indica labs

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Converting spectrally unmixed image tiles from PerkinElmer® Vectra™ 3 or Polaris™ into pyramidal whole slide tifs to enable immune cell quantification within the tumor and invasive margin

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.[1,2,3] 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 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. To facilitate faster viewing of the whole slide image, the resulting fused image was converted to a pyramidal tif format using HALO™ as outlined in Figure 1A-B. Tissue 1 and tissue 2 were created from fusion of 27 component tif image tiles and took approximately 1 minute each to fuse; Tissue 3 is composed of 393 component tif image tiles and took approximately 20 minutes to fuse. The final fused images as viewed in HALO are shown in Figure 1C. After fusion, the images were subjected to image analysis in HALO using Highplex FL module (cell-based analysis) and results were 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 as shown in Figure 3C. HALO's Highplex FL module was used to identify all CD8+ cell (Figure 3D) 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 3E-F).

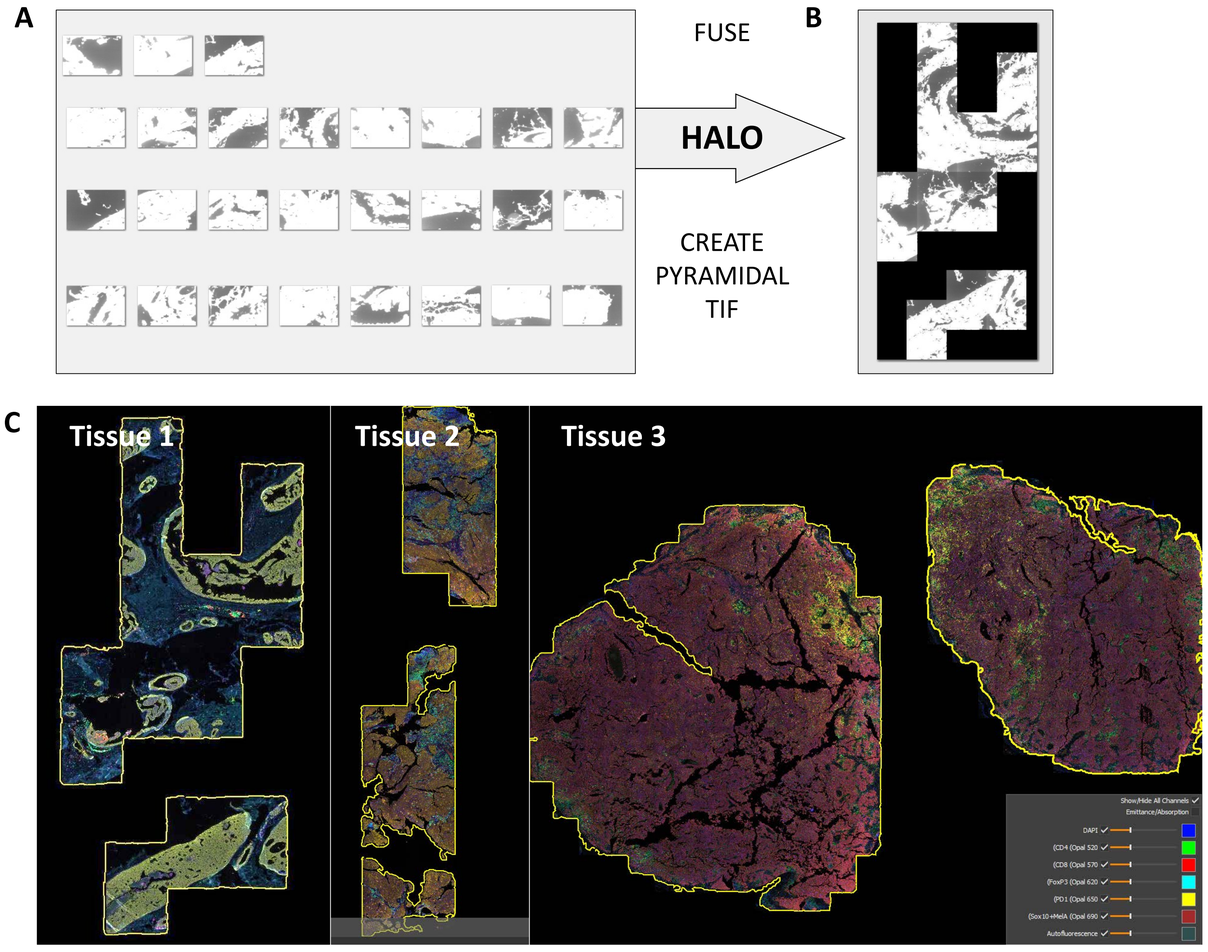
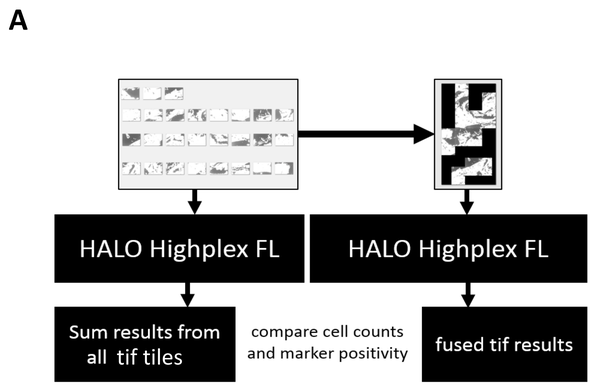
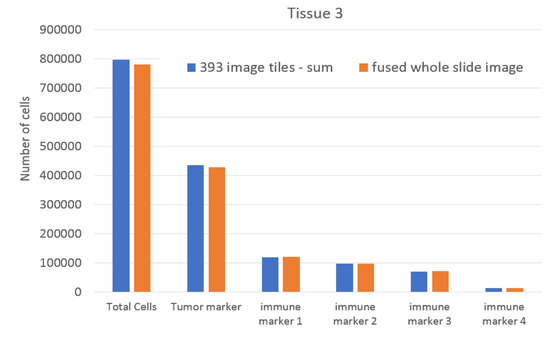


