Miltenyi Biotec



Analyzing complex spatial data for deep insights with MACS® iQ View Image Analysis Software

Realize the full potential of spatial biology analysis

Background

MACSima™ System is a highly multiplexed spatial proteomic technology that provides a tremendous amount of complex data. Analysis of large multidimensional data stacks, such as these, requires advanced tools to evaluate the spatial relationships of expressed markers and reveal deep insights. MACS iQ View is a powerful image analysis software that was specifically designed to do just that, and enable researchers to realize the full potential of spatial biology. With its versatile and intuitive user interface, this software solution gives researchers the ability to extract the underlying information in a convenient and comprehensive manner, revealing complex phenotypic subtypes, cell-to-cell communication, as well as the spatial relationships and substructures.

Methods

Sample preparation

FFPE samples of human breast carcinoma and human lung squamous cell carcinoma were sectioned with a thickness of 3 µm using a microtome. On the day of the experiment, slides were processed for (a) deparaffinization using xylol and (b) antigen retrieval using a heat-induced epitope retrieval protocol with TEC Buffer at pH 9. Frozen samples of human colon carcinoma and tonsil were embedded in optimal cutting temperature (OCT) compound then sectioned with a thickness of 8 µm and fixed with 4% paraformaldehyde (PFA).

MACSima Imaging workflow

Samples were stained with DAPI, then placed in the MACSima System and regions of interest (ROIs) were selected. Antibody panels were defined selecting antibodies with the specificities of interest and in accordance with sample fixation type and species. Antibody working solutions were prepared in MACSwell™ Deepwell Plates, sealed with MACSwell Sealing Foil and placed in the instrument. The experiment was started and the MACSima System performed cyclic staining and erasure, acquired and processed images, and saved them in the common TIFF format.

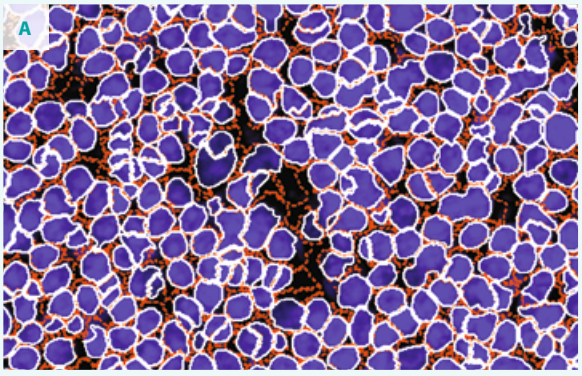
Image analysis

Raw data from the ROI of the tissues were processed for registration and background correction. The dataset was then opened in MACS iQ View Analysis Software, and boundaries of cells and nuclei were defined using the segmentation tool. After segmentation, several gating strategies were applied and the workflow editor was employed to perform various analyses.

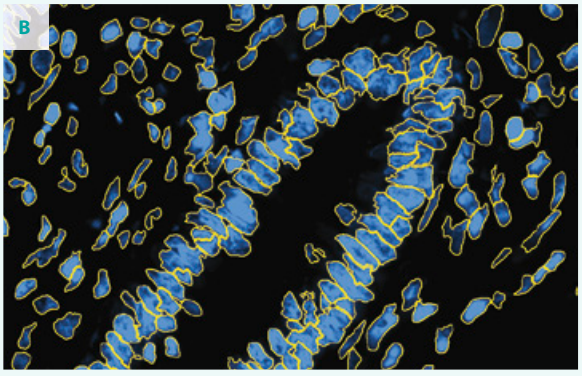
Results

Single-cell identification

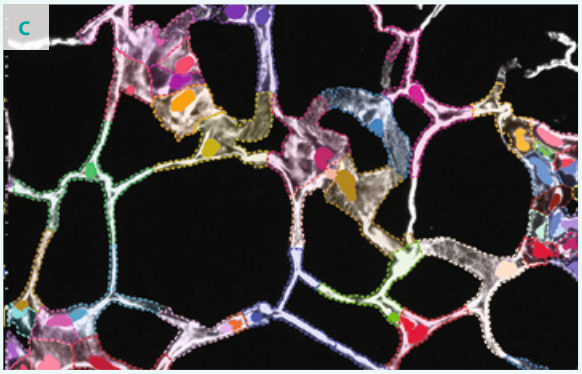
Equipped with a variety of specialized image analysis methods, MACS iQ View can obtain high-quality single-cell data particular to an experiment, using much more than just histogram intensity-based models (fig. 1). Single-cell segmentation works with customizable parameters that help direct and optimize the automatic algorithm for numerous tissue types. Superpixel-based segmentation is an alternative method for non-nucleated cells or samples requiring analysis of extracellular proteins. For ultimate flexibility, users are able to import their own segmentation mask or labeled image. Multiple biomarkers overcome common challenges in cell segmentation such as dense or irregularly shaped nuclei and cells.



