Highly Multiplexed Single-Cell Phenotypic and Spatial Profiling Using Multiplexed Ion Beam Imaging (MIBI™) Technology

The Need for Multiplexed Imaging in Translational Research

Disease progression and response to treatment are complex processes driven by the interplay between many discrete cell types. This includes an array of diseased cell phenotypes, as well as numerous immune cells, stromal cells, and resident normal cells present in the host tissue. Further, it is now established that cell function is driven by intricate spatial relationships with other cell types in the tissue. As a result, measuring disease progression and response to therapy requires a highly multiplexed imaging modality that preserves tissue histology and spatial information.

The MIBI Technology platform, with its flagship MIBIscope™ system, achieves these goals —providing a highly multiplexed imaging platform that can capture 40+ simultaneous markers with high resolution (down to 0.25 µm/pixel) and retain spatial and structural information. The MIBIscope platform is minimally destructive to tissue, thus permitting subsequent tissue re-scans—either for different resolutions or for Z-stack generation of three-dimensional imaging. With these features, the MIBIscope platform is a unique addition to the molecular imaging field. This document will describe the instrument workflow, its imaging potential, and the advanced analyses enabled by the high-dimensional spatial data produced by the MIBIscope platform.

MIBI Workflow: Tissue Staining and Data/Image Acquisition

Tissue preparation for the MIBI process follows standard immunohistochemical slide preparation techniques, making it easily adopted by researchers with immunohistochemistry (IHC) or immunofluorescence (IF) experience. Similar to traditional IHC and IF methods, tissue is sectioned onto MIBI slides using standard techniques (**Figure 1**). Prepared tissue is then stained overnight with a cocktail of metal isotope-tagged antibodies. Stained slides are then fixed and dehydrated, extending the lifespan of the detection signal to months post staining.

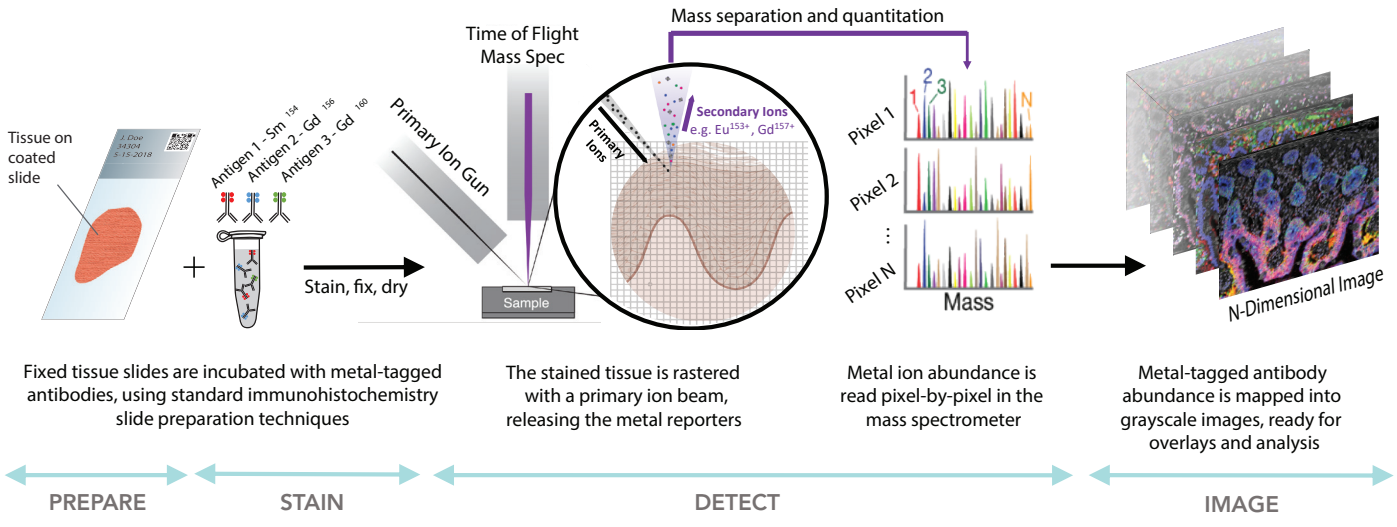

FIGURE 1: Tissue preparation and image acquisition process of the MIBIscope platform.

