Characterizing Immune and Cancer Cell Proximity and Distribution in the Tumor Microenvironment Using HALO® Spatial Analysis Tools

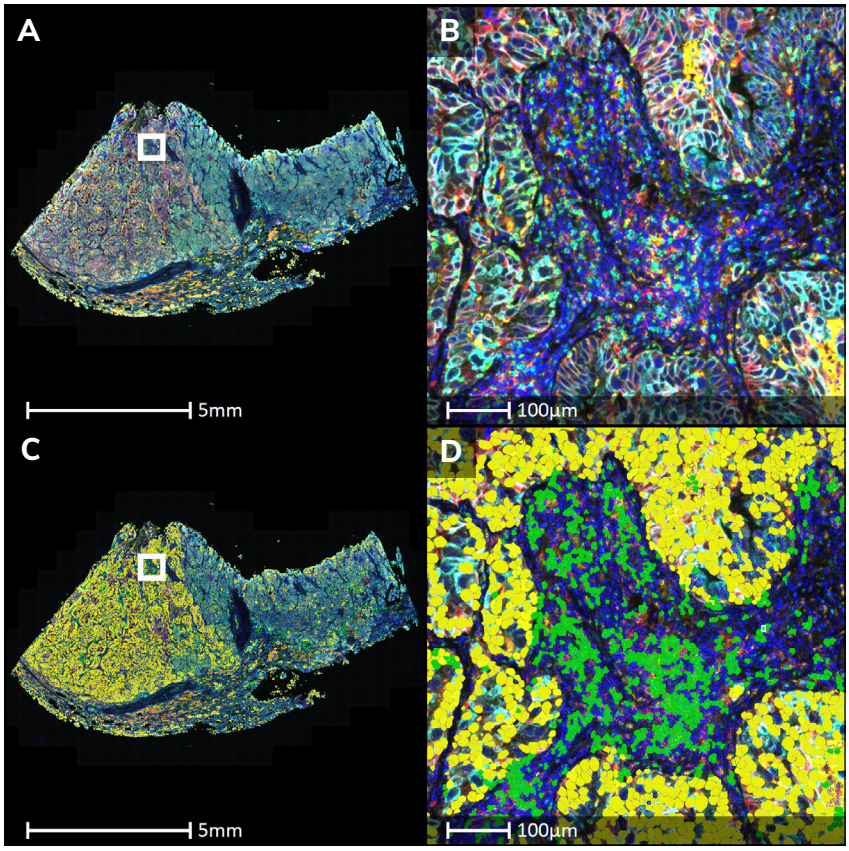

Figure 1. Phenotypic analysis of NSCLC sample. **A, B:** Whole slide image and magnified section of the slide, showing CD8 (green), CD68 (yellow), PD-L1 (red), Pan-CK (teal), and DAPI (blue). **C, D:** The same views are shown after analyzing cell phenotypes using the Highplex FL module, with cytotoxic immune cells in green and immune evasive carcinoma in yellow.

Introduction

Understanding how cells interact within the tumor microenvironment (TME) is essential for advancing cancer research and improving therapeutic strategies. In situ spatial analyses of cancer cells, immune infiltrates, and stromal components enable researchers to evaluate cell-cell interactions, map immune infiltration patterns, and identify immunologically active regions within the tissue context. Over roughly two decades, numerous studies have illustrated the promise of leveraging such spatial analyses to predict disease progression, immune evasion, and treatment response.[1,2,3]

From the study of immune infiltration in the TME to proximity analysis of microglia and amyloid plaques in the brain, the HALO® Spatial Analysis module supports in-depth spatial analysis across varied applications and detection methods. This module provides a suite of four algorithms—nearest neighbor, proximity, infiltration, and density heatmap—that identify proximity and relative spatial distribution of cells and objects across whole slide images, tissue boundaries, or serial sections. The algorithms can be used in conjunction with any cell- or object-based analysis module for brightfield or fluorescence.