Identification of dense nuclei using classic DAPI segmentation.



Broad detection of a variety of cell sizes and shapes.



Definition of cytoplasmic boundaries of irregularly shaped cells with a structural marker (vimentin).

Figure 1: Segmentation strategies for the identification of individual cells in various fixed tissues. (A) Segmentation of dense nuclei in acetone-fixed human thymus tissue. (B) Detection of variable nuclei sizes and shapes in 4% PFA-fixed human colon carcinoma tissue. (C) Identification of irregularly shaped cells using DAPI (multicolor) and cytoplasmic staining with vimentin (white) in FFPE human breast tissue.

Visualizing tumor microenvironment and tissue architecture

Although the specific composition may vary, most tumor microenvironments share hallmark cell types and tissue regions including tumor cells, immune cells, stromal cells, extracellular matrix, and vessels. The interplay of this cellular landscape and heterogeneity can affect tumor progression, prognosis, and possibly treatment response in many cancer types. Using a variety of markers to span all these cell types allows for the visualization and compartmentalization across tissue samples. In the colon carcinoma shown in figure 2, single-cell analysis was performed on the total area and cells classified into three broad categories of immune, tumor, or stroma, based on specific marker expression within the 36-marker panel. Staining patterns of any combination of markers within the panel may be visualized together. Immune cells were classified as CD45 positive, tumor cells as EpCAM (CD326) positive, and stroma as collagen IV positive (fig. 2).

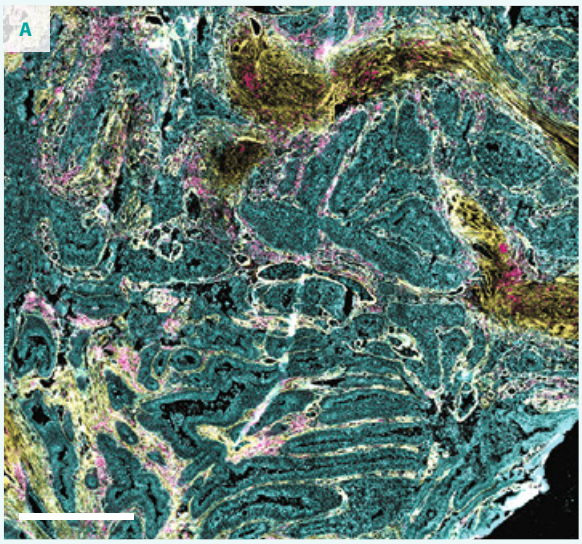
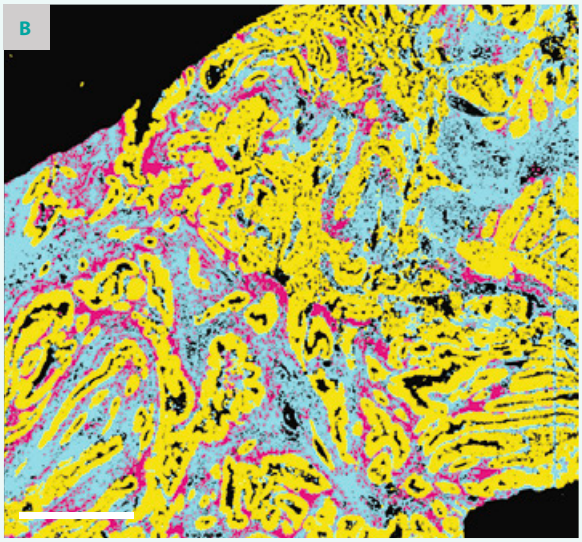


Image of CD45 (magenta), EpCAM (CD326, cyan), and collagen IV (yellow) co-expression on colon carcinoma.



