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Precise spatial multiplexing of protein biomarkers for immune profiling in tissue samples with ChipCytometry™

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Abstract

Immunohistochemistry is the most widely used diagnostic technique in tissue pathology. However, IHC is associated with several limitations including the labeling of just a few markers per tissue section and limited quantification of cell populations. As a result of plex limitations, key insights about tumor biology are missed, which could be important for advancing our understanding of tumor biology and ultimately improving patient outcomes. ChipCytometry™ is a novel image-based platform for precise spatial multiplexing that addresses these challenges by combining iterative immuno-fluorescent staining with high-dynamic range imaging to facilitate quantitative phenotyping with single-cell resolution. The platform enables simultaneous detection of dozens of markers on a single tissue section and enables accurate quantification of protein expression levels necessary to deeply profile single cells, understand interactions between key immune cells, and identify topographic biomarkers. Here we demonstrate how standard FCS files are generated from multichannel OME-TIFF images, enabling identification of cellular phenotypes via flow cytometry-like hierarchical gating. Quantification of results reveal precise expression levels for each marker in the assay in each individual cell in the sample, while maintaining spatial information about each cell. ChipCytometry has the potential to advance precision medicine in immuno-oncology and inform the discovery of novel biomarkers by enabling quantitative analysis of cellular phenotypes in the spatial context. The ChipCytometry platform enables simultaneous detection of multiple protein markers on a single tissue section for deep immune cell profiling in the tumor microenvironment. Combined with the single-cell spatial information, such data sets provide an opportunity for the discovery of new complex multiplexed biomarker signatures to inform therapeutic development.

Methods

Highly multiplexed image data was collected via the ChipCytometry™ workflow (Fig. 1) on the CellScape™ instrument using a 21-plex antibody panel (Table 1). After data collection, images were analyzed with built-in CellScape software with hierarchical gating to classify key immune cell types. A multi-channel stitched OME-TIFF was generated and uploaded to Enable platform as a proof of concept and for additional analyses including unsupervised clustering.

