

# Fusion of Spectrally Unmixed Image Tiles from PerkinElmer® Vectra™ 3 or Polaris™ into Whole Slide Tifs to Enable Analysis of the Tumor Microenvironment

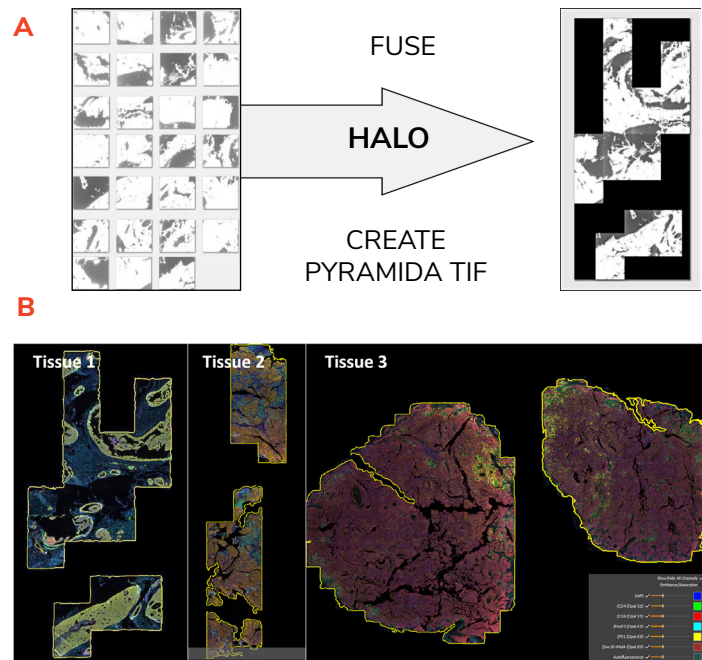
## INTRODUCTION

Numerous investigations have identified a correlation between patient outcomes, such as survival or response to immunotherapy, and the density of specific immune cell subtypes within the tumor and within the invasive margin around the tumor.<sup>1,2,3</sup> Immune cell subtypes express a specific biomarker or combination of biomarkers. As the list of relevant biomarkers increases, so grows the need for multiplexing technologies. The PerkinElmer® Vectra™ 3 and Polaris™ multispectral systems have become a popular choice in immuno-oncology. While these platforms allow up to seven biomarkers to be probed on a single tissue, only field-of-view image tiles can be captured for spectral unmixing. While some quantitative endpoints can be extracted using these image tiles, they are not amenable to automated or manual annotation of the tumor boundary as required for analysis of the invasive margin.

In this study, we describe a tiling method using HALO® which converts image tiles captured using the PerkinElmer Vectra 3 or Polaris platform into pyramidal, whole slide images that are spectrally unmixed and amendable to annotation and analysis. We validate the method by comparing cell-based image analysis results from individual tiles to the fused whole slide image. Finally, we analyze the spatial distribution of immune cells in these whole slide images within the tumor core and at the invasive margin using a novel infiltration analysis tool.

## METHODS

**Validation Study.** Three formalin-fixed paraffin embedded tissue slides probed using PerkinElmer Opal Discovery 7 kits utilizing different antibody combinations were used for the validation study. All three tissues were imaged using the PerkinElmer Vectra 3 imaging platform and the resulting .im3 image tiles were spectrally unmixed and exported as component tif image tiles using PerkinElmer's Inform™ software. Component tif image tiles from the same tissue were fused together using the x-y coordinates for each image and the resulting fused image converted to a pyramidal tif format for faster viewing using HALO (Figure 1A). Tissue 1 and tissue 2 are composed of 27 component tif image tiles,



**Figure 1. Image fusion using HALO.** A) Image tiles covering a large tissue area captured using the Vectra™ 3 system were exported from Inform after unmixing as component tif files. These unmixed FOV images were then imported into HALO where they were fused and a new pyramidal tif file was created. The black areas represent FOVs that were not included/captured. B) Tissues 1, 2, and 3 included in the validation study as viewable at 1x magnification in HALO.

tissue 3 is composed of 393 component tif image tiles. The final fused images as viewed in HALO are shown in Figure 1B. After fusion, the images were analyzed in HALO using Highplex FL module (cell-based analysis) and results compared to the individual component tif image tiles as outlined in Figure 2A.

**Spatial Analysis.** As shown in Figure 3B, a tissue classifier to separate tumor from stroma was created using the random forest classifier (tissue classifier add-on) in HALO. Classified tumor regions were converted to annotation regions. HALO's Highplex FL module was used to identify all CD8+ cell (Figure 3C) and the infiltration analysis tool which is part of the spatial analysis module was used to calculate CD8+ cell densities 100 µm inside and 100 µm outside of the annotated tumor boundary (Figure 3D-E).

## RESULTS

To validate the current fusion method, the number of cells and cells positive for each marker were compared between the fused tif and component tifs. For tissue

3, 2.14% fewer total cells and 1.62% fewer tumor cells were counted in the fused tif compared to the sum of all cells counted in the 393 component tifs, as shown in **Figure 2B**. A similar result was obtained for tissue 2 (**Figure 2C**). Overall the differences range from -2.14% to 1.62% and on average 0.43% fewer cells were counted in the fused image which is within the range of acceptability. Reduced cell numbers in the fused image is most likely due to overcounting of cells at the boundary of image tiles which can be visualized by lining up tiles side-by-side (**Figure 2D**).

