

# Quantifying immune cell distribution in the tumor micro-environment using HALO™ spatial analysis tools

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## INTRODUCTION

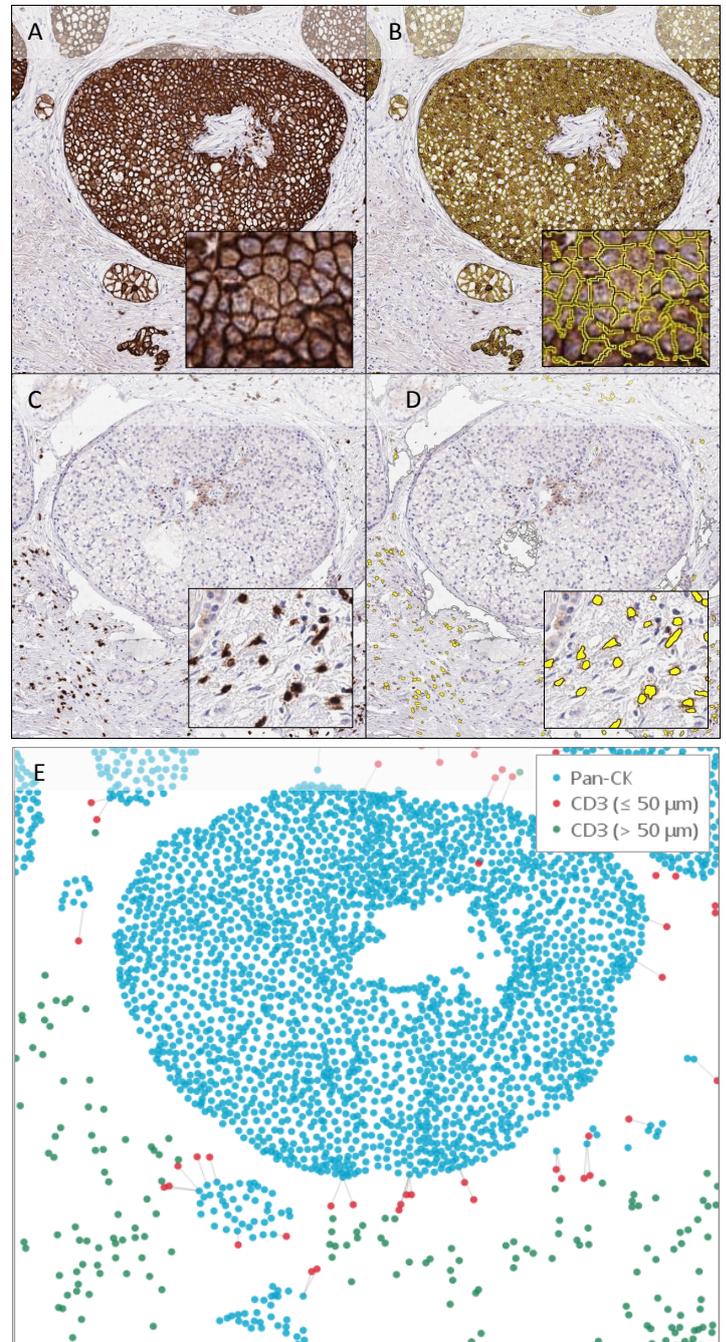
A positive correlation between survival and T-cell density in the tumor core and invasive margin was first reported in colorectal cancer (Galon, J. *et al.*, 2006). Independent investigations have confirmed the predictive value of 'immune contexture' with respect to survival (as reviewed by Fridman, W.H. *et al.*, 2011). Using HALO™ to analyze immunohistochemically (IHC)-labelled and immunofluorescently (IF)-labelled immune cell markers, Tumeh *et al.* reported that CD8+ cell density at the invasive margin could predict response to anti-PD1 therapy in aggressive melanoma (2014). These studies highlight the importance of technologies which facilitate *in situ*, spatially-resolved analysis of immune markers to enable our understanding of the complex interactions between the immune system and the tumor, and ultimately for predicting patient survival and selecting appropriate treatments.