Tumor microenvironment compartments of tumor (yellow), immune cells (magenta), and stromal and other cells (cyan).

Figure 2: Tumor, stroma, and immune landscape across issue. A panel was designed with 36 Miltenyi Biotec antibodies characterizing the phenotypic profile of the tumor, immune, vascular, and structural components of the tumor microenvironment (A). Single-cell analysis was performed, and cell expression levels used to characterize the three displayed populations (B). Scale bar is 1 mm.

Deep phenotyping of immune cells

Intratumoral heterogeneity in tissue is a major obstacle in understanding and treating many cancers. Immune profiling at the single-cell level has enabled the characterization of intratumoral immune cells and cell subsets in the tumor microenvironment. The MICS technology, with its ability to analyze hundreds of markers on a single piece of tissue, allows for the basic phenotypical identification of cells in tissue, but also cell type subsets and functional diversities of these cells. Tonsil tissue has many of these cell types. In order to characterize the numerous phenotypic and functional variations in the over 38,000 identified cells in figure 3, basic populations were classified on a few markers within the 35-marker panel. This amount of information can also be reduced into a t-distributed stochastic neighbor embedding (t-SNE plot) to better visualize clusters in the data not immediately recognizable by a few markers. For example, figure 3C had a well-defined cluster of cells that corresponded to a specific area within the tissue. Similarly, although general T cells can be identified with only a few markers, there is no information on the function of these cells. The identified T cells were further classified according to the strategy in figure 4A. As can be seen from the enumeration of each population, some populations make up less than 1% of the total cell population but these cells can affect immune responses.