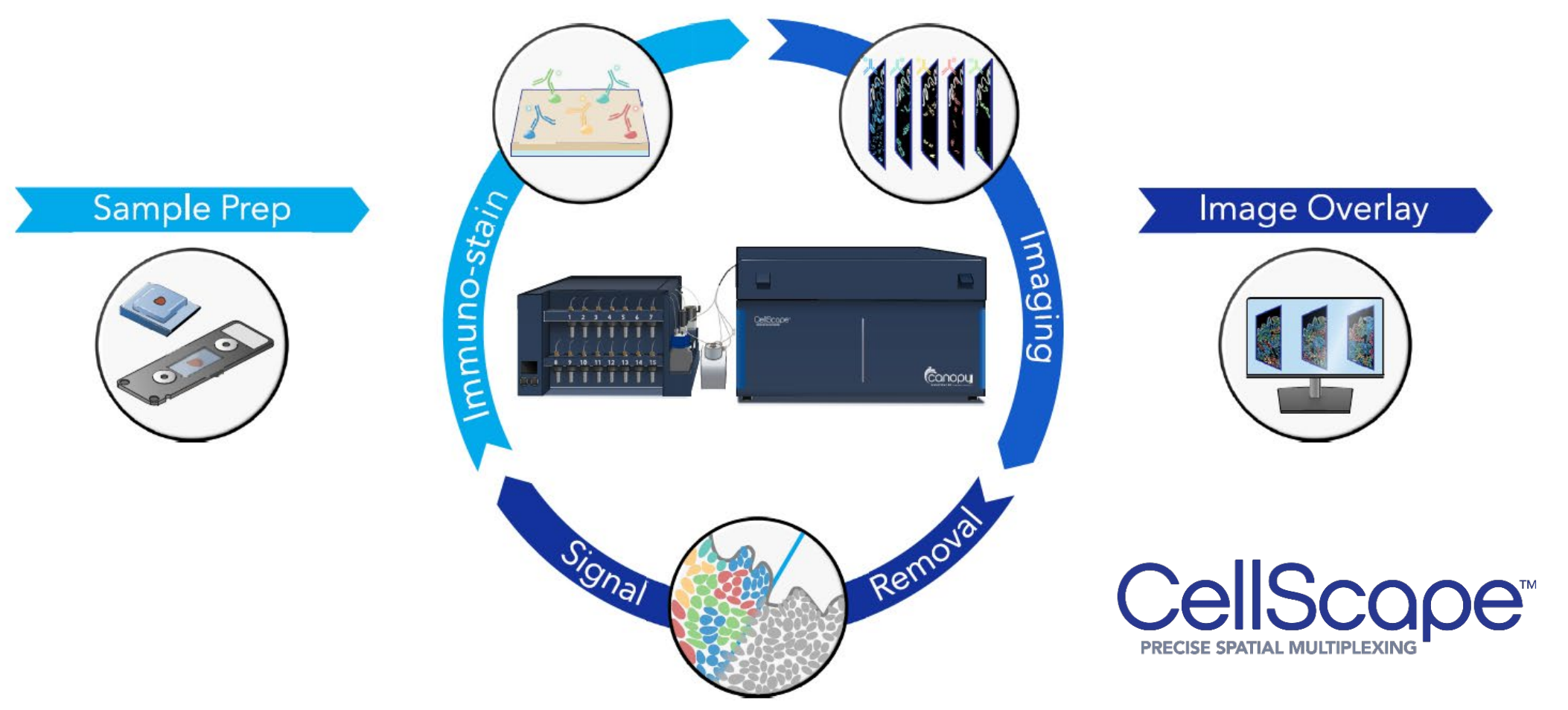

Figure 1. ChipCytometry workflow. After sample preparation, the ChipCytometry workflow consists of successive rounds of immuno-staining, imaging, and signal removal to profile virtually unlimited number of protein targets. An image overlay of each marker in the assay is created by aligning each channel to a reference channel. The process is fully automated with CellScape, the next-generation instrument for precise spatial multiplexing.

Table 1. Antibody Staining

Target	Dilution
CD3	1:100
CD4	1:100
CD8	1:200
CD11c	1:200
CD14	1:100
CD20	1:200
CD31	1:400
CD38	1:100
CD45	1:500
CD45RA	1:1800
CD45RO	1:900
CD56	1:100
CD68	1:300
CD123	1:100
PD1	1:100
EpCAM	1:1800
CD340	1:600
DNA	1:100000
HLA-DR	1:600
FoxP3	1:100
Pan-CK	1:1800

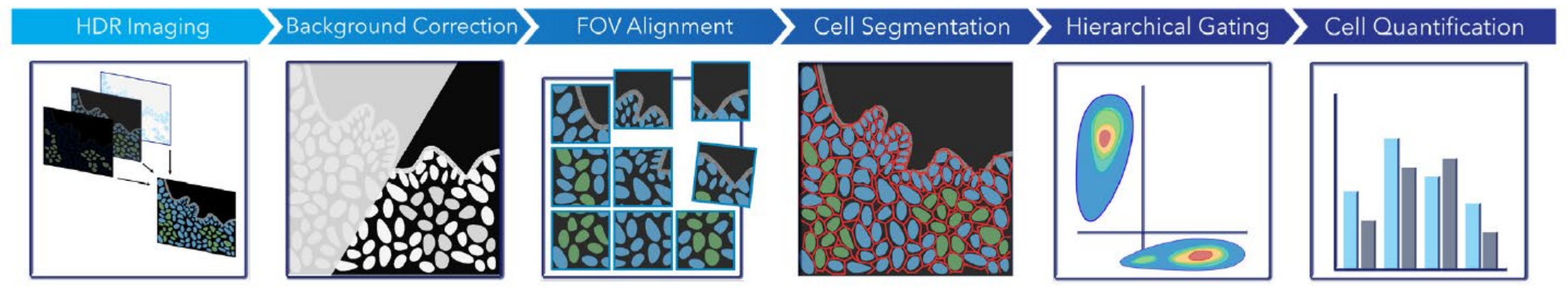

Figure 2. ChipCytometry image processing and analysis. Image process and analysis is managed the CellScape App and includes 6 key steps: (i) multi-exposure HDR image fusion, (ii) background correction, (iii) FOV alignment, (iv) cell segmentation, (v) hierarchical gating, and (vi) cell quantification.

Table 2. ChipCytometry Protocol

Sample Preparation	
Step 1	Five µm FF tissue sections were mounted onto glass coverslips
Step 2	Sections were loaded onto chips to preserve sample integrity during serial delivery of reagents
Data Collection	
Step 3	An initial autofluorescence scan was performed to identify ROIs in the tissue
Step 4	Tissue sections were stained with up to 5 fluorescent antibodies from commercial vendors and incubated at 15 min at RT
Step 5	Tissue sections were imaged in up to 5 channels using HDR multi-exposure imaging and high-resolution optics
Step 6	Tissue sections were photobleached to remove fluorescence signal
Step 7	Steps 4-6 were repeated in successive rounds until all targets were imaged (Table 1)
Image Analysis	
Step 7	Multi-exposure HDR image fusion and background correction for individual FOVs was performed using CellScape software
Step 8	Cell segmentation, hierarchical gating, and cell quantification were performed using CellScape software
Step 9	A custom ImageJ pipeline was used to stitch FOVs to generate a whole-slide image
Step 10	Cell segmentation, hierarchical gating and clustering were performed using Enable platform

Results

Here we present the analysis of a roughly 8 mm^2 (Fig. 2A) of 15 mm^2 total area scanned. Most tumor cells (Pan-CK+) are also HER2+ (Fig. 2B) signifying a carcinoma malignancy of epithelial origin. We found a single region of HER2-/Pan-CK+ epithelium with relatively normal tissue architecture (Fig. 2C). Each of the 21 markers in this assay were used for cell phenotyping using a hierarchical gating strategy based on expression values using both CellScape Analysis System (Fig. 4 and Fig. 5) and Enable platform (Fig. 6). We demonstrate the ability to quantify key immune cell populations using both platforms and, in addition, perform higher order analyses including unsupervised clustering using the Enable platform.