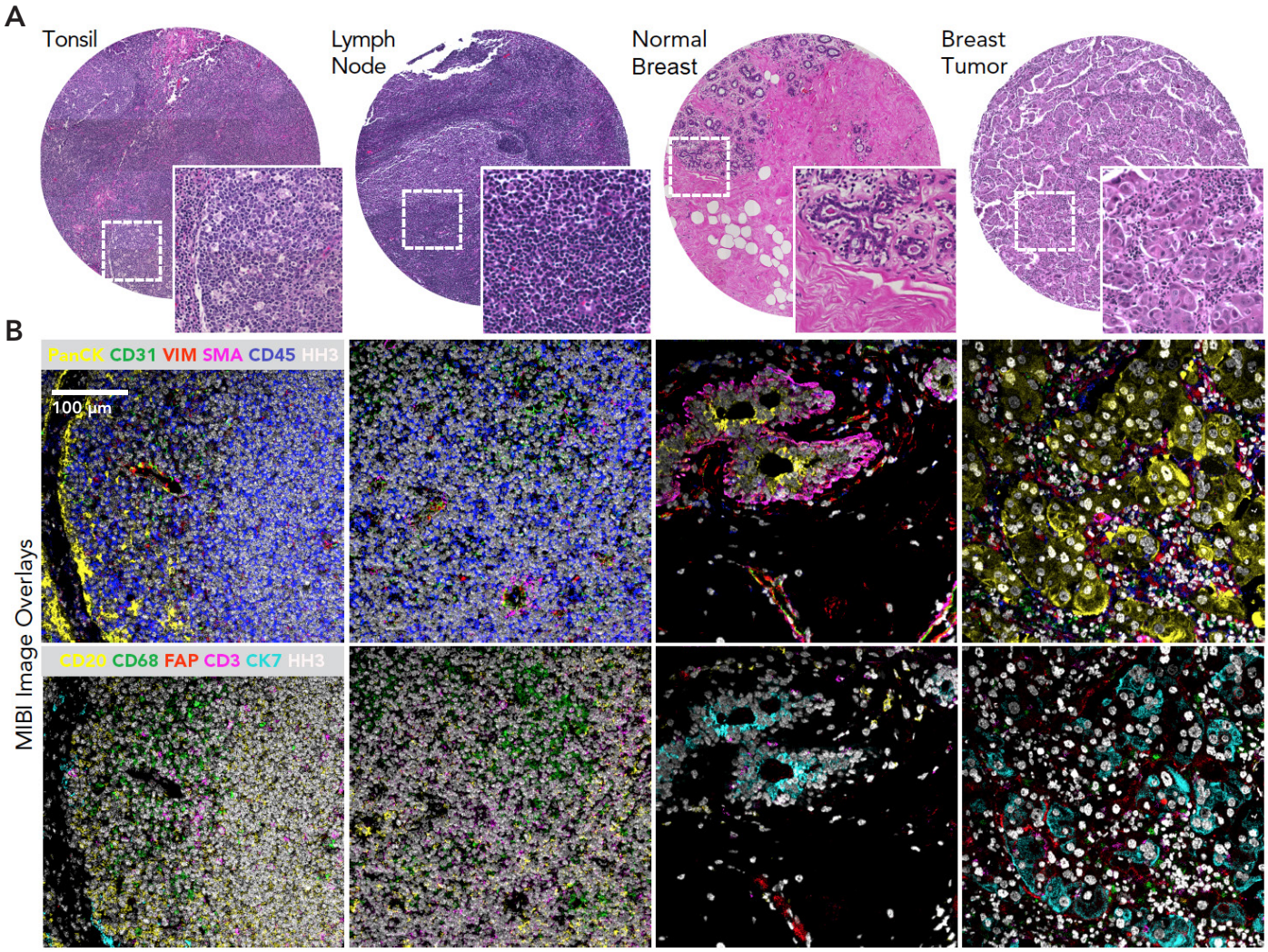

FIGURE 2: A) H&E staining of 4 different tissues types. B) MIBI image overlays for the same tissue types showing PanCK (yellow), CD31 (green), VIM (red), SMA (magenta), CD45 (blue), HH3 (white) markers for top section and CD20 (yellow), CD68 (green), CD3 (magenta), CK7 (blue), FAP (red), HH3 (white) markers for bottom section. Images in Panel B are all at the same magnification.

