Ultivue
Profiling Cancer Biology

Development of specific multiplexed immunofluorescence immune assays to study mouse models of tumorigenesis

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Ultivue E-Book

Introduction

Immunotherapy has transformed the treatment of metastatic and recurrent solid tumors. Advances in technology in the past few years have created unprecedented opportunities to identify biomarkers of disease processes, especially by using multi-omics technologies and datasets to derive valid and useful signatures of disease.

Today the use of pre-clinical mouse tumor models are widely used tools to demonstrate efficacy of novel immunotherapies. Specifically, to further our understanding of the immune response to the tumor and provide additional insights about cancer biology pathways and mechanism of drug action or test potentially different combinations of novel treatment in vivo, currently available syngeneic mouse models to a variety of tumor indications provide a critical pre-clinical resource.

Despite their widespread use, a comprehensive view of murine tumor immune compositions and their relevance to human tumors has only begun to emerge. Indeed, beyond the composition of the tumor immune microenvironment (TiME), most commonly addressed with flow-based approaches, studying the location and spatial distribution of immune cells can provide a framework for a better understanding of tumor biology and identifying potential predictive biomarkers.

To that end, the use of specific mouse tissue phenotyping and multiplex immunofluorescence (mIF) assays offer the unique advantage of preserving the architectural features of the tumor and also revealing the spatial relationships between tumor cells and immune cells. The urgency to discover and implement new biomarkers lays bare the need to integrate a variety of advanced pre-clinical tools using specific mouse models to probe the dynamic nature of events occurring in the TiME.

Methods

To investigate the microenvironment within immune competent murine tumor types we have utilized both normal spleen derived from a BALB/c mouse and two syngeneic mouse FFPE tissues (tumor indications: 4T1, Breast and B16 F10, Melanoma, implanted into the mammary fat pad by the SubQperior™ method, Reaction Biology, Freiberg, Germany). Murine specific InSituPlex® (ISP) technology (Figure 1a) was used to perform both single-plex 3,3'-Diaminobenzidine (DAB) staining and multiplex immune profiling on mouse whole slide FFPE tumor serial sections. Specifically, alternating serial sections were stained with a cocktail of optimized and verified primary antibodies for mouse (CD3, CD4, CD8 and FOXP3) or a single primary antibody, in parallel using an autostainer. Further, same slide sections for each sample were stained with H&E to provide additional morphological information.

Slides were then imaged on the Zeiss AxioScanZ1 providing high quality images of the four targets in just a single workday. The images were exported for downstream analysis with Indica Labs HALOv3.1 software. An example post imaging whole slide annotation is indicated in Figure 1b. Concordance of the single plex DAB assay to the 4-plex mouse specific ISP assay was assessed qualitatively. Precision of the 4-plex assay was determined by quantifying the percent difference in cellular density and signal intensity of immune cell subtypes.

Figure 1

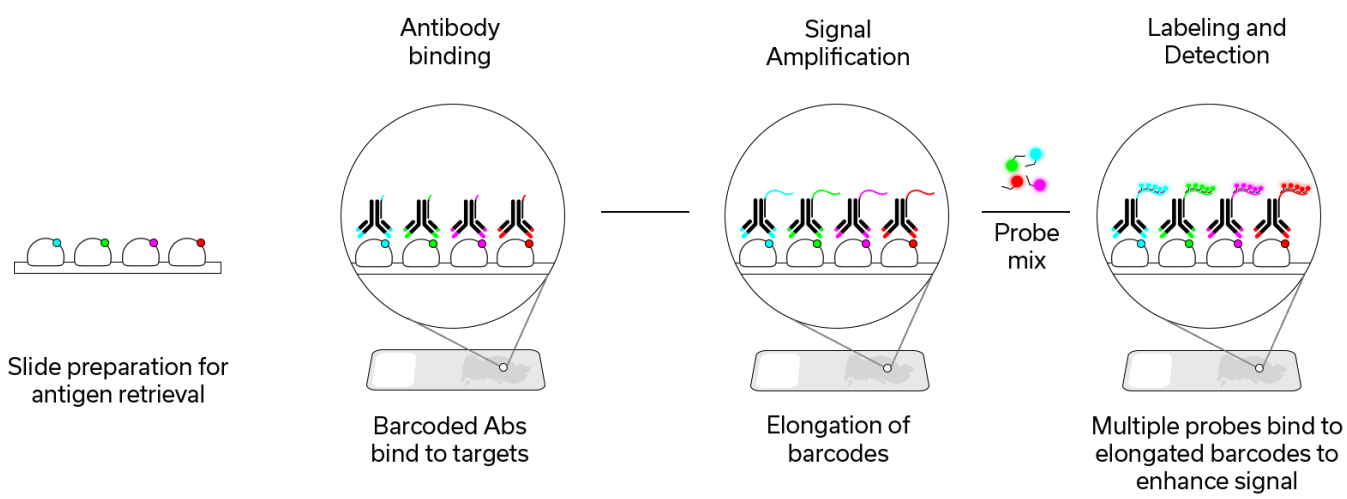


Figure 1a. Overview of InSituPlex technology to develop multiplexed mouse specific panels