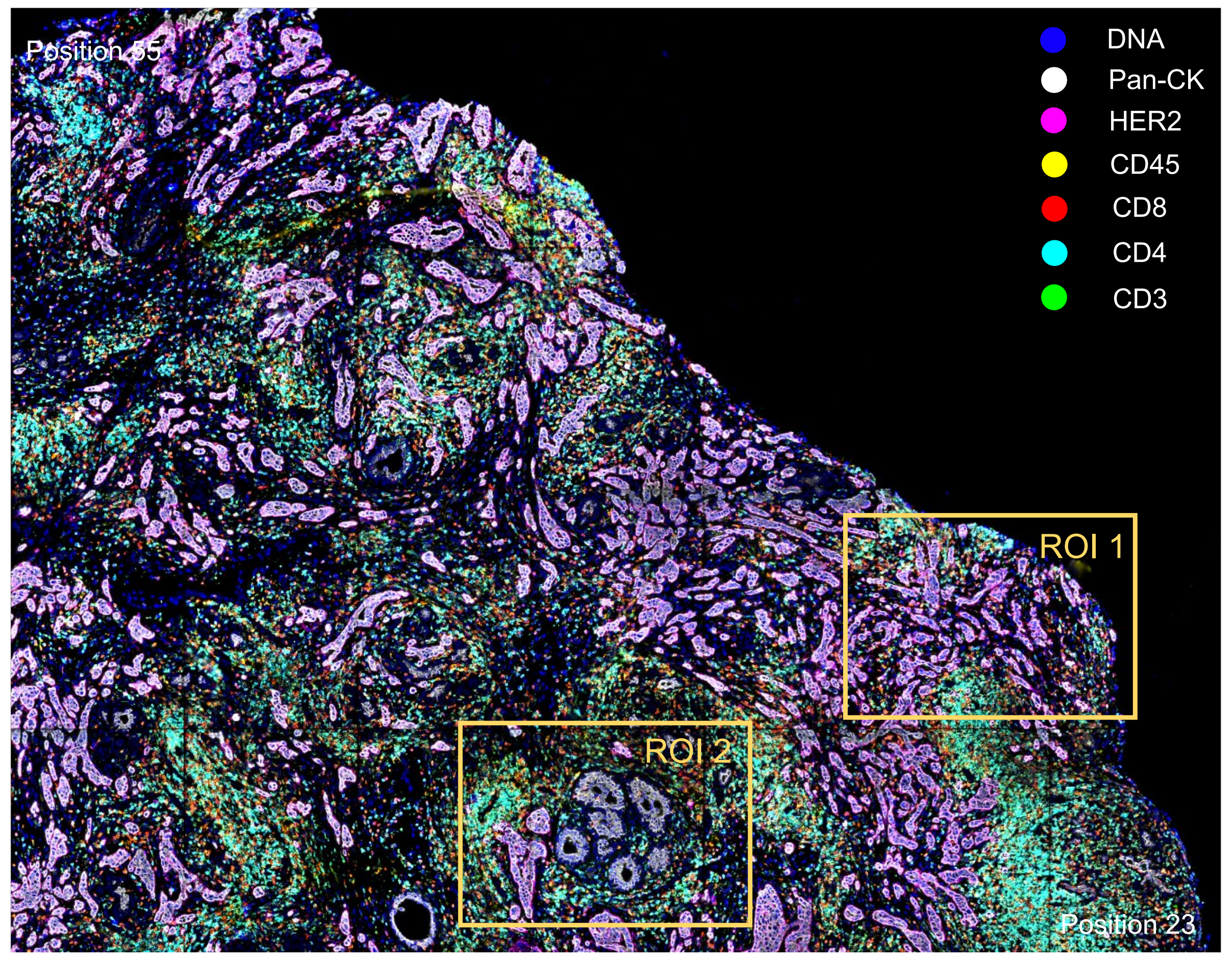

Figure 2A. Highly multiplexed image of HER2+ breast carcinoma. A 21-plex antibody stain was applied to a HER2+ breast cancer tissue specimen. Here, we show a subset of markers to highlight tissue architecture and immune cells across 23 scan positions (8 mm^2 area).