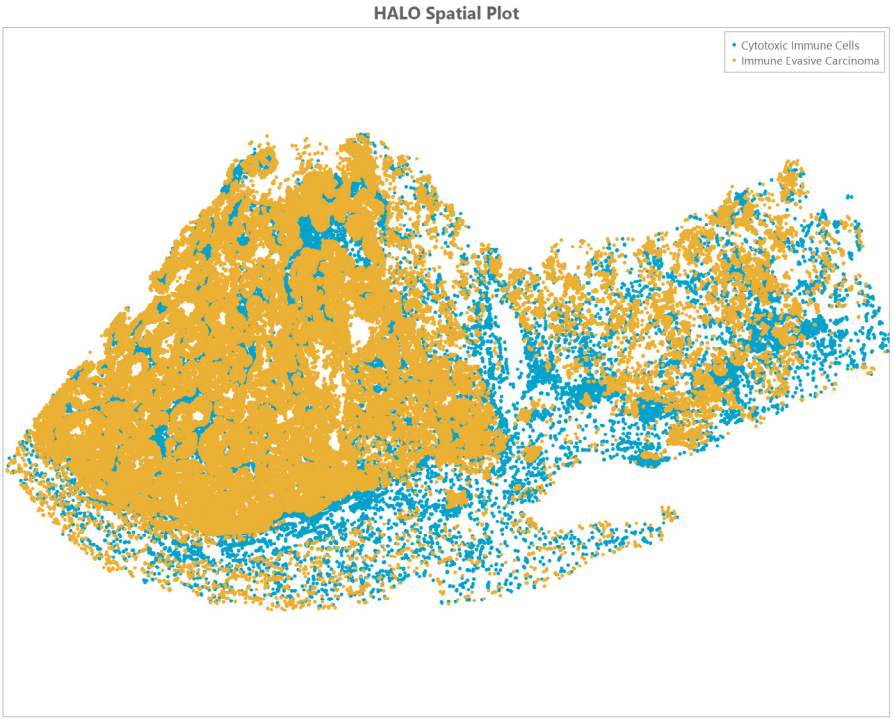

Figure 2. Spatial plot of phenotyped cells in NSCLC sample. Cytotoxic immune cells (teal dots) and immune evasive carcinoma (yellow dots) are plotted using their x-y coordinates in per-cell object data.

In this application note, we explore the four algorithms and walk through sample applications using these tools to analyze the distribution of immune and cancer cells in the TME of a non-small cell lung cancer (NSCLC) sample. While the tools included in the Spatial Analysis module can be used in myriad contexts, the discussion here highlights some of the common uses and considerations for each algorithm.

Nearest Neighbor

The nearest neighbor algorithm measures the spatial relationship between two defined cell or object populations by calculating the distance between each object in the first population and its closest counterpart in the second. This analysis provides two key metrics, the average distance between the populations and the number of unique neighbors. These metrics offer insight into how intermixed or segregated two populations are, such as assessing immune cell proximity to cancer cells or the extent of immune clustering. This algorithm is also useful as an initial step in a larger spatial analysis workflow, with its average distance output helping define the proximity or range parameters for subsequent Spatial Analysis algorithms.

For this study, an NSCLC sample stained with DAPI, probed for CD8, CD68, PD-L1, and pan-cytokeratin (Pan-CK) was first analyzed using the HALO Highplex FL module, as shown in **Figure 1 C**, **D**. Pretrained AI algorithms were used to identify cell nuclei and membranes, and biomarker positivity thresholds were set manually. Two phenotypes were defined, cytotoxic immune cells (CD8+) and immune evasive carcinoma cells (PD-L1+, Pan-CK+). Analysis was performed across the entire tissue and per-cell object data was generated, with the x-y coordinates of cells belonging to the two phenotypes mapped on a spatial plot, depicted in **Figure 2**.