To acquire images, a primary ion beam is rastered across regions of interest on the stained tissue. This process releases the associated metal-isotope mass reporters from their respective antibodies, as well as the many meaningful tissue-endogenous metals, in a pixel-by-pixel manner. Ionized metal-isotopes are then detected by a time-of-flight mass spectrometer. Mass reporter abundance is mapped to each pixel to generate multiplexed images of the abundance of each marker across the imaged tissue region. Imaging data is captured in a single tissue scan and sputters only nanometers of stained tissue, allowing users to perform additional re-scans of remaining tissue or multi-depth scans to provide information in the Z-dimension.

Compatibility with Diverse Archival Tissues

The MIBIscope platform is compatible with a wide range of formalin-fixed, paraffin embedded (FFPE) tissue types, enabling interrogation of a spectrum of human diseases and developmental processes (**Figure 2**). It is also compatible with fixed frozen tissue as well as tissue from model systems using species-specific antibody reagents.

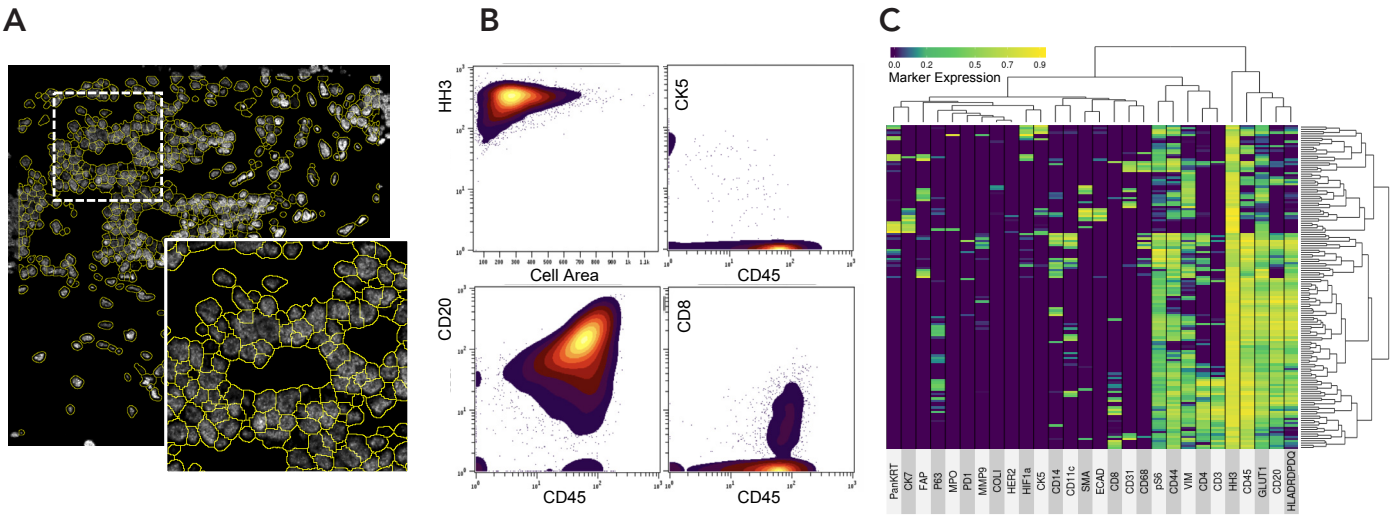

FIGURE 3: A) Segmented cells and their boundaries are shown in a normal breast tissue sample. Here a convolutional neural network (DeepCell) was trained to identify individual nuclei using the expression of histone H3. B) Output common to flow cytometry sorting (FCS) generated from the single-cell segmentation can be readily used by cytometry software to identify cell phenotypes through classic gating strategies. Bivariate plots from the tonsil are shown. C) Unsupervised clustering (FlowSOM) of all cells across all tissues is shown. Expression scaled to 99.9th percentile for each marker. By using the clustering of both markers and cells, populations with unique cell phenotypes can be annotated according to the expression of key lineage markers.

