Sequential same slide multiplex immunofluorescence and H&E staining for combined phenotypic and morphologic characterization of formalin-fixed paraffin-embedded tissue sections

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BACKGROUND

Multiplex immunofluorescence (mIF) has become an established method for characterizing the tumor immune microenvironment. Currently, hematoxylin and eosin (H&E) staining is typically performed on a serial tissue section to provide morphologic context for the mIF data. Serial sections do not represent identical cell populations, however, and are not always possible to obtain when the amount of available tissue is limited. Consequently, a method to examine the same tissue section by both fluorescence and brightfield imaging would be advantageous. Here we present a streamlined method and system for serial mIF and H&E staining on a single tissue slide for a comprehensive analysis to support high-throughput tissue immunophenotyping.

METHODS

The UltiMapper® I/O PD-L1 kit was used for multiplex immunofluorescence staining of CD8, CD68, PD-L1, and pan-Cytokeratin in formalin-fixed, paraffin-embedded (FFPE) samples from 3 serial sections of human tonsil and primary colon and lung tumor biopsies using the Leica® Biosystems BOND RX autostainer. Stained tissues were imaged in five spectrally distinct fluorescence channels (DAPI, FITC, TRITC, Cy5, Cy7) on the RareCyte CyteFinder® II HT Instrument. Slides were de-coverslipped and stained with H&E, then imaged with brightfield using the CyteFinder II HT instrument. To segment the tumor and stroma tissue regions, a HALO® AI classifier was created for the lung and colon H&E images. Fluorescence images were analyzed using the HALO Highplex FL module to identify CD8+ cytotoxic T-cells, CD68+ macrophages, CD68+/PD-L1+ immuno-suppressive macrophages, pan-CK+ tumor cells, and pan-CK+/PD-L1+ immune-evading tumor cells within the tumor and stromal regions identified by the H&E stain. As a comparison, a classifier was also trained on the fluorescent CK and DAPI signal.

RESULTS

The serial sections from each tissue type demonstrate the expected variability in numbers of cells. The classifier trained on the H&E images was able to distinguish between tumor and stroma regions of tissue equally as well as the classifier trained on the fluorescent cytokeratin and DAPI signal. PD-L1 expression was localized to the expected tumor and crypt areas of the lung tissue.

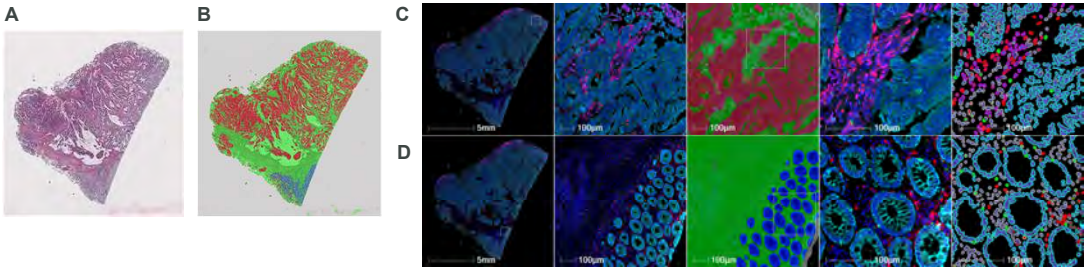

Figure 3. Colon tissue classified from H&E image

(**A**) H&E image of colon tissue (**B**) classified into tumor epithelium (red), stroma (green), and normal epithelium (blue) regions. (**C**) Tumor region and (**D**) normal region of the fluorescence image shown in closeup and segmented with color-coded phenotypes outlines: CK-positive (cyan), CD8-positive (green), CD68-positive (red), PD-L1-positive (pink), negative cytoplasm (grey).