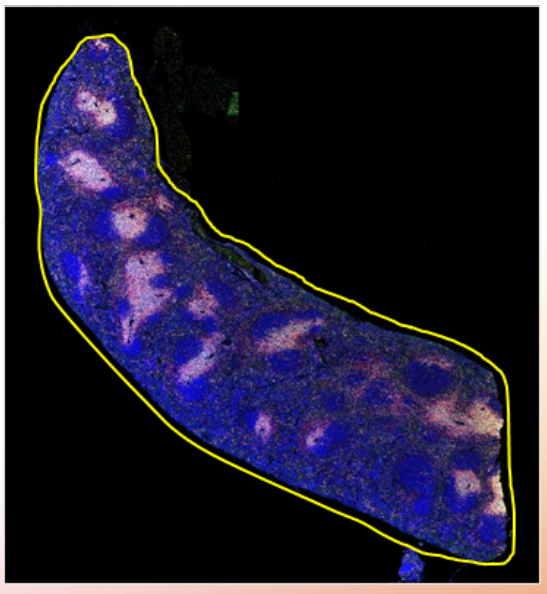


Figure 1b. Example of WSI annotation in spleen

Results

We initially sought to verify that our novel 4-plex (CD3, CD4, CD8 and FoxP3) mouse specific panel performed equally to monoplex DAB staining for each antibody. Serial sections were stained to qualitatively compare staining patterns of each antibody in both a conventional DAB IHC test and using an ISP stain.

Qualitative assessment and quantitative analysis from whole slide imaging of normal splenic tissue revealed a high level of reproducibility for the novel mouse assay by performing intra-assay staining on serial sections, with each single markers' coefficients of variation falling within an acceptable range (range is <30CV, Figure 2a-b). Each single marker testing verification showed an expected level of expression and expected staining pattern of the cellular phenotypes (Figure 2c). Importantly, the 4-plex multiplex assay was highly concordant to the DAB single plex assay (Figure 2d). The use of H&E images can be used to delineate tissue architectural regions such as tumor and stroma, eliminating the need for a tumor-specific biomarker in the fluorescence panel. One key attribute of the ISP chemistry, means the tissue only needs to undergo mild antigen retrieval conditions during the staining process. Precise co-registration of multiplex images with the same H&E-stained tissue section used for pathologic diagnosis then allows a pathologist to directly correlate the appearance of every cell in the tissue section with its marker profile. An example of same slide H&E is highlighted for normal splenic mouse tissue whole slide imaging and zoomed in is indicated in Figure 2e.

Figure 2

DEMONSTRATION OF ASSAY REPRODUCIBILITY IN MOUSE SPLENIC TISSUE

a.

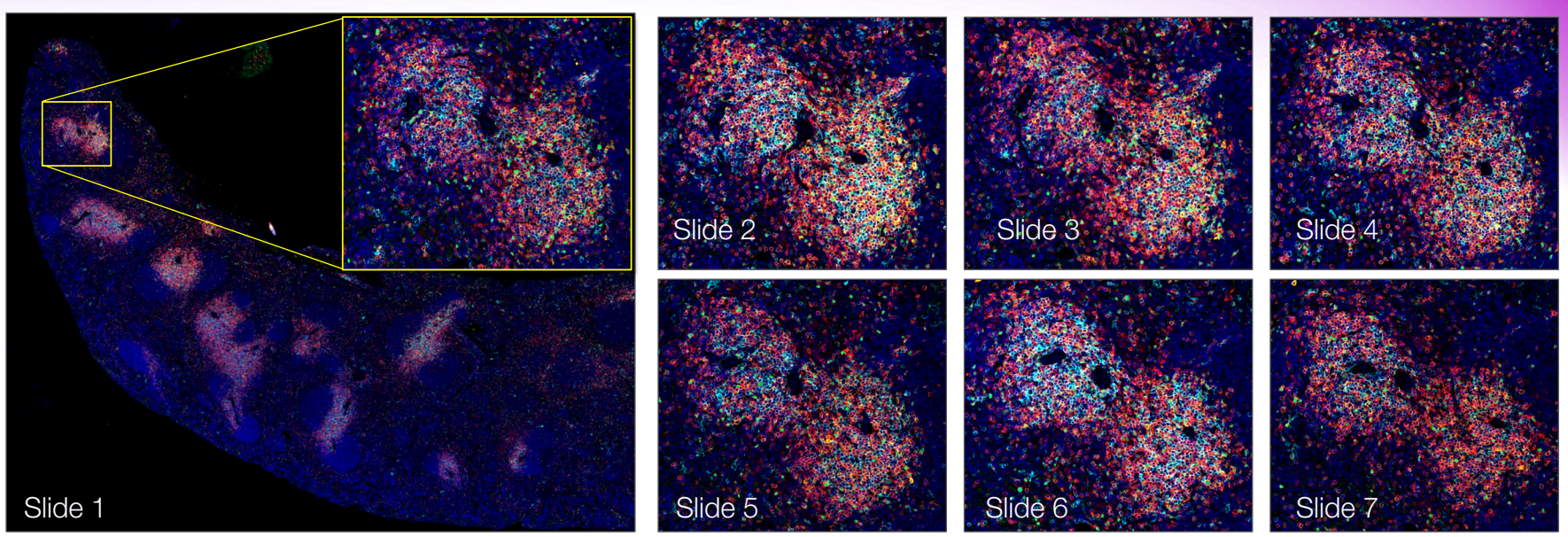
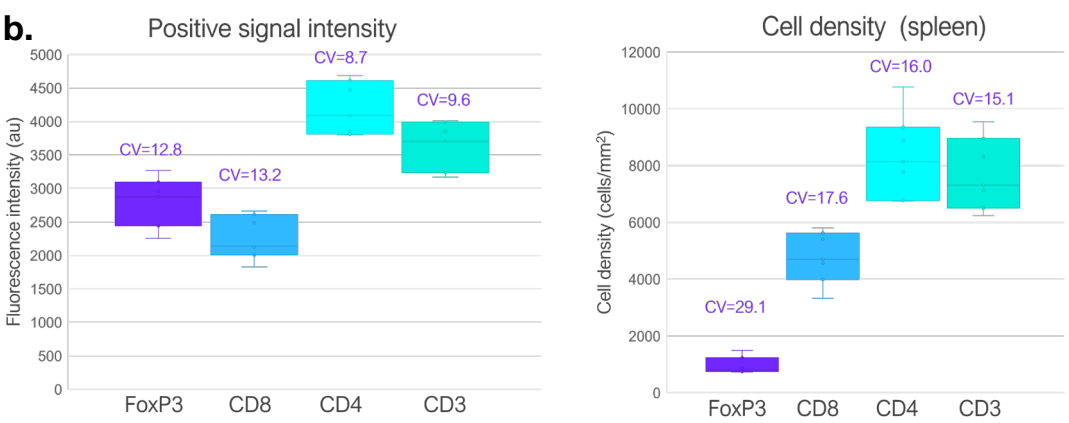