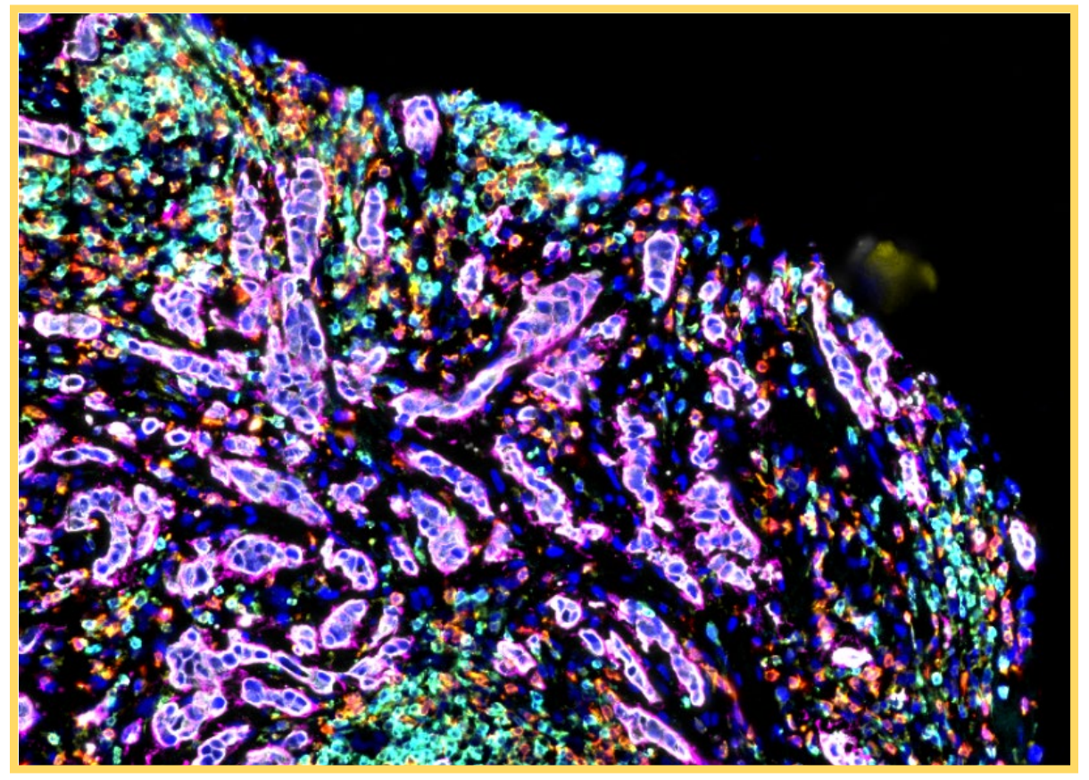

Figure 2B. Zoomed in view of ROI 1. HER2+ region of epithelial tissue.

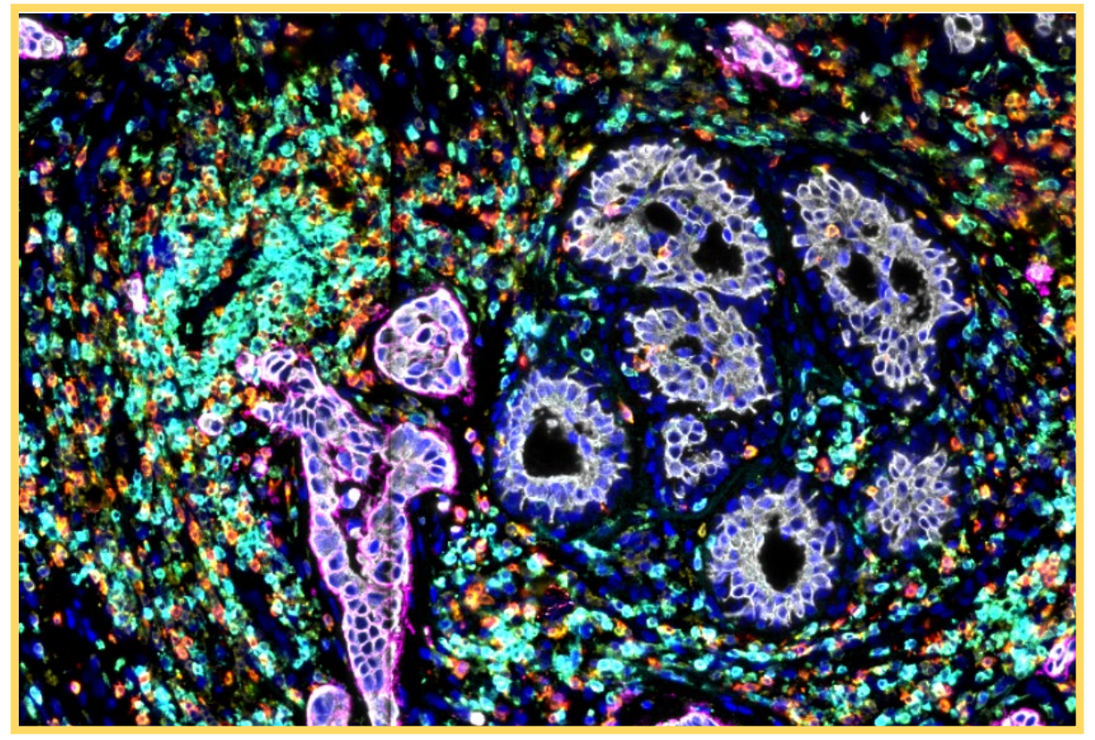

Figure 2C. Zoomed in view of ROI 2. HER2- region of epithelial tissue (Pan-CK+).