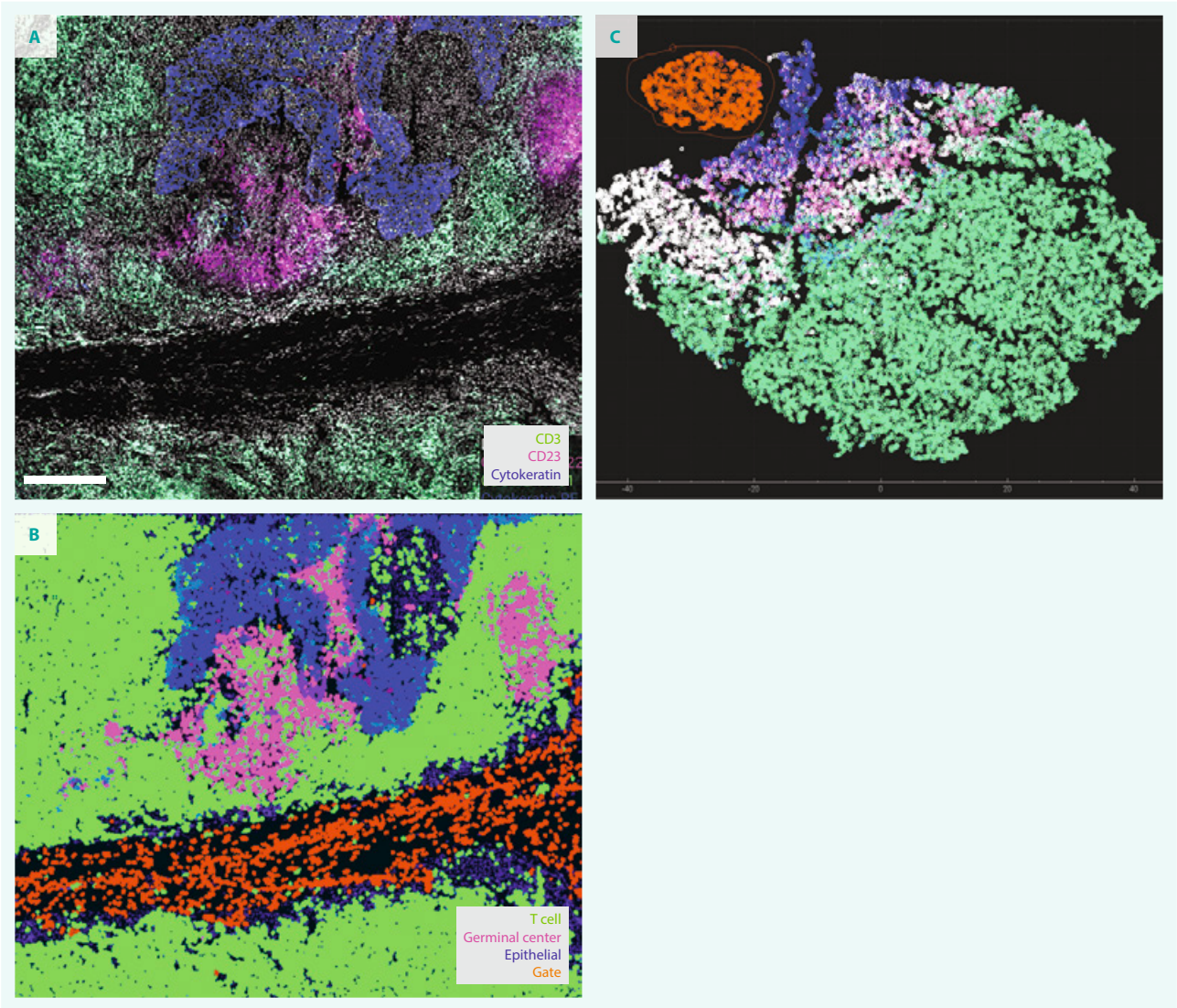


Figure 3: Classification of cell populations

A panel was designed in human tonsil tissue fixed with 4% PFA with 35 antibodies (28 Miltenyi Biotec and 7 other providers to characterize T cell classes). (A) CD3, CD23, and cytokeratin expression on human tonsil. Nuclear DAPI stain is displayed in white. (B) Classification of single-cell analysis (shown in orange) to identify common populations found in tissue. T cells are $CD3^+$ and $CD45^+$, germinal centers are $CD23^+$, and epithelial cells are $cytokeratin^+$. The "gate" is a cluster found in t-SNE plot derived from all cell expressions (C). The populations are color-coded on the t-SNE as described in (B). Scale bar is 200 µm.