Single-Cell Segmentation and Phenotyping

The simultaneously captured images produced by the MIBIscope system may be visualized as single-channel images or can be overlaid in any combination to assess the phenotypic diversity and spatial organization of the tissue. By combining these visualizations with single-cell analyses, the true power of MIBI is realized to measure multiplexed marker expression and localization across the tissue microenvironment.

Quantitative single-cell information can be generated using readily available software packages including DeepCell, Halo, CellProfiler, QuPath, Fiji, and Visiopharm (**Figure 3**). Single-cell data generated from MIBIscope images can be analyzed and interpreted using approaches similar to those used for single-cell cytometry data. Such methods include cell phenotyping, enumeration and relative abundance of defined cell types, multi-dimensional clustering, dimensionality reduction, and more. These analyses can be performed using traditional approaches, such as drawing gates on histogram- or variate-plots, or through more recently developed machine learning approaches. Depending on the needs and resources of users, either commercial software platforms (e.g., Cytobank, FlowJo, FCS Express, etc.) or freely available bioinformatics packages developed with open-source programming languages (e.g., R, Python) can be used to analyze and interpret MIBI multiplexed imaging data.

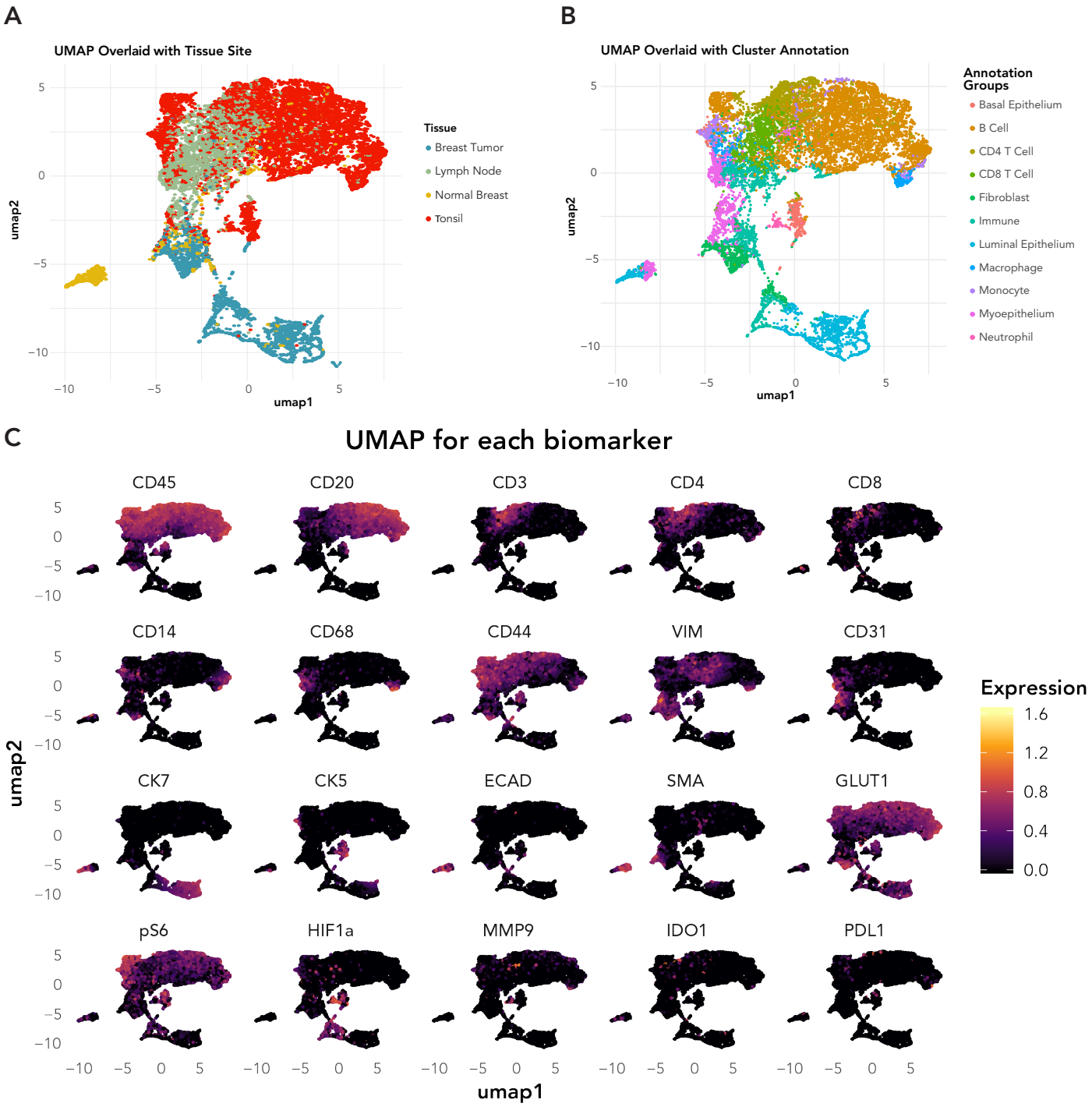

FIGURE 4: Uniform Manifold Approximation and Projection (UMAP) plots showing all combined cells from the imaged tonsils, lymph nodes, normal breast samples, and breast tumors. Cells are then colored by tissue of origin (top left), by annotated phenotype from FlowSOM metaclustering (top right), or by selecting protein marker expression (bottom).

Utilizing Data Dimensionality Reduction to Identify Important Phenotypes