Figure 2a. Mouse spleen intra run concordance. Shown are zoomed in regions from whole slide images (WSI) of seven serial sections stained with multiplex ISP. Qualitative analysis showed a high level of reproducibility in all 4 markers.

b.



c.

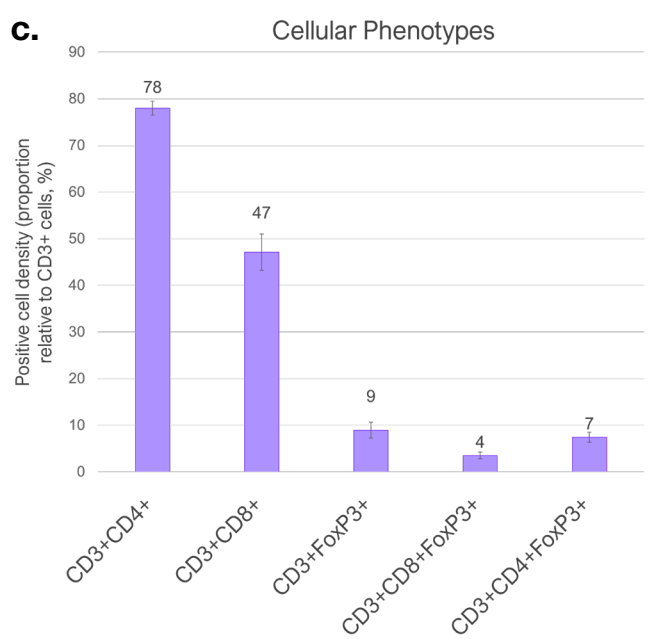
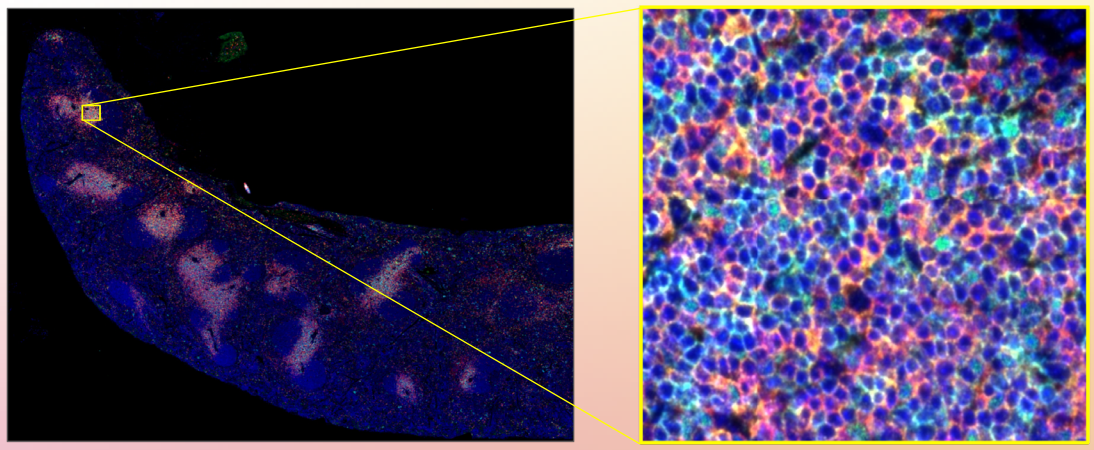


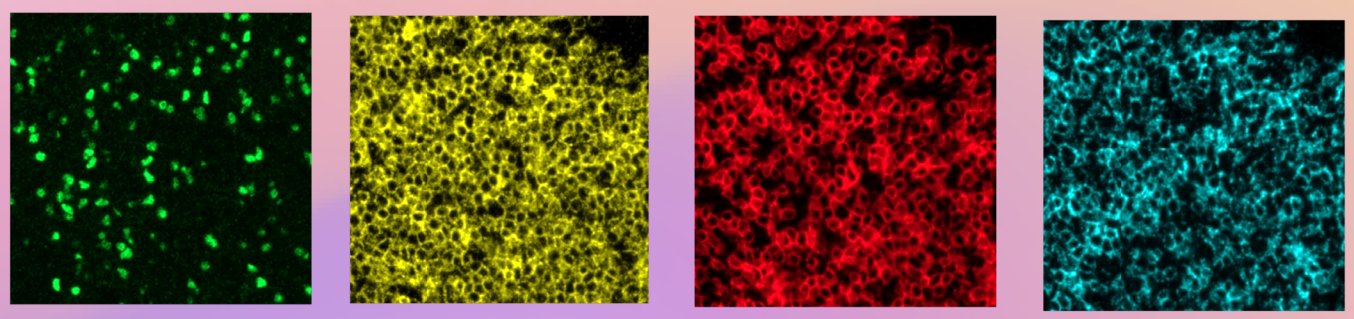
Figure 2b. Box Plot analysis of intensity and cell density for each marker in the seven serial sections. **c.** Bar graph showing the % of double or triple positive cells, relative to total CD3+ cells.

d.