Figure 1. Image fusion using HALO. A) Image tiles covering a large tissue area captured using the Vectra™ 3 system were exported from Inform after unmixing as component tif files. B) These unmixed FOV images were then imported into HALO where they were fused and a new pyramidal tif file was created.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

Results

To validate the current fusion method, the total number of total cells and cells positive for each marker were compared between the fused tif and its component tif images. 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





C

% difference in cell counts sum of component tifs vs. respective fused image

	Tissue 1 (27 tifs)	Tissue 2 (27 tifs)	Tissue 3 (393 tifs)
Total cells	0.00%	-1.49%	-2.11%
Tumor cell marker	0.00%	-0.92%	-1.62%
immune marker 1	0.00%	-1.34%	0.15%
immune marker 2	0.01%	0.75%	0.00%
immune marker 3	0.00%	1.56%	0.29%
immune marker 4	0.00%	1.62%	-0.75%
immune marker 5	N/A	-1.04%	N/A

D

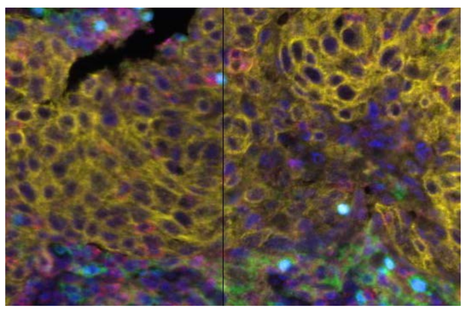


Figure 2. Validation results using HALO. A) Validation workflow. B) Total number of cells and total cells positive for each marker, sum of 393 image tiles (blue bar) that compose tissue 3 and fused pyramidal whole slide tif of tissue 3 (orange). C) Percentage difference number of cells in fused image 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).

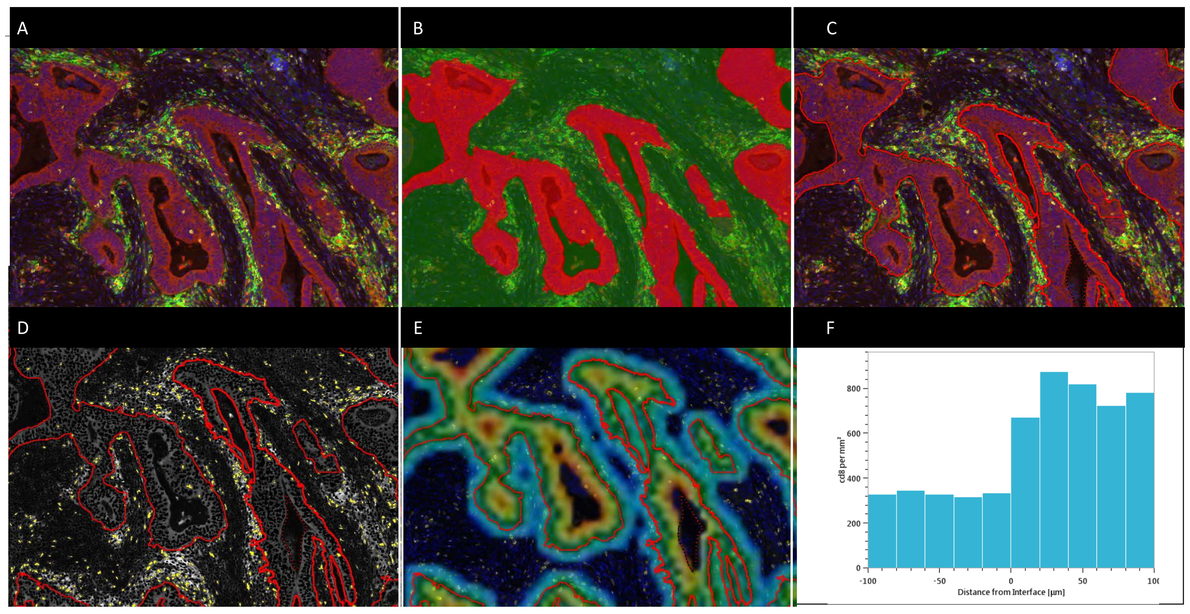


Figure 3. Spatial analysis using HALO. A) Original image B) Classifier segments tumor (red) from other tissue (green). C) Classified tumor regions are converted to annotations. D) Image is analyzed to identify all immune cells of interest (here CD8+ cells). D) CD8+ cell density is counted 100 µm inside and 100 µm 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 µm is area outside the tumor up to 100 µm and <0 is area inside the tumor up to -100 µm.

which is within the range of acceptability. Reduced cell numbers in the fused image is most likely due to overcounting of cells that fall at the boundary of the image tiles which can be easily 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 µm inside and 100 µm outside of the annotated tumor boundary using HALO's infiltration analysis tool. The invasive margin distance can be set by the user depending on the tumor type and application. The histogram shows an accumulation of cells outside of the tumor boundary. A separate analysis was performed with invasive margin 500 µm inside and 500 µm outside of the tumor boundary and a peak accumulation of CD8+ cells was observed at 175 µm outside of the tumor (data not shown).

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, most likely due to reduction in the number of cells that are double counted at the border between image tiles. 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

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

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