In this application note, we describe two HALO analysis tools that are used to quantify the distribution of immune cells relative to tumor cells in the tumor microenvironment, **Proximity Analysis** and **Tumor Infiltration Analysis**. The analysis tool used ultimately depends on the staining methods employed, and more importantly, on the research objectives and outputs required as described in more detail in the following sections.

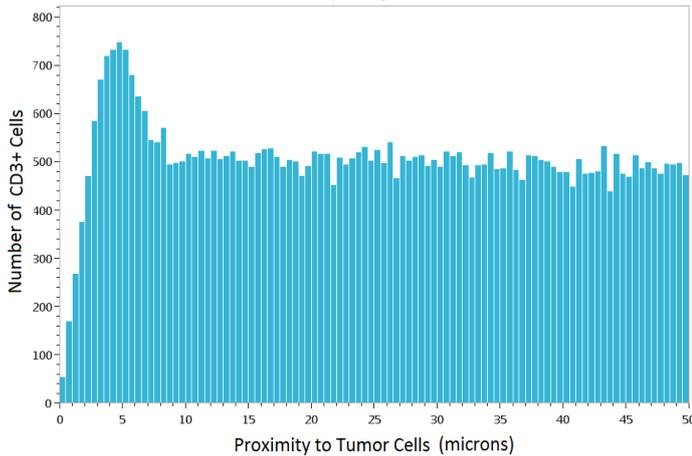
## PROXIMITY ANALYSIS

Proximity analysis is used to quantify the spatial relationship between any two cell or object populations detected using HALO. In immuno-oncology, the primary applications for proximity analysis are to 1) count number of cells labelled with an immune cell marker (e.g. CD8, CD3, or CD4) within a certain distance of tumor cells (identified with tumor-specific marker) and 2) measure the distance between the two cell populations. Immune cells and tumor cells can be stained together on a single section using dual IHC, multiplex IHC, or multiplex IF or can be stained separately in parallel using serial sections.

An example of proximity analysis using serial sections from pancreatic tumor is shown in Figure 1. Here, the two serial section images probed individually for the tumor marker pan-cytokeratin (Pan-CK) and T-cell marker CD3 are aligned using whole-slide elastic registration. The Pan-CK-stained section is analyzed with membrane IHC and the CD3-stained section is analyzed with cytonuclear IHC to identify stained cells and map their x-y coordinates on each tissue section. Next, the x-y



**Figure 1. Proximity analysis of CD3+ and Pan-cytokeratin+ cells across serial sections.** Representative areas of pan-cytokeratin (Pan-CK) (A and B) and CD3 staining (C and D) with and without analysis mark-up are shown. Stain-positive cells are labelled yellow in mark-up images (see 20x inset images). E) Pan-CK+ tumor cells (blue) and CD3+ cells are plotted spatially and analyzed with proximity analysis tool. CD3+ cells within 50 μm of Pan-CK+ tumor cells are labelled red and CD3+ cells more than 50 μm from Pan-CK+ tumor cells are labelled green in spatial plot.



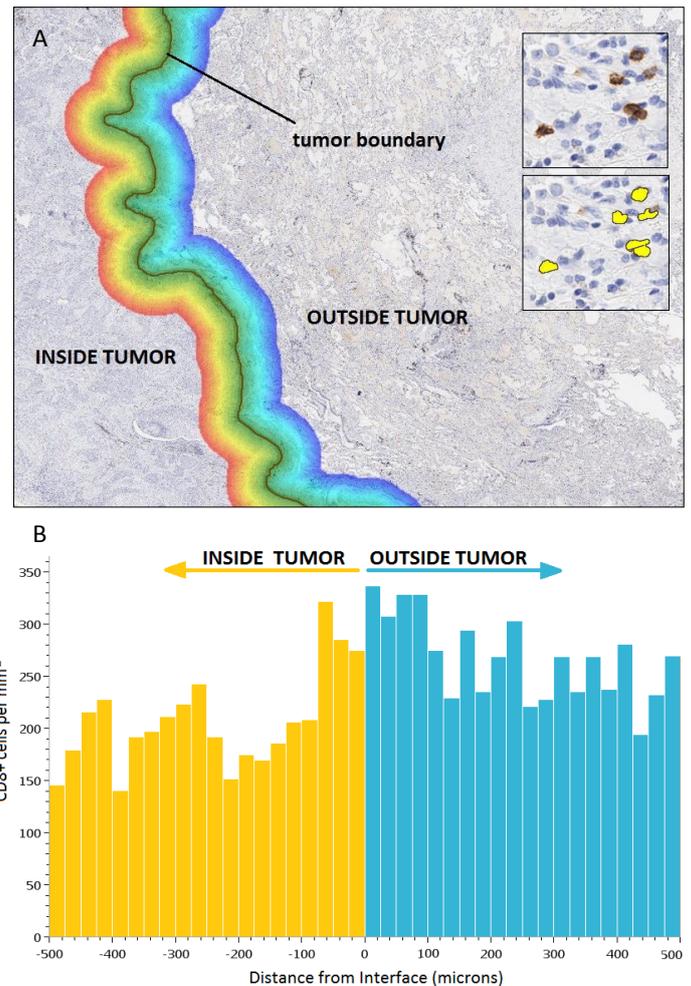
**Figure 2. Immune cell proximity histogram.** CD3+ cell number binned according to distance away from Pan-CK+ tumor cells.