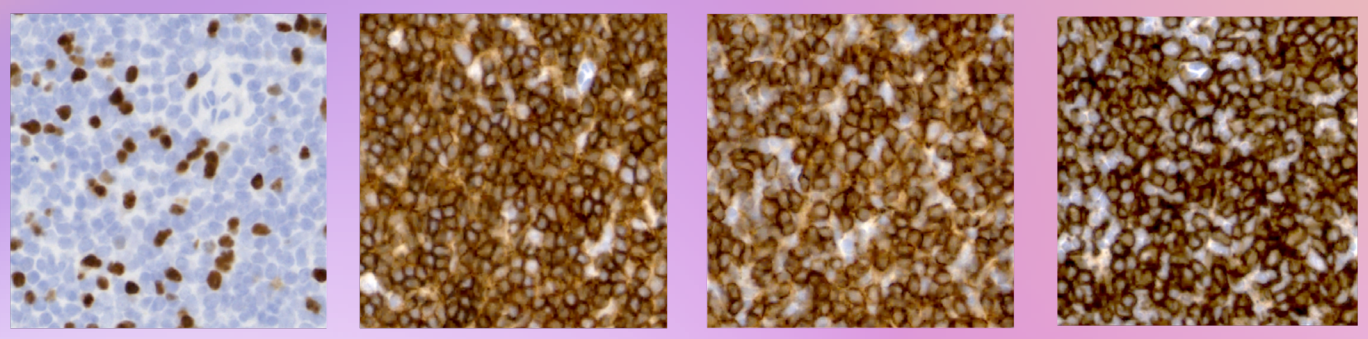


FoxP3 CD3 CD4 CD8

ISP



cDAB



uDAB

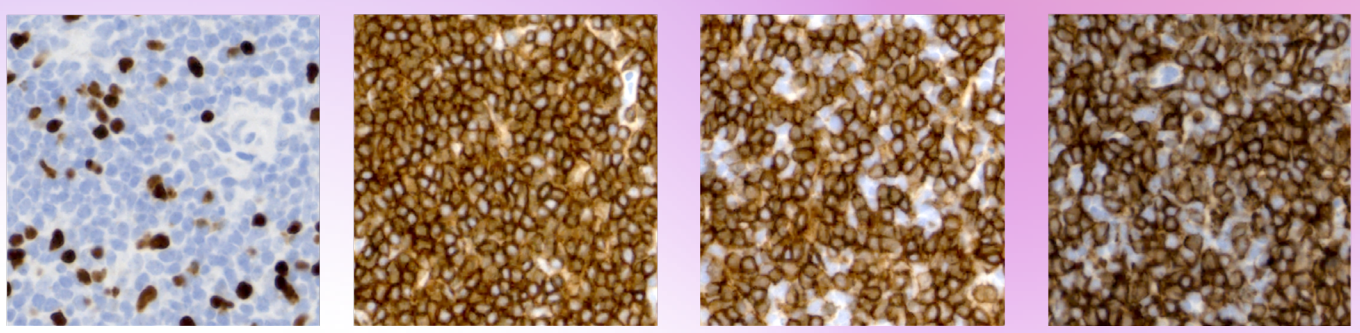


Figure 2d. Multiplex image of mouse spleen. The whole slide and zoomed in region. Top panel single channel ISP, middle panel barcode-conjugated DAB, bottom panel unbarcoded DAB. Qualitative assessment of predicate comparison results reveals a high level of concordance between the DAB and ISP staining for each marker in this panel.

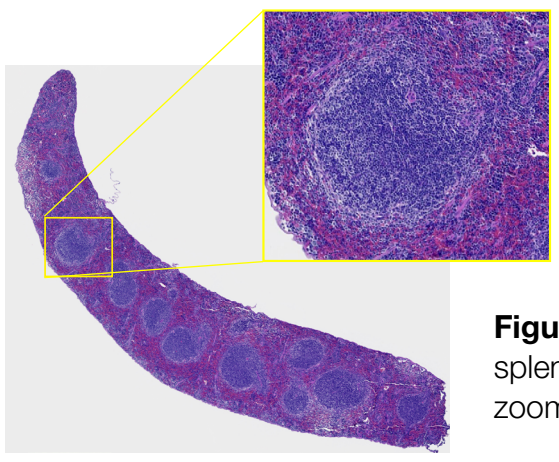


Figure 2e. H&E stained splenic tissue with zoomed in region.

Syngeneic mouse models are tumors derived from murine cancer cells engrafted on genetically identical mouse strains. They have been widely used tools in cancer studies especially for studying tumor immunity and immunotherapy response in the context of a fully functional murine immune system. The use of the murine B16 syngeneic melanoma tumor model has been extensively used to determine the mechanisms associated with the metastatic process and the development of novel therapeutics for this tumor indication.