To visualize how cells relate in high dimensional space, users can employ data dimensionality reduction tools such as tSNE or UMAP (**Figure 4**). Thus, for example, in a study where several cell types are simultaneously examined (e.g., T cells, B cells, endothelium, fibroblasts, etc.), one can quickly identify cell types and examine how the expression of functional markers varies among cell populations. This can be leveraged to identify phenotypes that are unique to distinct patient groups, outcomes, tissue sites, or other criteria.

Identifying Spatial Relationships Between Phenotypes

With its high-resolution tissue scans and single-cell phenotyping, MIBI enables detailed spatial analyses of the imaged tissue. Using approaches including nearest-neighbor calculations, researchers can assess the spatial relationships among cell phenotypes (e.g., tumor cell and immune cell proximity, **Figure 5**) or specific histologic features (e.g., tumor border vs. interior). This feature of multiplexed images acquired with the MIBIscope platform enabled Keren and co-authors to robustly characterize the spatial expression and organization of tumor and immune cells across a cohort of primary tissue samples from breast cancer patients (Keren *et al.*, *Cell*, 2018). Keren et al. identified tumors with varying degrees of tumor and immune cell mixing while additionally observing tumor-border specific cell phenotypes and expression of functional markers. This led the authors to observe an increased expression of histone modifications positively associated with transcriptional activity in tumor cells found in close proximity to the tumor-immune border, relative to that found in cells far from the border. Importantly, these applications highlight the ability to investigate the expression of multiple functional molecules in distinct cell types as a function of spatial location and/or relative distance to histologic features from a single MIBIscope image.

A typical MIBIscope image analysis workflow is illustrated in **Figure 6.** Investigators in this study were able to study the relationship between the expression of epithelial and mesenchymal molecules in tumor cells relative to their distances from the tumor-immune border.