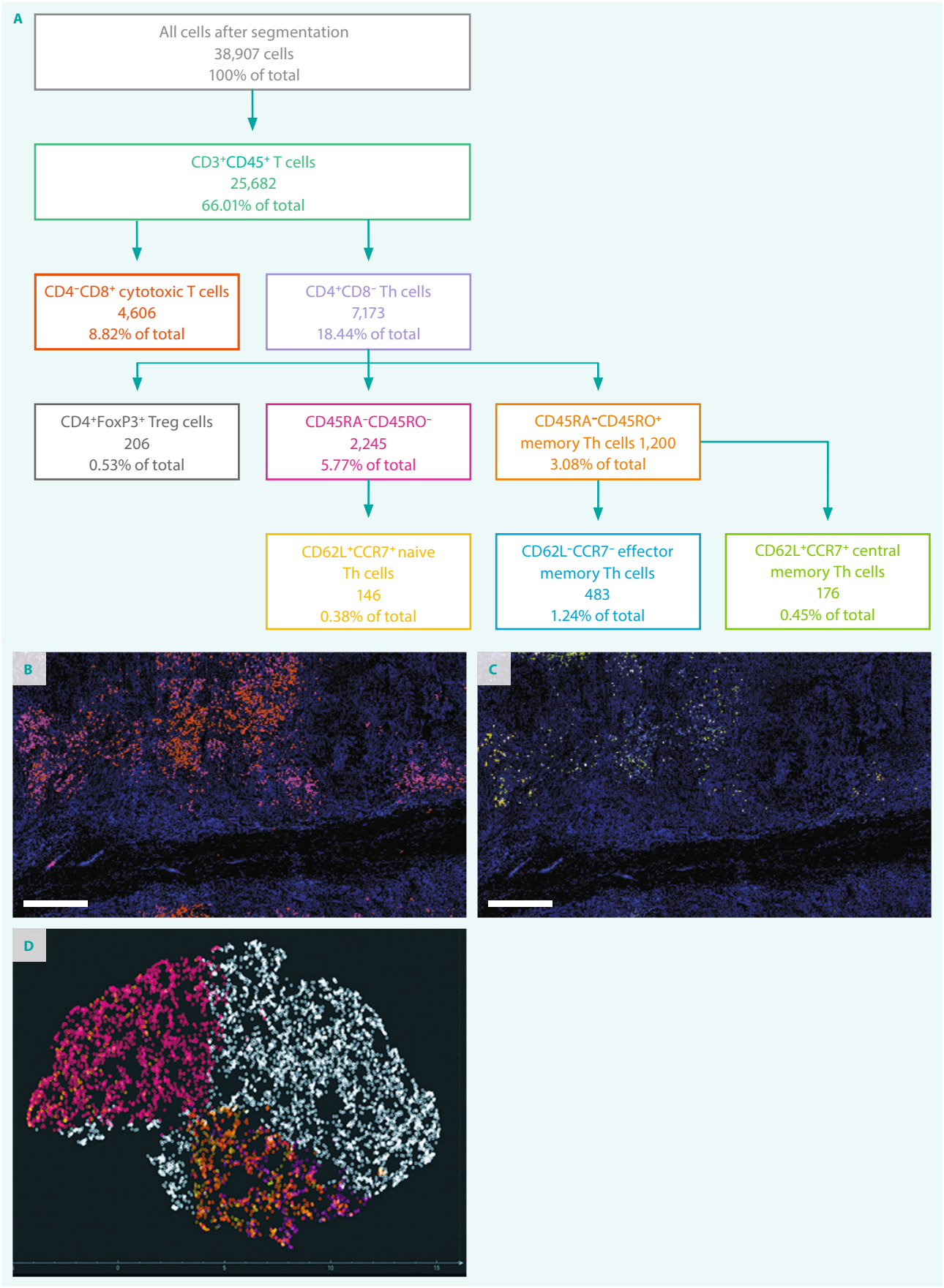


Figure 4: Classification of T cell population
T cells identified in figure 3 were further classified into the subsets that have unique functional and activation states. (A) Classification strategy. Each population was numerated, and the percentage of the total identified cells was calculated. (B) T helper cells divided into memory T cells and CD4SRA+/CD4SRO- cells and displayed on the tissue. These are further divided into activation status which is displayed in (C). Display of the cell population is the same as (A) and DAPI is in blue. (D) UMAP generated from T helper cells. The highlighted cells are the populations identified in (B) and (C). Scale bar is 200 µm.

Profiling differentiation of tumor cells and immune checkpoints

The differentiation status of particular epithelial tumors has been shown to be correlated to progression and prognosis. Understanding and characterizing the basal and luminal subtypes can dictate more effective treatment avenues. One current immunotherapy pathway is to target immune checkpoints. To uncover possible treatment responses or pathways, characterizing the tumor subsets as well as cell status becomes a powerful tool. Human lung squamous cell carcinoma was probed with 112 antibodies from Miltenyi Biotec and other providers to examine the basal and luminal characteristics of the tumor epithelial cells. After single-cell analysis, k-means clustering was performed with 38 features unique to the cytokeratin-positive cells. Each cluster exhibited certain signatures in their cytokeratin expression (fig. 5C). While cluster 2 was high in both basal and luminal features, the other clusters had signatures for one of the luminal cytokeratins (cytokeratin 7 or cytokeratin 14). Within these clusters, the PD-L2 expression per cell was mapped and seemed highest in cluster 2 (fig. 5D).