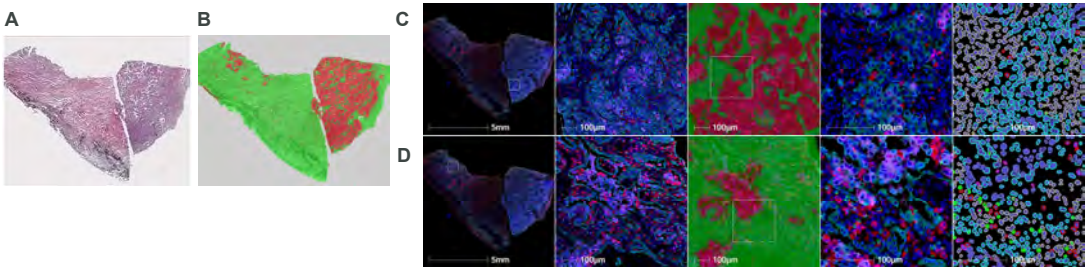

Figure 4. Lung tissue classified from H&E image

(**A**) H&E image of lung tissue (**B**) classified into tumor epithelium (red), and stroma (green). (**C**) Tumor region and (**D**) stromal region of the fluorescence image shown in closeup and segmented with color-coded phenotypes outlines: CK-positive (cyan), CD8-positive (green), CD68-positive (red), PD-L1-positive (pink), negative cytoplasm (grey).

***Figure 1*: Sequential staining and analysis workflow**

Stain slides with the UltiMapper I/O PD-L1 kit on the Leica BOND RX

Image fluorescence on the CyteFinder II HT Instrument

Decoverslip and stain with H&E

Image brightfield on the CyteFinder II HT Instrument

Align H&E image to fluorescence image using the Serial Stain Registration tool in HALO

Classify tumor and stroma regions from H&E image using HALO AI

Phenotype and quantify cell types within regions using HALO Highplex FL

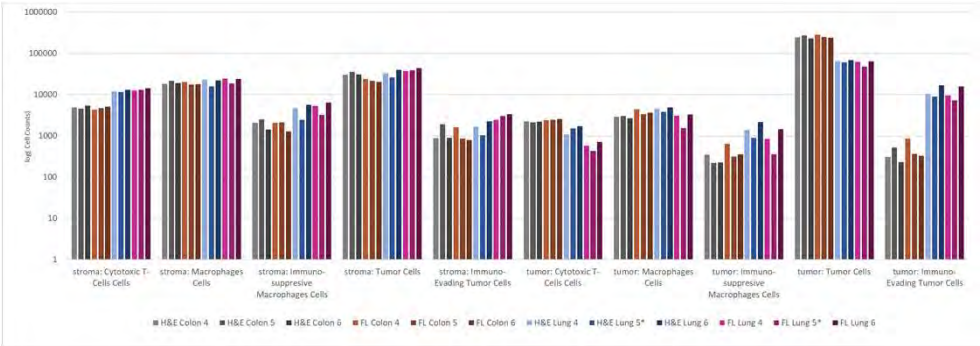

Figure 2. Classifier Comparison

Cell counts/region comparing a classifier trained on the H&E image vs the fluorescence CK+DAPI signal are comparable. Both classifiers provide equivalent results, indicating that the tumor marker could be dropped from the fluorescence panel if using the H&E for tissue architecture classification. *Lung 6 tumor/stroma segmentation was applied for Lung 5, since the tissue was damaged during decoverslipping.

CONCLUSIONS

- Here we demonstrate a tissue-preserving workflow to generate H&E images from a slide that is previous stained and imaged in fluorescence on the CyteFinder II HT Instrument.
- These H&E images can be used to delineate tissue architectural regions such as tumor and stroma, eliminating the need for a tumor-specific biomarker in the fluorescence panel.
- H&E trained classifiers perform equivalently to classifiers trained on fluorescent signal from DAPI and pan-CK.
- The UltiMapper I/O PD-L1 kit produces very reproducible staining results across multiple serial sections.
- HALO Highplex FL can be used to identify phenotypic cell populations and expression levels within tissue architectural regions.

CONTACT INFORMATION

- To learn more about UltiMapper I/O kits, contact Katir Patel at katir.patel@ultivue.com
- To learn more about the CyteFinder II HT Instrument, contact Kyla Teplitz at kteplitz@rarecyte.com
- To learn more about HALO image analysis workflows, contact Anne Hellebust at ahellebust@indicalab.com