Figure 3a demonstrates the utility of our 4-plex panel to determine immune infiltrates from whole slide imaging in melanoma. Qualitative assessment and quantitative analysis from whole slide imaging of melanoma tissue revealed a high level of reproducibility for the novel mouse assay by performing intra-assay staining on serial sections, with each single markers' coefficients of variation falling within an acceptable range (range is <30CV, Figure 3b). Each single marker testing verification showed an expected level of expression and expected staining pattern of the cellular phenotypes (Figure 3c) and morphology as revealed by H&E staining (Figure 3d).

Figure 3

VALIDATION IN A SYNGENEIC MODEL OF MELANOMA

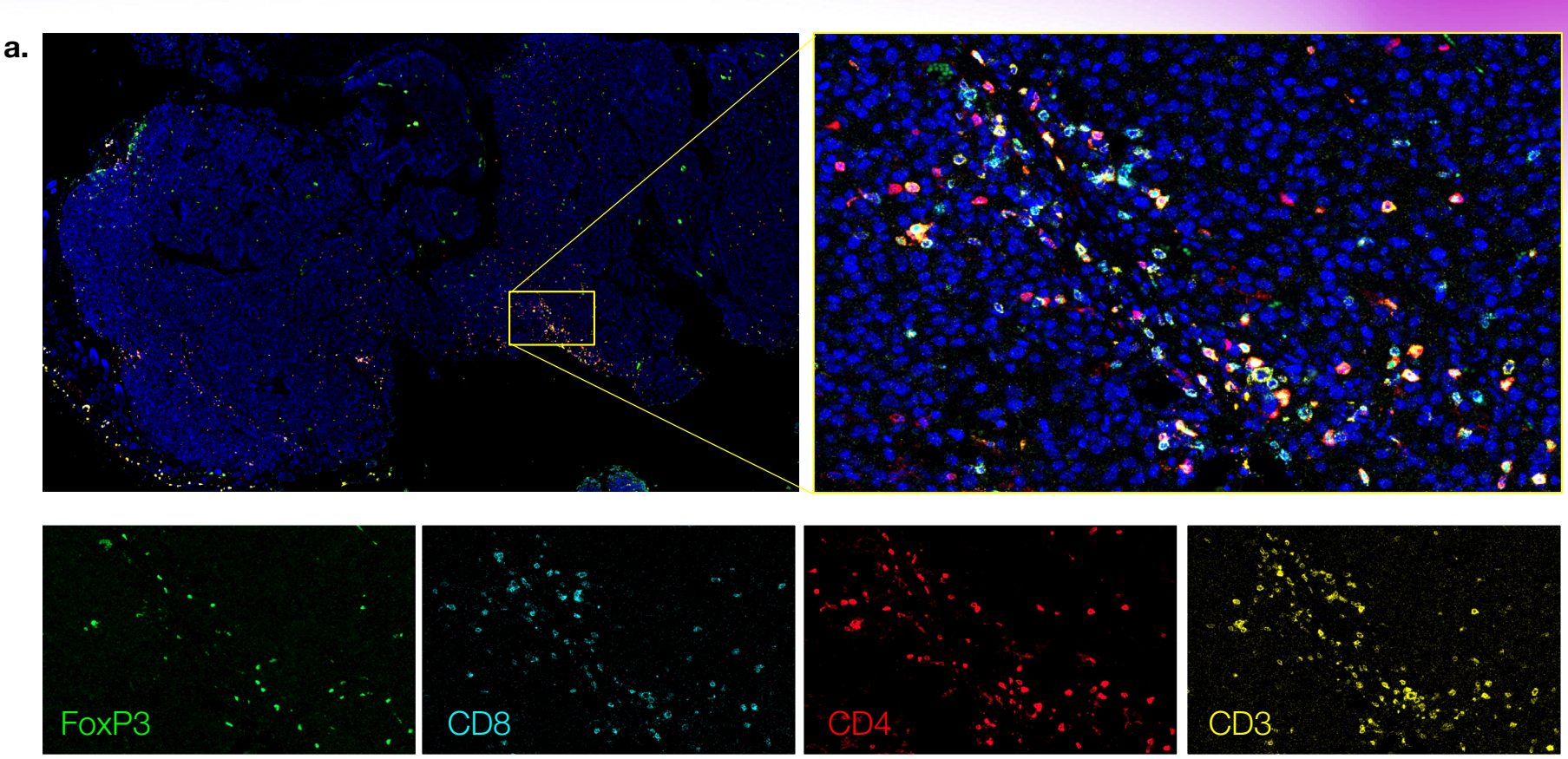
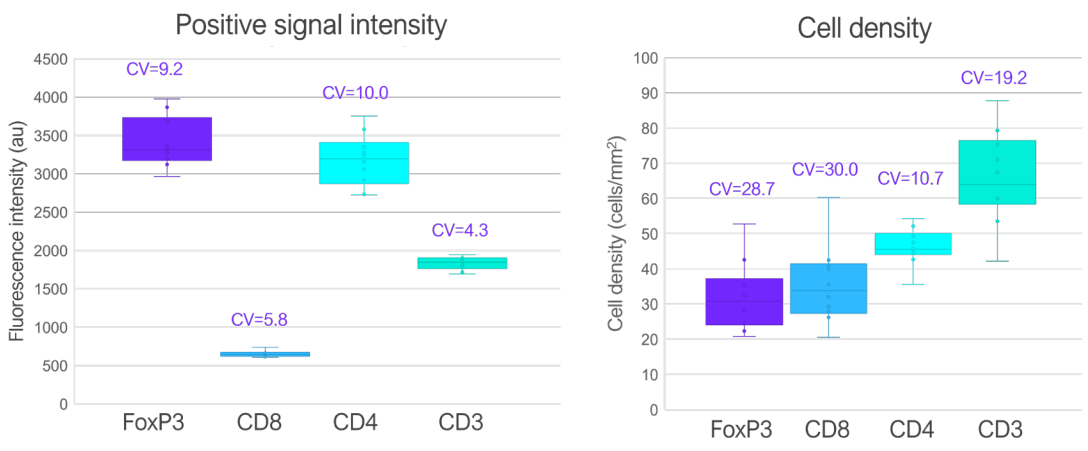
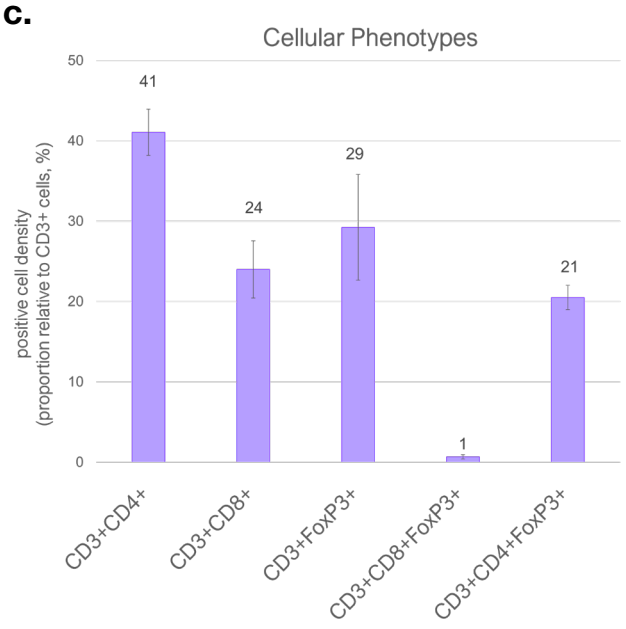