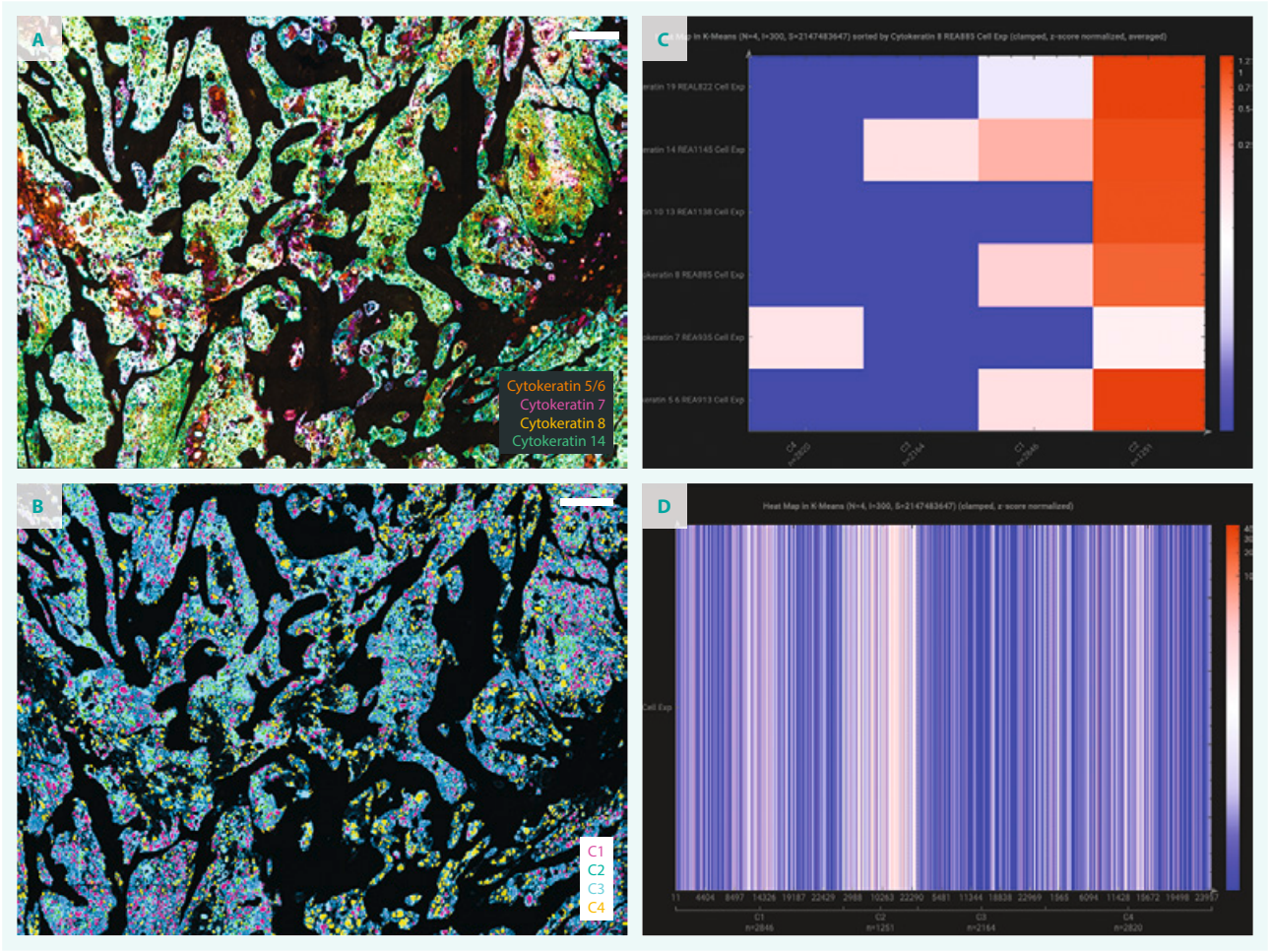


Figure 5: Differentiation profile and cluster characterization.
A panel was designed with 112 antibodies to examine the basal and luminal characteristics of the tumor epithelial cells in FFPE lung squamous cell carcinoma tissue. (A) Various cytokeratin markers displayed within the scanned tissue region. (B) K-means clustering was performed on the cytokeratin-positive cells. Color-coded clusters overlaid on cytokeratin-stained cells. (C) Averaged heat map of signature cytokeratin markers for basal and luminal epithelial cells per cluster. (D) Heat map of PD-L expression for each cell in each cluster. Scale bar is 200 µm.

Mapping spatial relationships

Spatial analysis of protein markers has great potential in evaluating the single-cell composition of the tumor microenvironment while simultaneously preserving the spatial landscape and inherent interactions between adjacent cells. The local neighborhood of immune and tumor cells can dictate differentiation, activated molecular pathways, and treatment response. In addition to the spatial location being recorded and observed, conclusions can be drawn from signatures in the different populations and neighborhoods where the cells reside.

In the breast cancer dataset below, T cell distance from the defined tumor region (double-positive for cytokeratin 5 and cytokeratin 7) was calculated and organized into "zones" around the tumor (fig. 6B). T cells were defined as CD3 positive and actin (smooth muscle) negative for this study. When the T cells in each zone were examined for other marker signatures, it was noted that the T cells furthest away from the tumor region had the lowest expression of CD8 indicating that these cells are likely T helper cells and the T cells closer to the tumor region likely contain a greater proportion of cytotoxic T cells (fig. 6C).