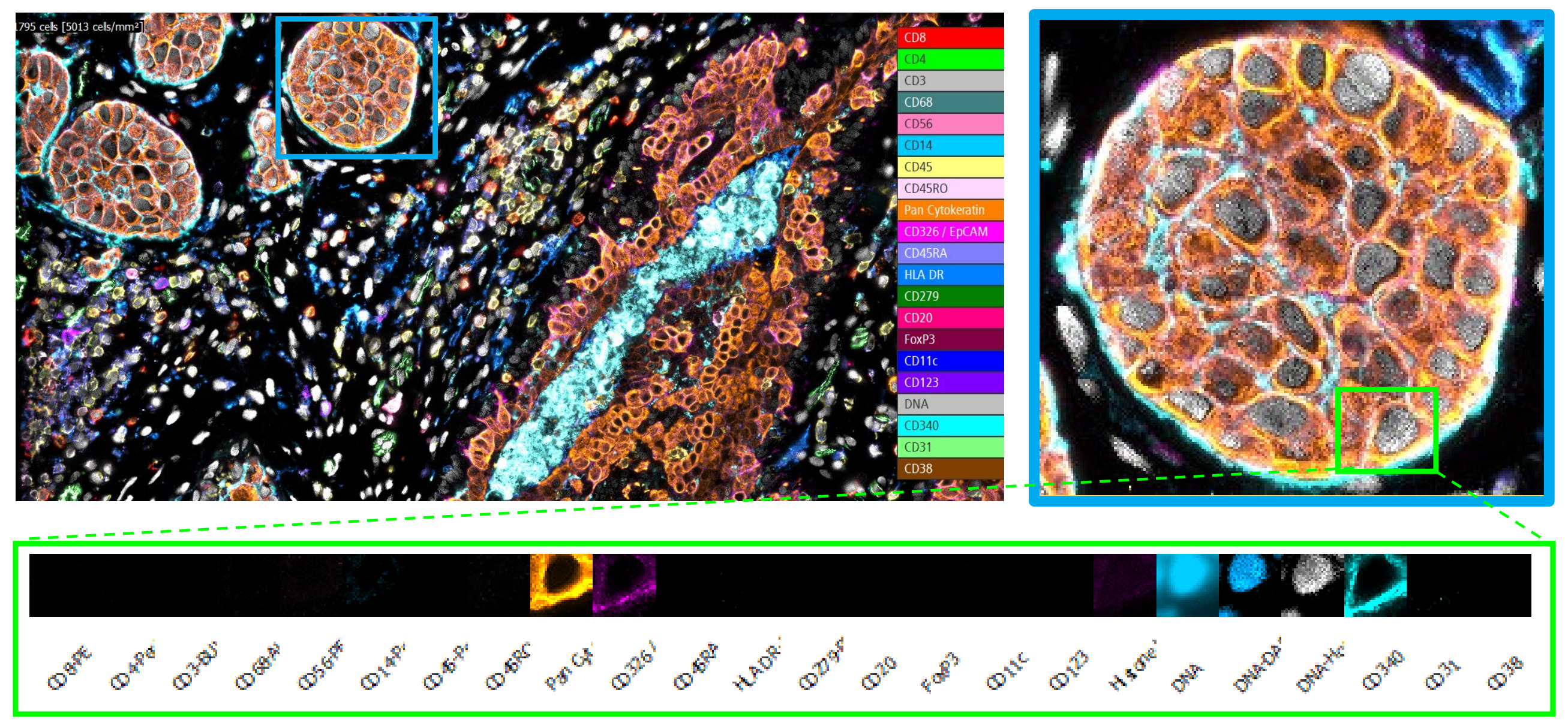

Figure 3. Single-cell resolution imaging. The CellScape imaging system combines high-quality optical components with advanced analysis software to quantify protein expression and cell populations at the single-cell level. Here, we show expression in all channels for a single cell expressing Pan-CK, EpCAM, and HER2.