Figure 3a. Multiplex image staining of B16 melanoma. The whole slide and zoomed in region.

b.



c.



d.

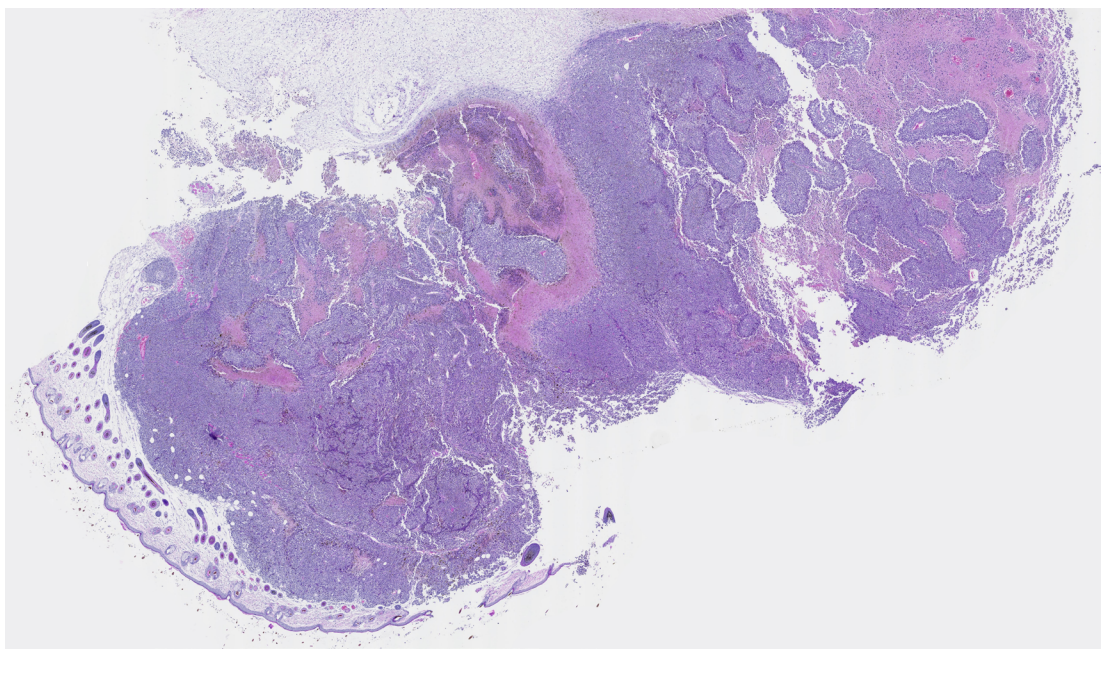


Figure 3b. Box Plot analysis of intensity and cell density for each marker in the seven serial sections. **c.** Bar graph showing the % of double or triple positive cells, relative to total CD3+ cells. **d.** Whole slide H&E stained melanoma tissue.