coordinates and underlying elastic registration are used to align the cell populations on a common spatial plot as shown in Figure 1E. Once the cell data is plotted, proximity analysis is used to quantify total CD3+ cell counts across the image, number of CD3+ cells within specified proximity distance of tumor cells (50  $\mu\text{m}$ ), percentage of all CD3+ cells within proximity distance, and average distance between CD3+ and tumor cells. The cell counts are binned according to distance and a histogram is automatically generated, as shown in Figure 2. In this example, a peak in CD3+ cell counts is observed between 4.5 and 5  $\mu\text{m}$  away from tumor cells.

## TUMOR INFILTRATION ANALYSIS

Several publications have described a correlation between immune cell density at the tumor invasive margin (IM) and survival or therapeutic response (reviewed in Fridman et al, 2011; Tumeh et al., 2014), including the landmark study describing the all-important ‘immunoscore’ (Galon, J. *et al.*, 2006). The tumor infiltration analysis tool was created to streamline analysis of the invasive margin. Unlike proximity analysis, tumor cell analysis is not necessary with this tool, so there is no requirement for a tumor-specific marker, serial sections, dual IHC staining or multiplexing.

In Figure 3, invasive margin analysis with the tumor infiltration tool is demonstrated on a CD8-stained section from lung cancer. As with proximity analysis, the image is first analyzed with cytonuclear IHC to identify and map the x-y location of each CD8+ cell in the section. The tumor boundary is manually drawn using a pen annotation as shown in Figure 3A. The invasive margin on either side of the annotation line is highlighted with a ‘rainbow’ mark-up. In this example, the invasive margin is defined as 500  $\mu\text{m}$  inside and 500  $\mu\text{m}$  outside of the tumor boundary; however, different inside and outside margins can be specified by the user. Cells within the invasive margin are



**Figure 3. Infiltration analysis of CD8+ cell density around the invasive tumor margin.** A) Section analysed with cytonuclear IHC to identify CD8+ cells (20x inset images, with and without analysis mark-up, ). The invasive ‘tumor boundary’ is manually annotated using HALO annotation tools. CD8+ cells are counted within the invasive margin, 500  $\mu\text{m}$  inside and 500  $\mu\text{m}$  outside of tumor as identified with rainbow mark-up. B) CD8+ cell density is binned according to distance from the margin and a 40 bin histogram is generated. The middle of the histogram is the tumor boundary, to the left is inside tumor (orange bars), and to the right is outside tumor (blue bars).

counted and binned according to distance from the tumor boundary in order to generate a cell density histogram as shown in Figure 3B. Here we observe that the density of CD8+ cells is highest 0 to 100  $\mu\text{m}$  inside and outside of the invasive margin. CD8+ cell density decreases significantly as you move toward the tumor core and overall CD8+ cell density is higher outside of the tumor.

## REFERENCES

- Galon, J, *et al.* Science. 2006; 313: 1960-4.
- Fridman, W.H., *et al.* Nat Rev Cancer. 2012; 12:298-306
- Tumeh, P.C., *et al.* Nature. 2014; 515:568-71

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