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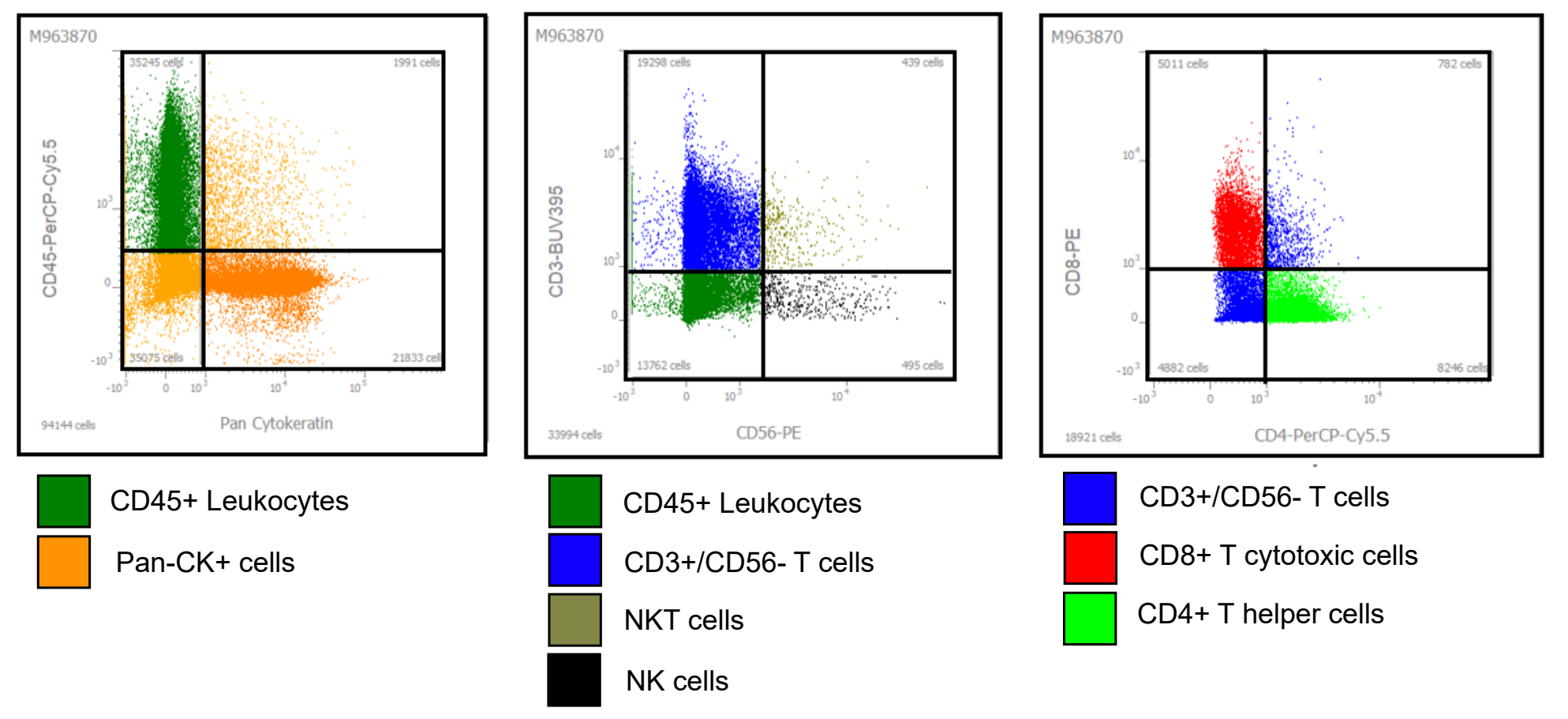

Figure 4. Hierarchical gating with CellScape App. Cell phenotyping was performed via flow cytometry-like hierarchical gating to identify key immune cell populations.

Table 3. Quantification of cell populations

Cell Population	Parent Gate	Gating Strategy	% of Total Leukocytes	Absolute Count
All cells	Hoechst Stain for DNA	N/A	-	**94184**
Leukocytes	All	CD45+ Pan CK-	**100.00%**	**35245**
T cells	CD45+ Leukocytes	CD3+ CD56-	**54.75%**	**19298**
NK cells	CD45+ Leukocytes	CD3- CD56+	**1.40%**	**495**
NKT cells	CD45+ Leukocytes	CD3+ CD56+	**1.24%**	**439**
T cytotoxic	T cells	CD4-CD8+	**14.22%**	**5011**
T helper	T cells	CD4+CD8-	**23.40%**	**8246**
T regulatory	T helper	FoxP3+	**1.33%**	**468**
B cells	CD45+ Leukocytes	CD3-CD20+	**1.57%**	**554**
Macrophages	CD45+ Leukocytes	CD68+	**9.11%**	**3210**
Dendritic Cells	CD45+ CD3- CD20- CD14-	HLA-DR+ CD56-	**18.44%**	**6499**
Myeloid DCs	Dendritic Cells	CD11c+ CD123-	**13.24%**	**4666**
Plasmacytoid DCs	Dendritic Cells	CD11c- CD123+	**0.21%**	**74**
Tumor cells	All	CD45- Pan-CK+	-	**21833**

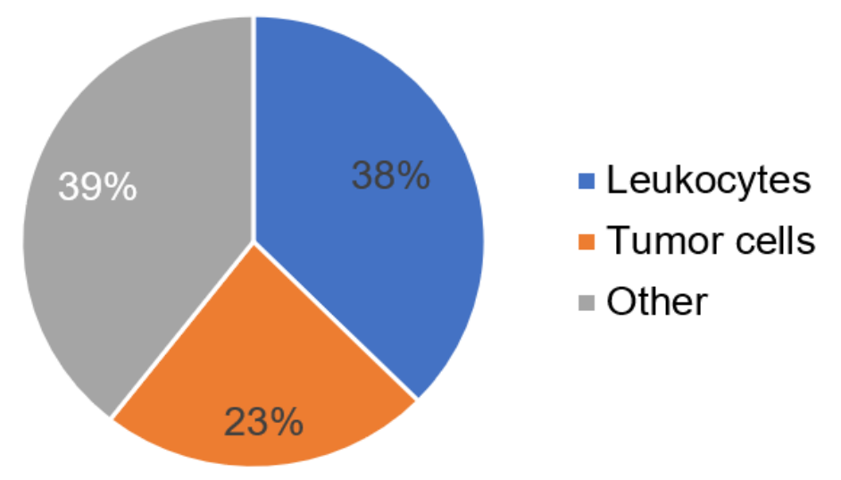

Figure 5A. Percent of all cells. The majority of cells were classified as leukocytes (38%) or tumor cells (23%).