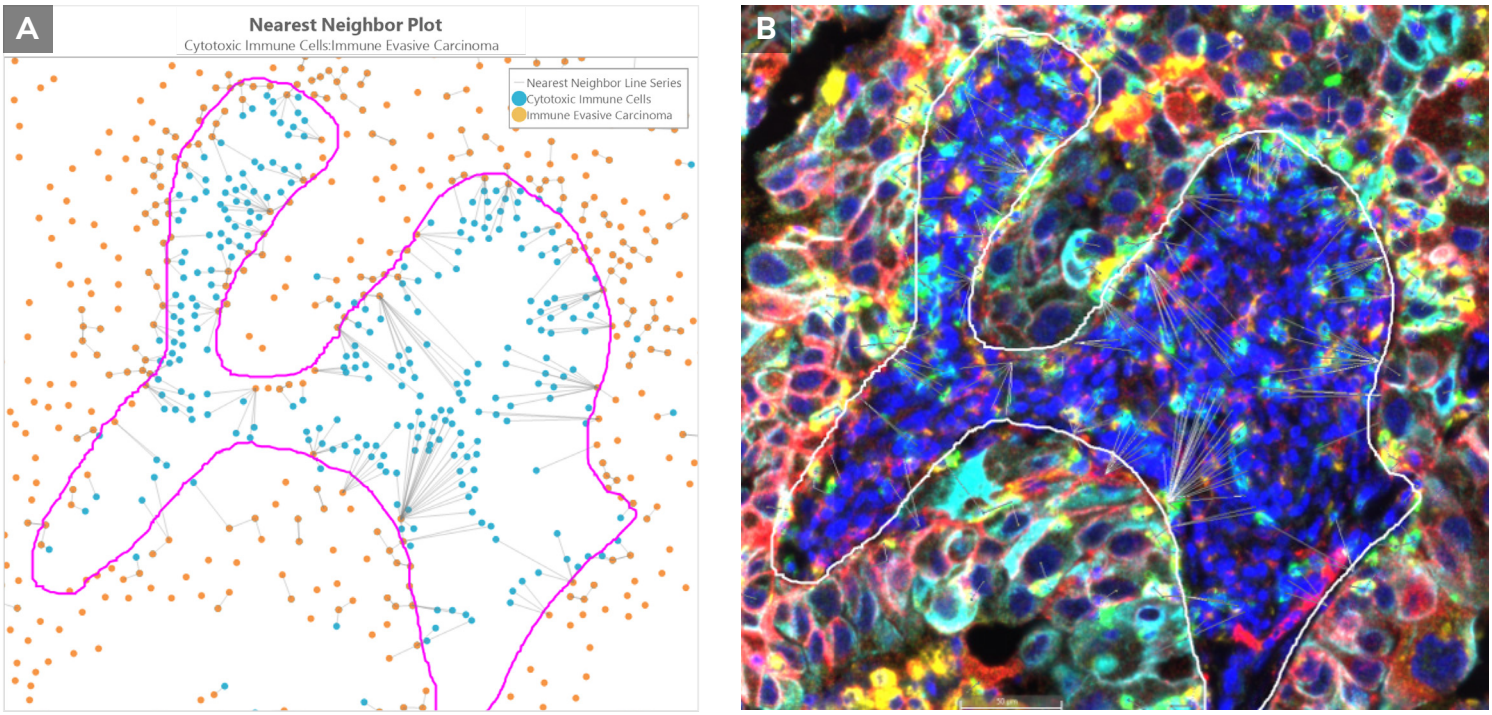

Figure 3. Nearest neighbor analysis of cytotoxic immune cells and immune evasive carcinoma. **A:** The spatial plot and **(B)** image markup show immune cells (teal dots) and cancer cells (yellow dots) and can assist with locating regions where immune cell numbers and/or proximity relative to cancer cells differ from the average. The tumor boundary is annotated in both images.

Analysis of the plotted phenotypes was subsequently performed using the nearest neighbor algorithm to measure the nearest immune evasive carcinoma cell to each cytotoxic immune cell. As with all Spatial Analysis module algorithms, this analysis produced a new spatial plot layer and image markup, shown in **Figure 3**, with a line connecting each immune cell and its nearest cancer cell. The calculated average distance from immune to cancer cells was 48.5 µm, with an average of approximately three cytotoxic immune cells per nearest neighboring immune evasive carcinoma cell.

Proximity Analysis

The proximity analysis algorithm quantifies the number of objects from one population that are within a defined radius of objects from a second population. This approach enables researchers to investigate how frequently specific cell types are found within a certain distance. The results are more granular than those provided by nearest neighbor analysis, offering a direct readout of

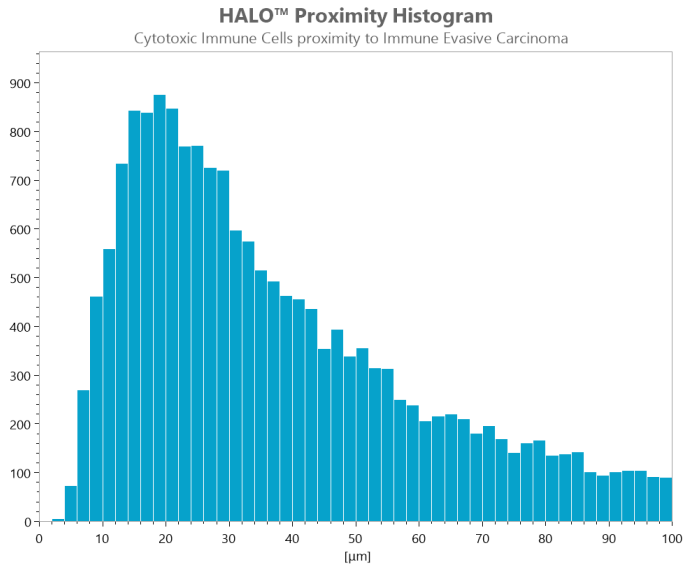

Figure 4. Proximity analysis of cytotoxic immune cells. CD8+ cells are binned according to their distance away from PD-L1+, Pan-CK+ cancer cells and show a peak before 20 µm.