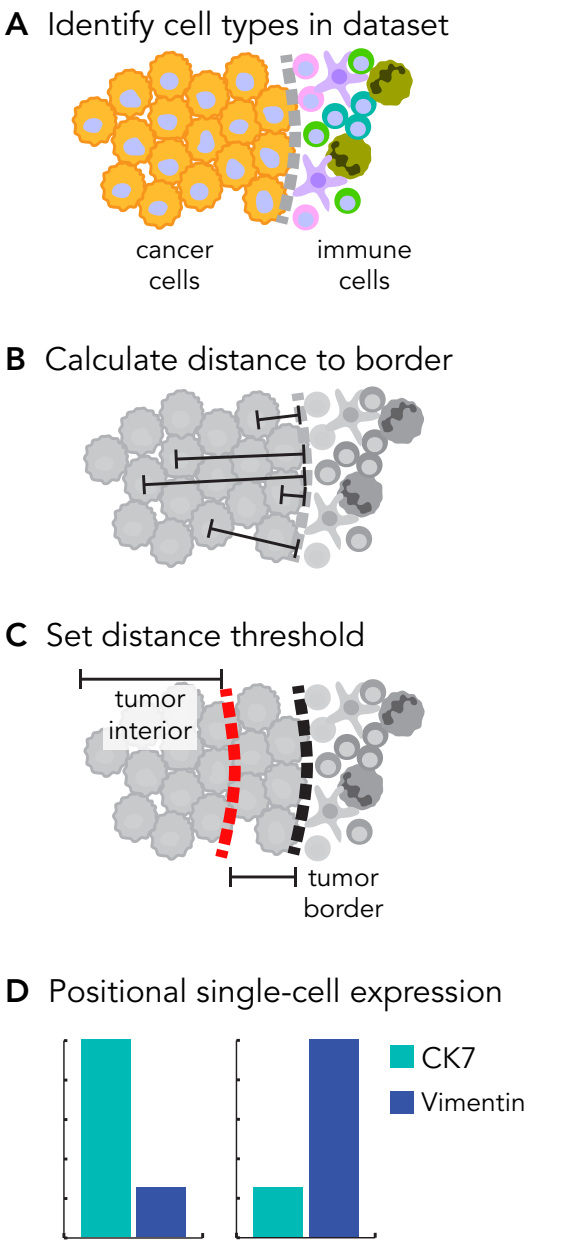

FIGURE 5: Tumor border spatial analysis. As demonstrated and performed by Keren, *et al.* (*Cell*, 2018), annotated single-cell data derived from multiplexed images is used to analyze spatially-restricted and cell-type specific biomarker expression. A) All cell types are annotated, and a tumor-immune interface is identified for the given field-of-view. B) Distance is calculated between each cell and the identified tumor border. C) User-selected distance threshold determines single cell spatial categories, 'tumor interior' or 'tumor border'. D) Single-cell expression compared across spatially defined sub-anatomical structures.