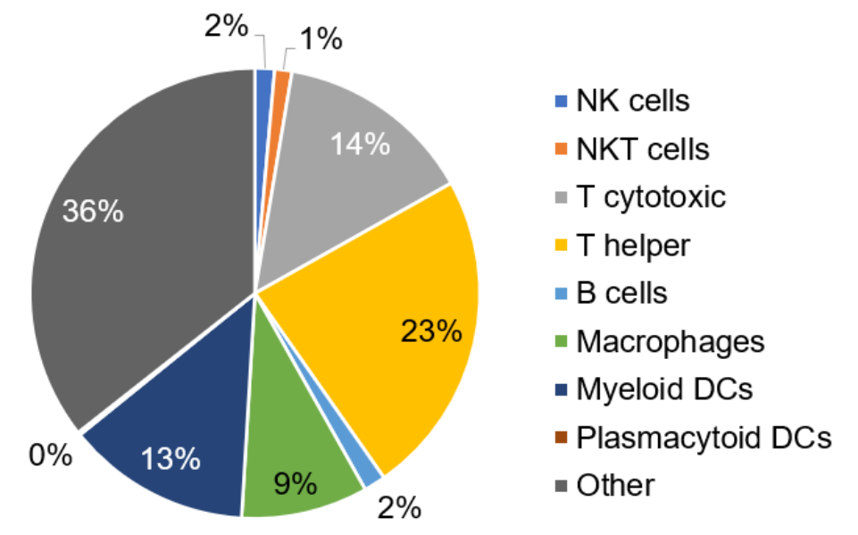

Figure 5B. Percent of leukocytes. The majority of leukocytes were T helper (23%) or T cytotoxic (14%) cells.

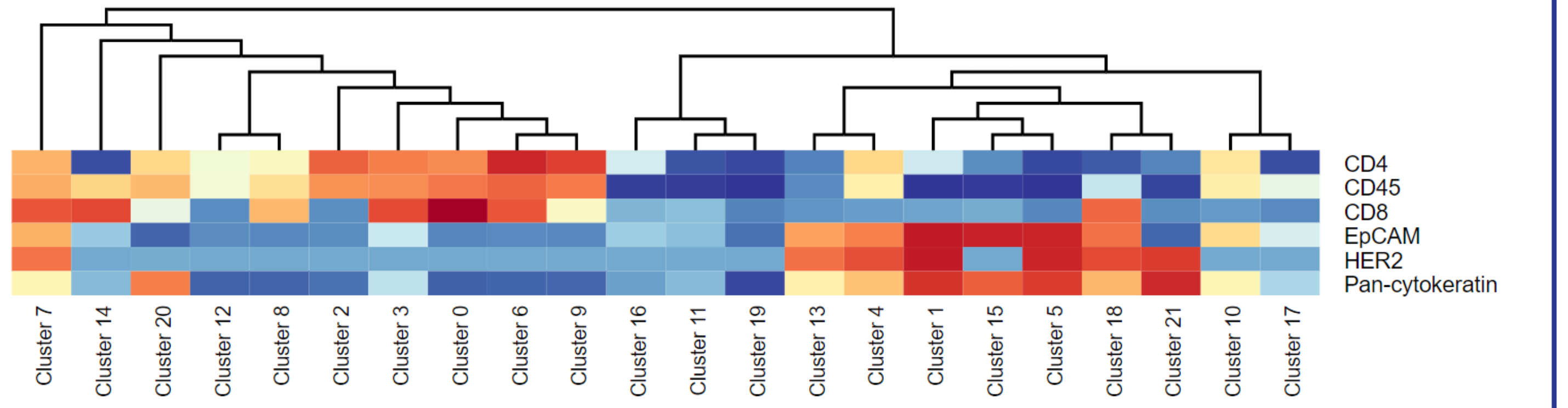

Figure 6A. Unsupervised clustering with Enable platform. Heatmap showing biomarker expression profile for each of the 21 clusters identified with unsupervised clustering (Leiden method).