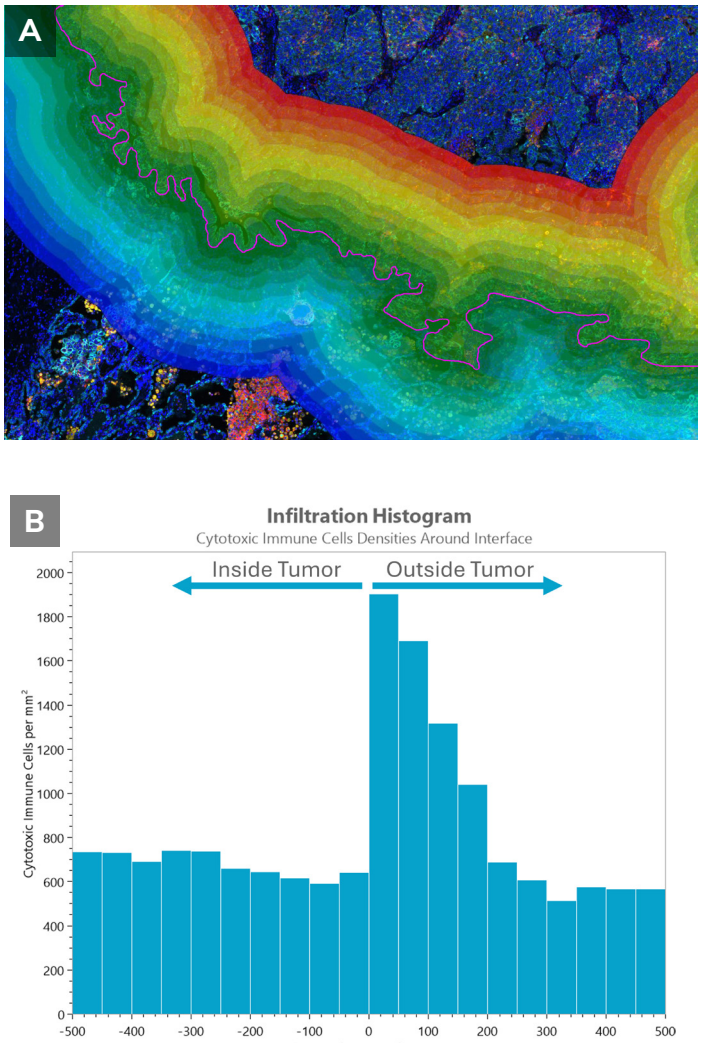

Figure 5. Infiltration analysis of cytotoxic immune cells. **A:** The "rainbow" markup along the annotated tumor boundary (in pink) enables quick review of the user-defined distance bands, which are 50 µm wide in this analysis. **B:** The infiltration histogram depicts the density of cytotoxic immune cells in each of the bands and shows a sharp increase on the exterior face of the boundary.

potential cell-cell interactions, and outputs include both a plot and proximity histogram. However, proximity analysis does require users to define a distance cutoff and the number of bands into which cells will be placed according to distance.

Common use cases include evaluating how many immune cells are located near cancer cells, how often T cells co-localize with antigen-presenting cells, or the spatial organization of marker-positive cells around vasculature. As with nearest neighbor analysis, proximity analysis requires that both populations of interest be labeled as well as detected using a HALO cell or object analysis module.

For this study, proximity analysis of the cytotoxic immune cells and immune evasive carcinoma cells in the NSCLC sample was performed. The algorithm quantified the total number of cytotoxic immune cells across the image, along with the number and percentage of cytotoxic immune cells within the specified distance (100 µm) of cancer cells and the average distance between the cell types. This analysis also produced the proximity histogram shown in **Figure 4**, where immune cells were binned into 50 distance bands, with a peak observed between 18 and 20 µm away from cancer cells.

Infiltration Analysis

The infiltration analysis algorithm quantifies the number of objects located within specified distances of an annotated boundary, such as a tumor margin or other region of interest. It is well suited for studying how immune cells penetrate or accumulate around tumor boundaries, streamlining analysis of the invasive margin. This method is widely used in immuno-oncology to evaluate relationships between immune cell density and tumor boundaries, as in the Immunoscore method.[1]

The infiltration analysis algorithm is annotation-based and does not require tumor-specific markers or dual-labeling, making it compatible with simple, single-marker workflows. Like the proximity analysis algorithm, infiltration analysis requires users to define a width for the invasive margin (or other boundary) and the number of distance bands into which cells will be placed.