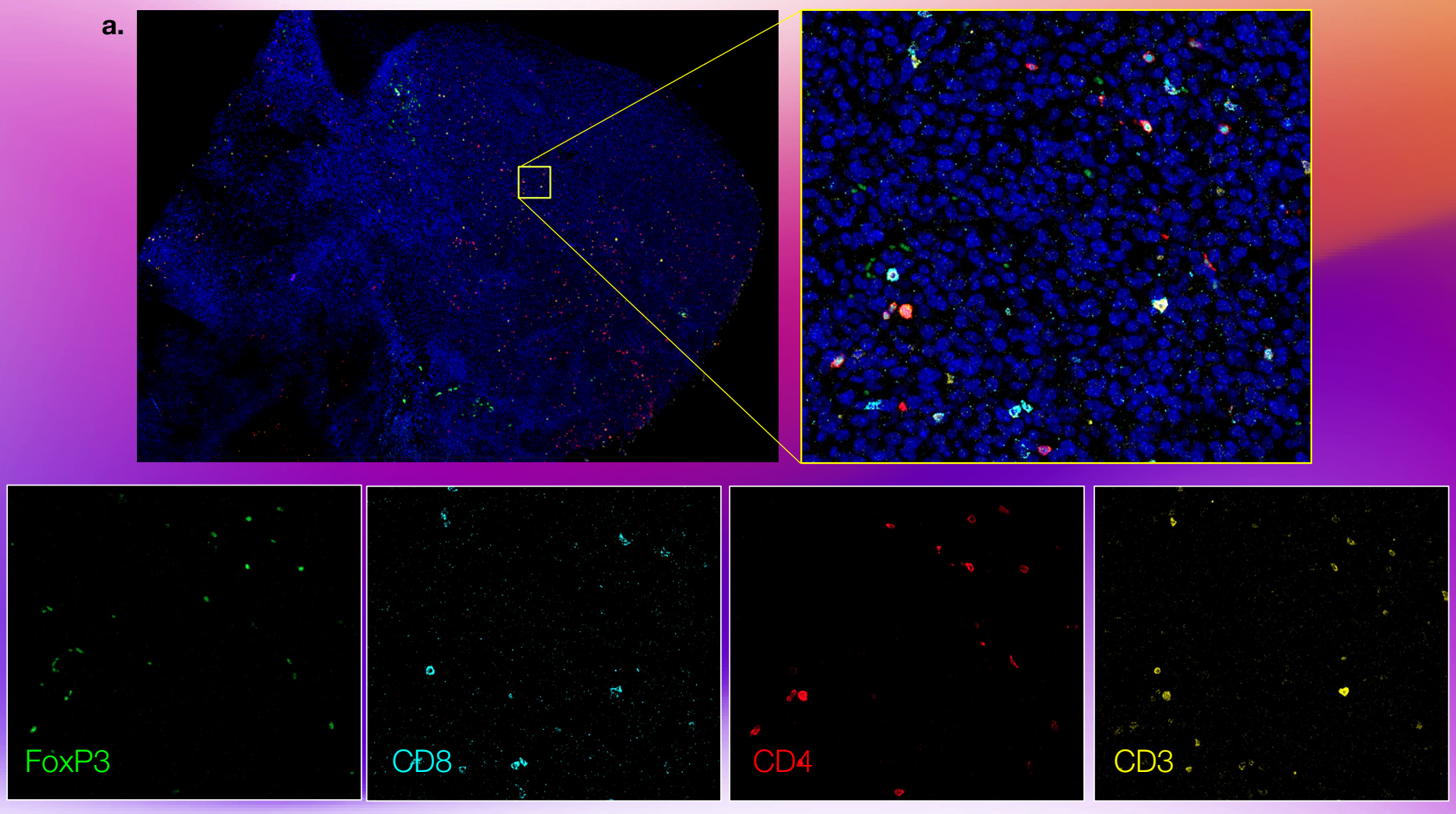
The use of the 4T1 mammary carcinoma is a transplantable tumor cell line that is highly tumorigenic and invasive and can spontaneously metastasize from the primary tumor in the mammary gland to multiple distant sites including lymph nodes, blood, liver, lung, brain, and bone. Therefore, syngeneic mouse models using this tumor line are a suitable experimental animal model for human mammary cancer. Similar to human breast cancer, 4T1 metastatic disease develops spontaneously from the primary tumor. Further, the progressive distribution of 4T1 metastases to the draining lymph nodes and other organs is very similar to that of human mammary cancer.

Figure 4a demonstrates the utility of our 4-plex panel to determine immune infiltrates from whole slide imaging in melanoma. Qualitative assessment and quantitative analysis from whole slide imaging of melanoma tissue revealed a high level of reproducibility for the novel mouse assay by performing intra-assay staining on serial sections, with each single markers' coefficients of variation falling within an acceptable range (range is <30CV, Figure 4b). Each single marker testing verification showed an expected level of expression and expected staining pattern of the cellular phenotypes (Figure 4c) and morphology as revealed by H&E staining (Figure 4d).