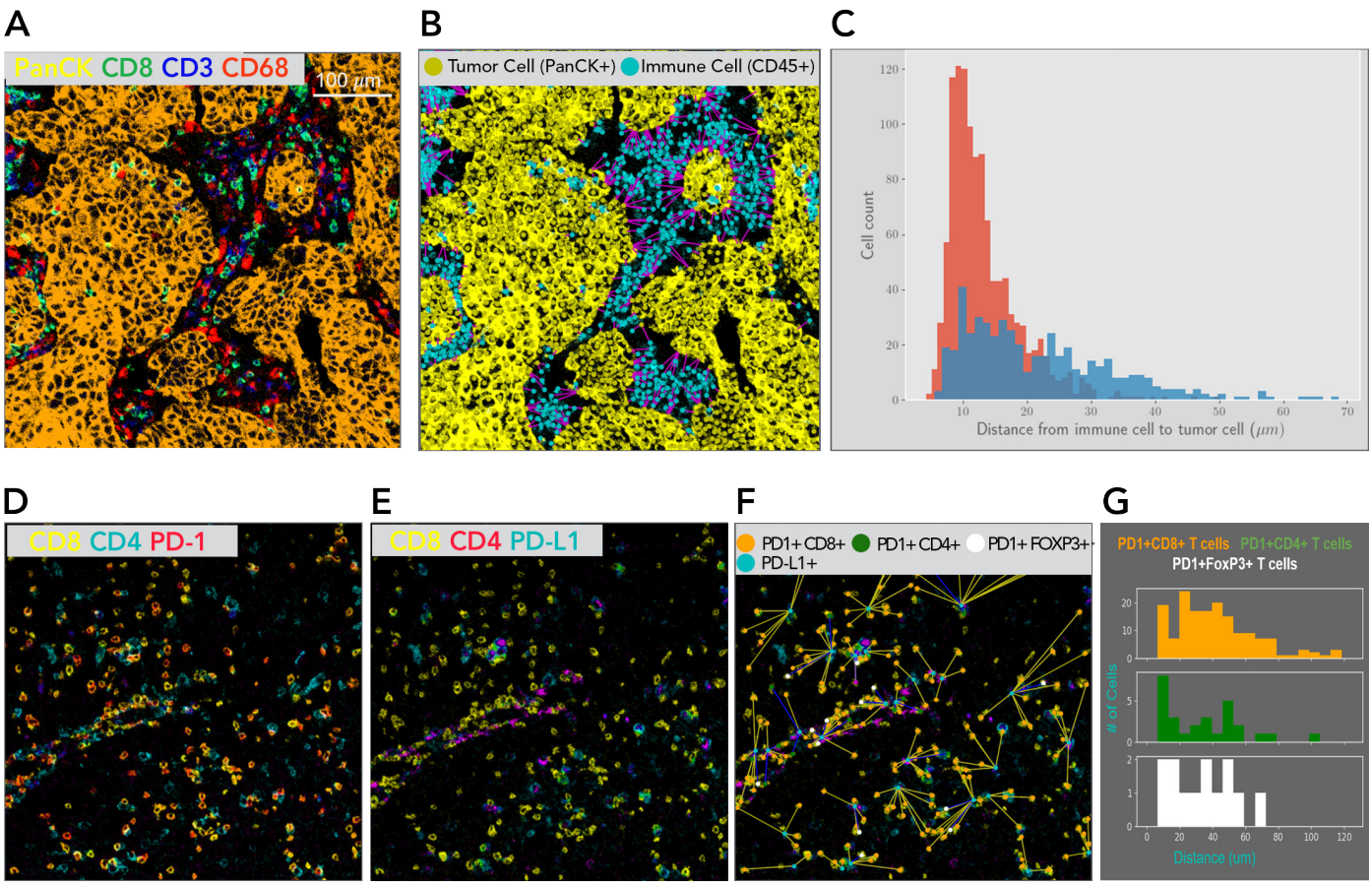

FIGURE 6: An ovarian serous carcinoma sample is shown in a four-color overlay representing a small subset of the markers within the high dimensional image (A), with distances calculated between immune cells and the nearest tumor cells (B), and histograms of tumor-immune cell distance between two different tumors are compared (C). On the bottom row, different immune cell populations from an urothelial carcinoma sample are shown in overlays (D, E) and the distances between PD-L1+ cells and PD1+ T cell subsets are shown analyzed (F, G).

Storage and Visualization of Data with MIBItracker

In order to facilitate data accessibility and storage of the multiplexed imaging datasets generated by the MIBIscope platform, IONpath developed MIBItracker—a powerful web-based software platform designed to process, visualize, and store MIBI data. MIBItracker also enables data management and collaboration with different access levels by user and project. A key feature of MIBItracker is the overlay display mode in which MIBIscope images can be evaluated and reviewed. Users can overlay various channels, select their pseudo-colors, and adjust their relative intensities for publication-ready visualization. Also, images can be exported as multi-layer TIFF files that can be analyzed in other software packages via compatibility with the Bio-Formats standard.

MIBI Image and Analysis Examples

Images from
Keren *et al.* (2018). A Structured Tumor-Immune Microenvironment in Triple Negative Breast Cancer Revealed by Multiplexed Ion Beam Imaging. *Cell* 174(6):1373-1387, E19,
are available in the MIBItracker demo.