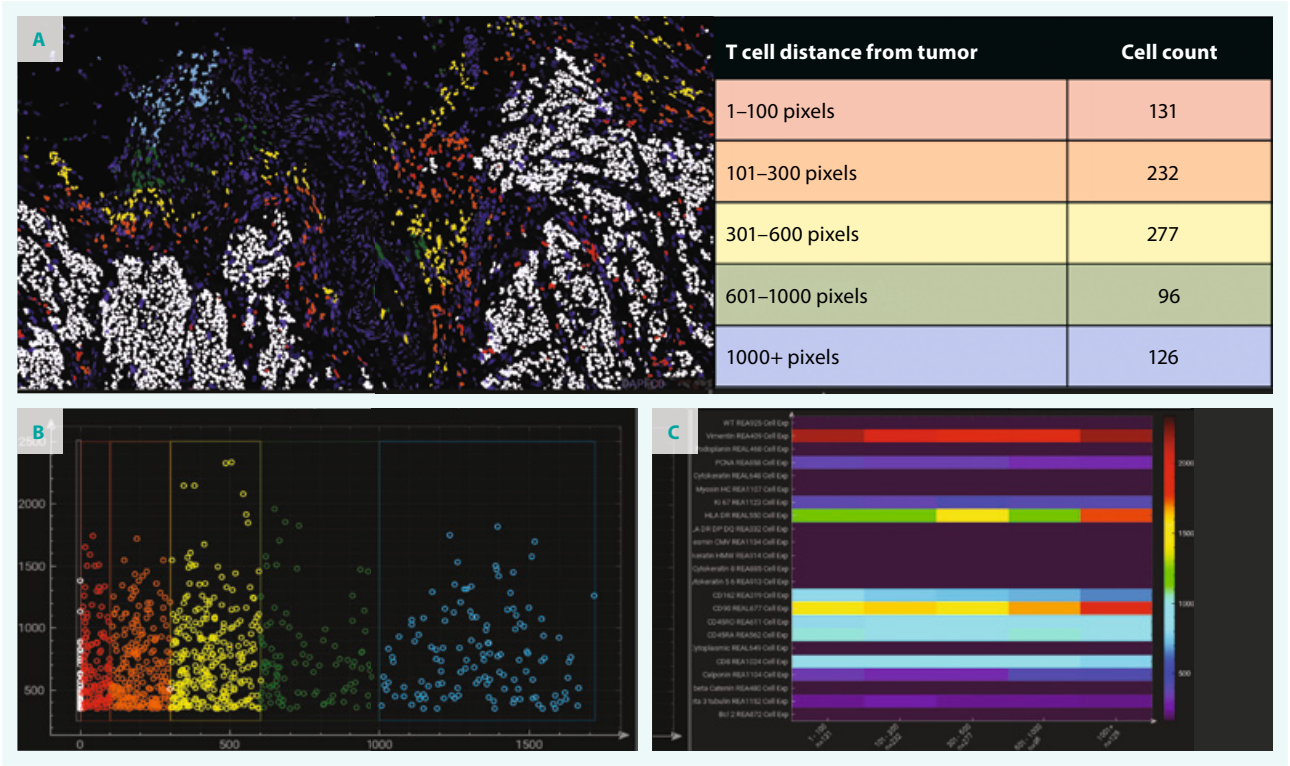


Figure 6: Spatial arrangement of T cells in relation to tumor cells.
FFPE breast cancer tissue stained with 45 Miltenyi Biotec antibodies. (A) Identified T cells color-coded via the distances gated in (B). Tumor cells are denoted in white and DAPI-stained nuclei are displayed in blue. Scale bar is 100 µm. (B) Distance zones created from calculated distance to $CD3^+$ cells from the identified tumor region. Numeration of $CD3^+$ T cells in each distance zone is tabulated in color-coded table. (C) Heat map of additional markers within the panel of the $CD3^+$ T cells in each distance region.

Conclusion

MACS iQ View provides the tools needed to effectively analyze complex imaging data. Specifically developed to manage large multidimensional data stacks generated by the MACSima System, MACS iQ View employs a number of analytical and visualization options that are simple, yet flexible, to enable deep insights.

Researchers can easily

- identify, quantify, and phenotype the individual cells in complex tissue,
- classify individual cell states and gain insights into differentiation and/or activation status, and
- locate and quantify spatial relationships amongst cell populations.



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