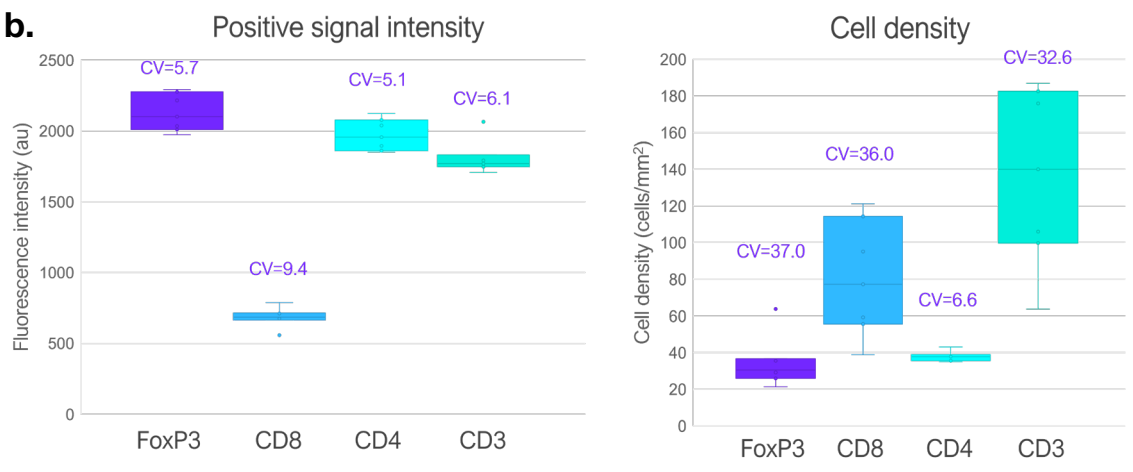
Figure 4

VALIDATION IN A SYNGENEIC MODEL OF BREAST CANCER

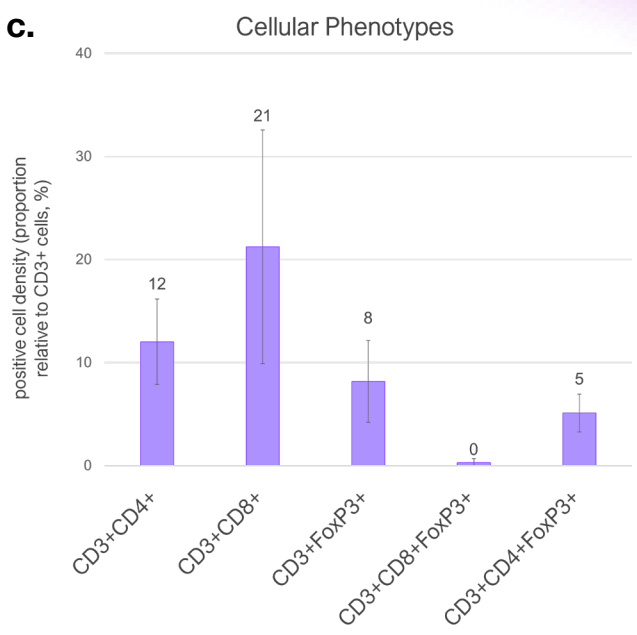
a.



b.



c.



d.

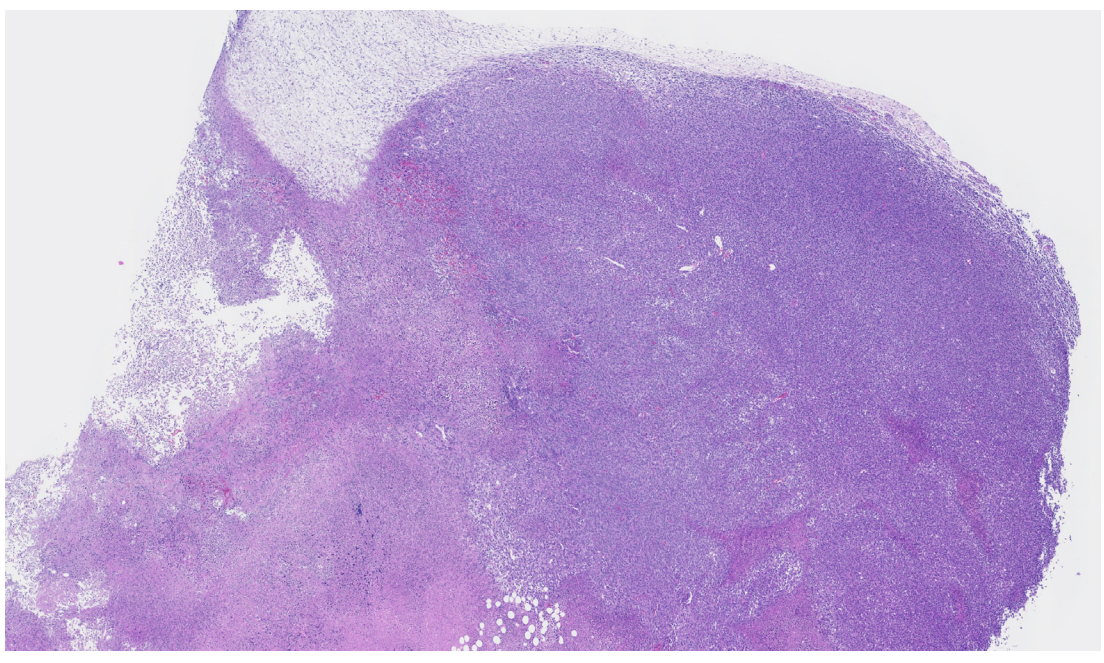


Figure 4a. Multiplex image staining of 4T1 breast. The whole slide and zoomed in region.

Figure 4b. Box Plot analysis of intensity and cell density for each marker in the seven serial sections. **c.** Bar graph showing the % of double or triple positive cells, relative to total CD3+ cells. **d.** Whole slide H&E stained melanoma tissue.

Conclusion

The underlying mechanisms of the response to current immune checkpoint blockade therapy remains unclear. While the use of clinical samples from patient tumors accurately represents the tumor microenvironment, they are difficult to source and conduct controlled experiments. Pre-clinical models that faithfully recapitulate the complexity of cancer cells and their interactions with the immune system are therefore essential for investigating potential resistance mechanisms. Our unique streamlined pre-optimized protocol enables multiplexed staining specifically for mouse tissues to provide an improved workflow to investigate the immune system, including the important spatial analysis of the tumor immune microenvironment and reveal potential mechanism of action of immune-related drugs in pre-clinical syngeneic or genetically modified mouse models.

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