Once fused, these spectrally-unmixed whole slide images are amenable to analysis with HALO's invasive margin tool as shown in **Figure 3**. While the tumor boundary can be manually drawn, in this case we automate the process by employing a train-by-example tissue classifier to identify tumor areas and then convert these to annotations. We then quantified the density of CD8+ immune cells within the invasive margin, 100  $\mu\text{m}$  inside and 100  $\mu\text{m}$  outside of the annotated tumor boundary using HALO's infiltration analysis tool. The histogram shows an accumulation of cells outside of the tumor boundary.

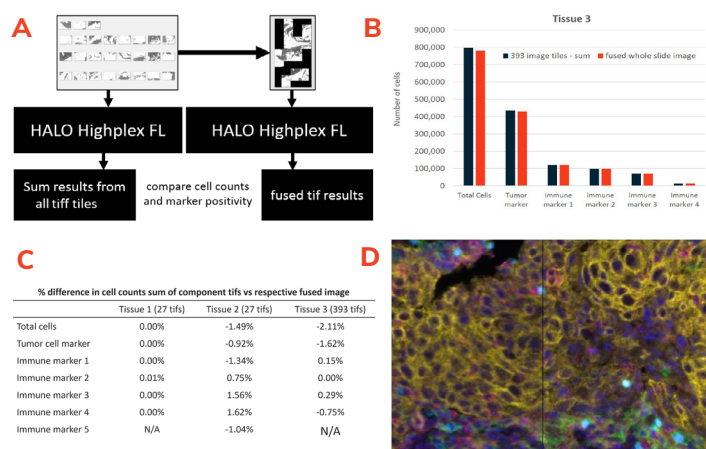
## CONCLUSIONS

In this study, we demonstrate a method for converting individual, field-of-view component tifs generated from the PerkinElmer Vectra or Polaris system and converting them into whole slide spectrally unmixed images using HALO. A small reduction in cell counts is observed in the fused image compared to the sum of the individual component tifs. Unlike the component tif images, these new spectrally-unmixed whole slide images are amenable to invasive margin analysis, an important application in immuno-oncology applications such as the immunoscore®.

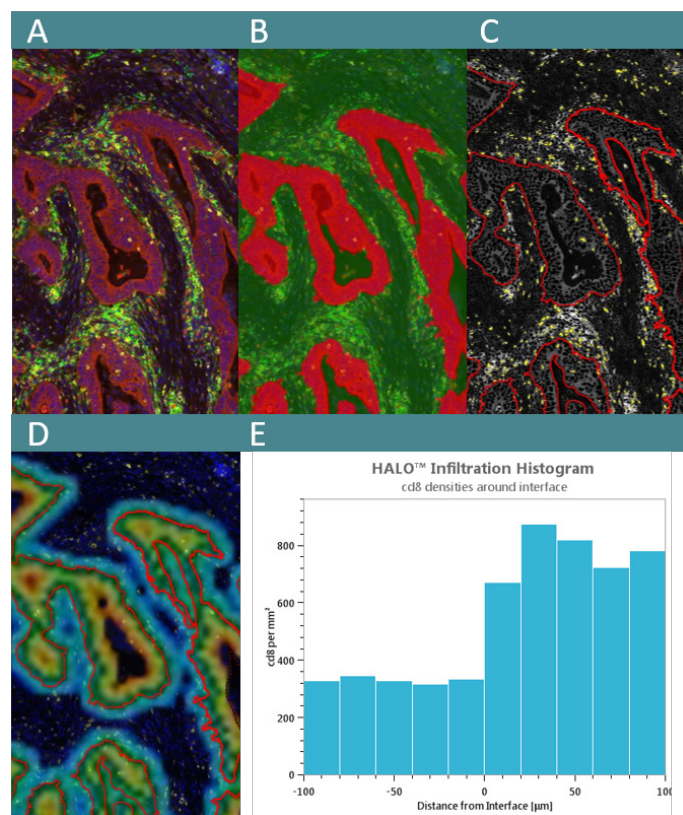
## REFERENCES

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**Figure 2. Validation results using HALO.** **A)** Validation workflow. **B)** Total number of cells and total cells positive for each marker, sum of 393 image tiles (blue bar) that compose tissue 3 and fused pyramidal whole slide tif of tissue 3 (orange). **C)** Percentage difference number of cells in fused image vs. sum of all component tifs for tissues 1, 2, and 3. **D)** Two neighboring image tiles where cells are artificially segmented (see blue cell).



**Figure 3. Spatial analysis using HALO.** **A)** Original image **B)** Classifier segments tumor (red) from other tissue (green). **C)** Image is analyzed to identify all immune cells of interest (here CD8+ cells). **D)** CD8+ cell density is counted 100  $\mu\text{m}$  inside and 100  $\mu\text{m}$  outside the tumor boundary (rainbow colored area); tumor boundary is identified by annotation. **E)** The density data is binned and used to create the histogram shown here. 0 is the tumor boundary,  $>0 \mu\text{m}$  is area outside the tumor up to 100  $\mu\text{m}$  and  $<0$  is area inside the tumor up to -100  $\mu\text{m}$ .

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