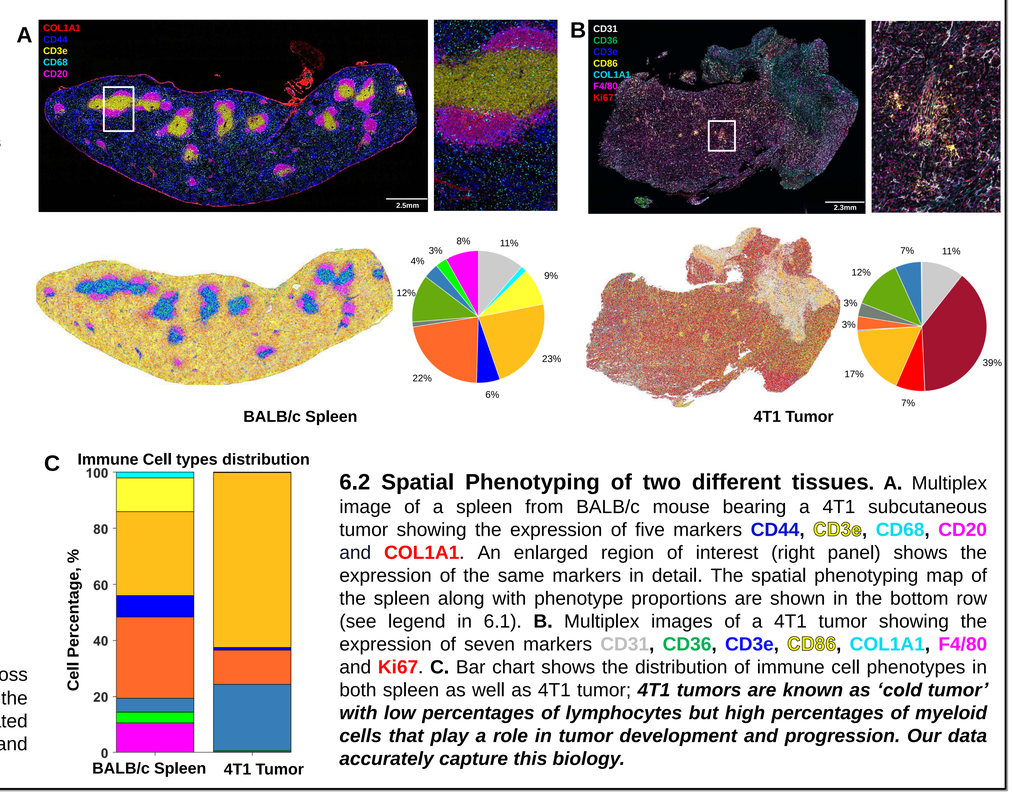


5.2 Validation of PhenoCycler Antibodies on FFPE Mouse Tissues. A) Staining with CST antibodies conjugated to PhenoCycler barcodes after optimization of titer and exposure time. **B)** Each antibody was screened on multiple tissues as shown for CD20 on three different tissues. **C)** Qualitative assessment confirms PhenoCycler antibody staining specificity and cellular morphology, appropriate overlap with counterstain (CD45R/B220+ CD20), and lack of overlap with negative counterstain (CD3ε vs. CD20). All images were acquired using the **PhenoCycler®-Fusion** platform.

6. Spatial Phenotyping of 4T1 Syngeneic Mammary Tumor and BALB/c Spleen



6.1 Single-cell Phenotyping identified 12 distinct cell phenotypes across 726,356 cells in two samples: 4T1 tumor and spleen. **A)** The heatmap shows the curated clustering dendrogram with twelve different cell phenotypes, each indicated by a color code. **B)** The t-SNE plot shows these phenotypes sorted by color and geographical distribution.



6.2 Spatial Phenotyping of two different tissues. A. Multiplex image of a spleen from BALB/c mouse bearing a 4T1 subcutaneous tumor showing the expression of five markers **CD44**, **CD3e**, **CD68**, **CD20** and **COL1A1**. An enlarged region of interest (right panel) shows the expression of the same markers in detail. The spatial phenotyping map of the spleen along with phenotype proportions are shown in the bottom row (see legend in 6.1). **B.** Multiplex images of a 4T1 tumor showing the expression of seven markers **CD31**, **CD36**, **CD3e**, **CD86**, **COL1A1**, **F4/80** and **Ki67**. **C.** Bar chart shows the distribution of immune cell phenotypes in both spleen as well as 4T1 tumor; ***4T1 tumors are known as 'cold tumor' with low percentages of lymphocytes but high percentages of myeloid cells that play a role in tumor development and progression. Our data accurately capture this biology.***

7. Conclusion

We present a newly developed 32-plex antibody panel for deep spatial phenotyping of FFPE mouse tissues. The panel was validated for multiple mouse tissues and captured known biological features. This panel can now be deployed for discovery and translational research and will engender future comparative mouse and human spatial phenotyping studies that can further our understanding of cancer biology.

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