In this study, the tumor boundary was manually drawn in the example NSCLC whole slide image using a pen annotation, as seen in **Figure 5A**. The

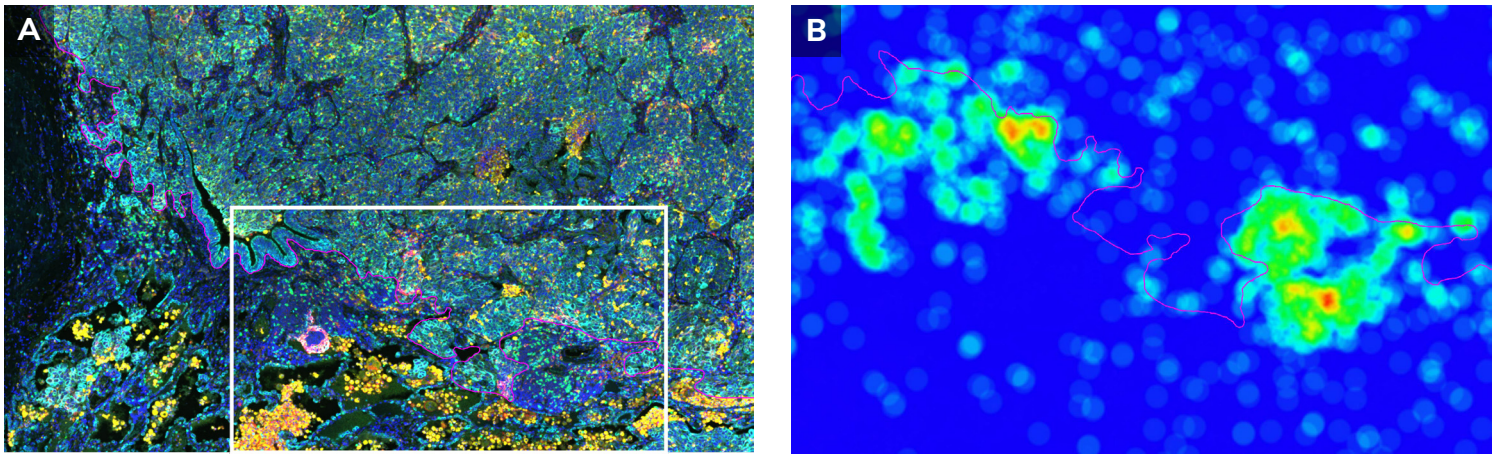

Figure 6. Density heatmap of cytotoxic immune cells at the tumor boundary. **A:** Along a portion of the tumor boundary shown in **Figure 4**, **(B)** two broad clusters of cytotoxic immune cells are visible.

infiltration analysis algorithm was then used to quantify cytotoxic immune cells within 500 µm of the boundary on each side, with cells divided into 20 bins based on distance to the boundary. The infiltration analysis produced the "rainbow" markup shown in **Figure 5A**, with differently colored distance bands, as well as the cell density histogram shown in **Figure 5B**. In our analysis, we observe the highest density of cytotoxic immune cells between 0 and 50 µm outside the tumor boundary, with a striking decline in immune cell density just inside the tumor.

Density Heatmaps

The density heatmap algorithm visualizes and quantifies the local density of a selected cell or object population across the tissue. It measures how tightly a population is clustered within a user-defined radius, revealing "hot spots" of activity that might indicate regions of immune activation or tumor aggression. Heatmaps are generated as image markups, where intensity reflects local object density, and can be quantified across entire images or within specific regions of interest. Users can also subdivide the maximum density value into a defined number of zones to generate annotations and summary data that captures the area and number of cells per density zone.

For this study, the density heatmap algorithm was used to measure cytotoxic immune cell density near the previously drawn tumor boundary in the NSCLC sample. The density radius was set to 25 µm for this analysis. As seen in **Figure 6**, the analysis markup shows areas of dense immune cell concentration at or near the exterior face of the tumor boundary, paralleling the quantitative results from the infiltration analysis. Additionally, the algorithm summary data noted a maximum of 36 cytotoxic immune cells within the defined 25 µm radius.

Conclusions

As spatial biology continues to shape our understanding of tissue organization, disease progression, and immune dynamics, the need to extract meaningful spatial insights from tissue images is only increasing. In this application note, we explored the analysis toolkit available in HALO's Spatial Analysis module and demonstrated how these algorithms enable robust, reproducible spatial analysis workflows that can drive discovery. Seamlessly integrated with the image segmentation and phenotyping capabilities of other HALO modules, the Spatial Analysis module empowers researchers to unlock new insights in spatial biology.

References

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