Register for Free at
www.ionpath.com/mibitracker/

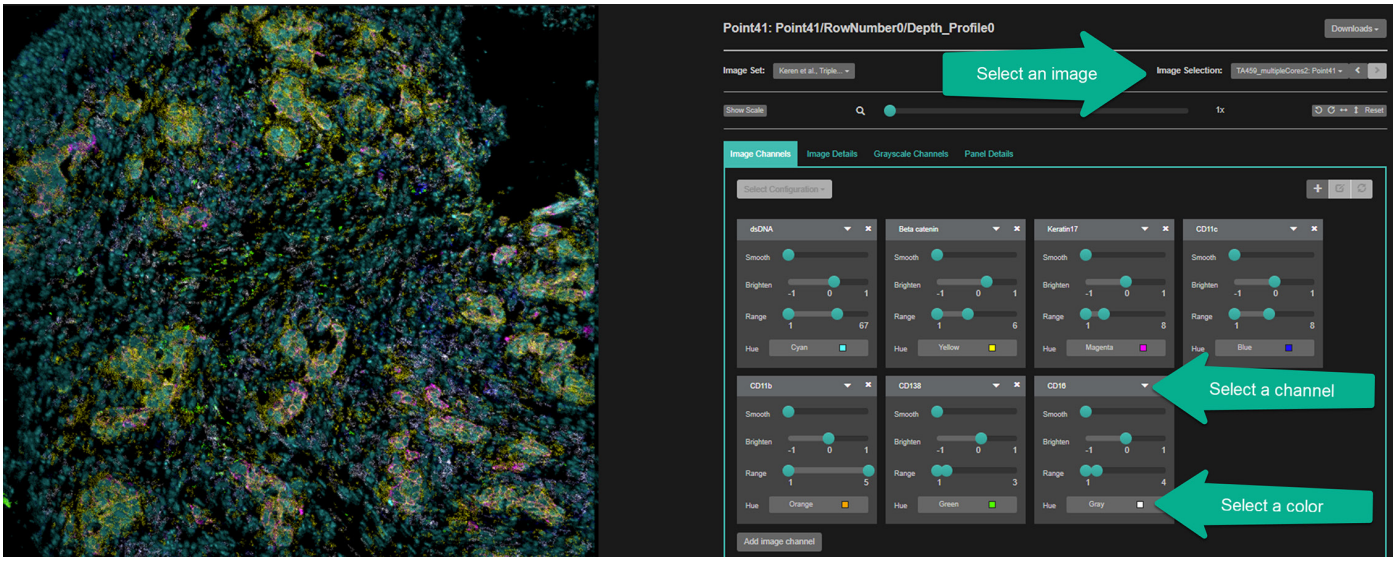

FIGURE 7: MIBItracker provides a software suite allowing image extraction, low-level processing, image assessment and storage, which grants users the ability to interact with their multiplexed image data and identify meaningful biology.

Summary and Future Directions

It is becoming increasingly evident that outcomes in disease and development are the culmination of a multitude of cellular interactions, driven by the presence and spatial relationships between distinct cell phenotypes from the diseased/developing tissue, the immune system, and tissue stroma. This has generated a previously unmet need for highly multiplexed imaging modalities that can obtain the resolution and spatial information of immunohistochemistry, but are not limited to the low-plex limitations of immunofluorescence techniques. The MIBIscope platform pairs the ease and familiarity of immunohistochemistry with the rigorous quantitation and precision of mass spectrometry. It generates high-resolution images that preserve the histology and single-cell spatial information in the tissue yet allows for highly multiplexed marker panel use (40+ markers), while still preserving the imaged tissue for further analyses.

The MIBIscope platform is compatible with a variety of diverse archival tissues and enables high-resolution, highly multiplexed images with key spatial information. The single-cell data produced from MIBI images is amenable with widely available single-cell phenotyping and spatial relationship analyses so that important discoveries in tissue biology can be further elicited. The current capabilities and future advancements of this technology make MIBIscope an unparalleled multiplexed imaging platform for scientific discovery.

Find out more at IONpath.com

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