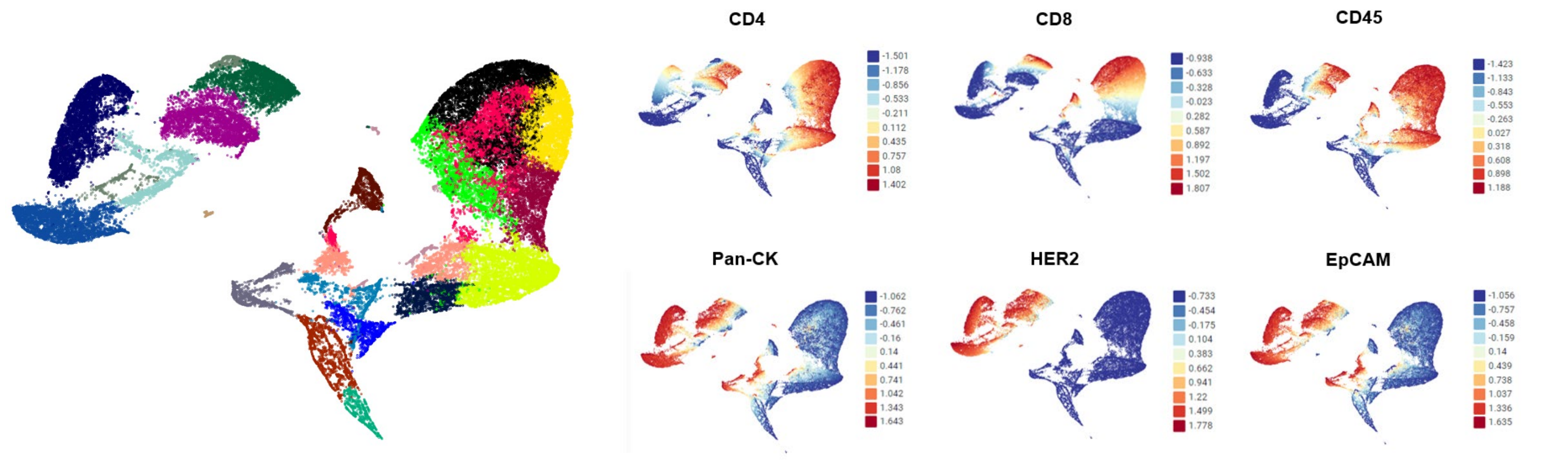

Figure 6B. UMAP plots with Enable platform. Plot shows the result of UMAP applied to the normalized biomarker expressions of each cell, colored by the selected feature.

Conclusions

- We demonstrate the utility of ChipCytometry to generate highly-multiplexed, spatially-resolved protein expression data from a clinical sample. We show quantitative measurement of 21 clinically relevant biomarkers at the single-cell level for every cell in this tissue specimen from a patient with HER2+ breast cancer.
- ChipCytometry is a multiplexed imaging method that uses commercial antibodies from any vendor to spatially resolve protein targets *in situ*. ChipCytometry does not require any additional abstractions (e.g., oligo barcoding), which enables a simpler validation workflow and greater target versatility.
- We quantify relevant populations of tumor and immune subpopulations, revealing high relative abundance of T helper and T cytotoxic cells in this HER2+ breast cancer tissue. Quantification of cell populations expressing very high or low levels of a single marker is more challenging and made possible through high-dynamic range (HDR) imaging.
- We demonstrate the compatibility of ChipCytometry datasets with Enable platform to perform custom image processing and analytics, including unsupervised clustering, to better understand cellular frequencies, interactions, and neighborhoods at single-cell resolution.

Selected Publications

1. Carstensen, S., Holz, O., Hohlfeld, J. M., & Müller, M. (2021). Quantitative analysis of endotoxin-induced inflammation in human lung cells by Chipcytometry. Cytometry Part A, 99(10), 967–976. https://doi.org/10.1002/cyto.a.24352
2. FitzPatrick, M. E. B., Provine, N. M., Garner, L. C., Powell, K., Amini, A., Irwin, S. L., Ferry, H., Ambrose, T., Friend, P., Vrakas, G., Reddy, S., Soilleux, E., Klenerman, P., & Allan, P. J. (2021). Human intestinal tissue-resident memory T cells comprise transcriptionally and functionally distinct subsets. Cell Reports, 34(3), 108661. https://doi.org/10.1016/j.celrep.2020.108661
3. Hagel, J. P., Bennett, K., Buffa, F., Klenerman, P., Willberg, C. B., & Powell, K. (2021). Defining T Cell Subsets in Human Tonsils Using ChipCytometry. The Journal of Immunology, 206(12), 3073–3082. https://doi.org/10.4049/jimmunol.2100063
4. Jarosch, S., Köhlen, J., Sarker, R. S. J., Steiger, K., Janssen, K.-P., Christians, A., Hennig, C., Holler, E., D'Ippolito, E., & Busch, D. H. (2021). Multiplexed imaging and automated signal quantification in formalin-fixed paraffin-embedded tissues by ChipCytometry. Cell Reports Methods, 1(7), 100104. https://doi.org/10.1016/j.crmeth.2021.100104
5. Schupp, J., Christians, A., Zimmer, N., Gleue, L., Jonuleit, H., Helm, M., & Tuettenberg, A. (2021). In-Depth Immune-Oncology Studies of the Tumor Microenvironment in a Humanized Melanoma Mouse Model. International Journal of Molecular Sciences, 22(3), 1011. https://doi.org/10.3390/ijms22031011

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