Introduction of a robust workflow for the whole-side acquisition and co-registration of multiplex immunofluorescence tissue images for analysis of a 9-color, 8-marker immunophenotyping assay

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Background

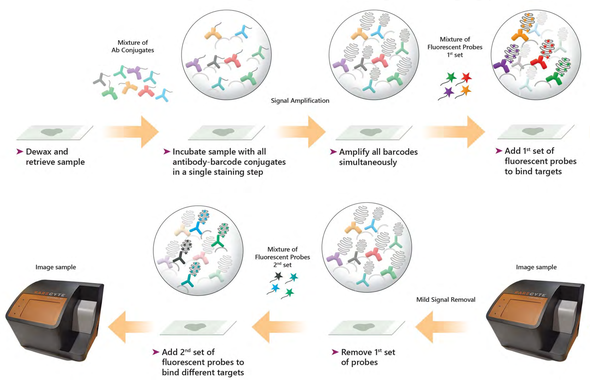
Profiling multiple cell types in tissue samples requires more markers than can be supported by conventional approaches to immunohistochemistry. InSituPlex® multiplex immunofluorescence (mIF) technology enables biomarker multiplexing in tissue samples with an automated workflow to support discovery of immune cell signatures in the tumor immune microenvironment (TiME). Here we have designed and tested an 8-plex immunophenotyping panel using the InSituPlex approach to detect and classify T cells, macrophages, and tumor cell in non-small cell lung cancer (NSCLC) and colorectal cancer (CRC) FFPE tissue.

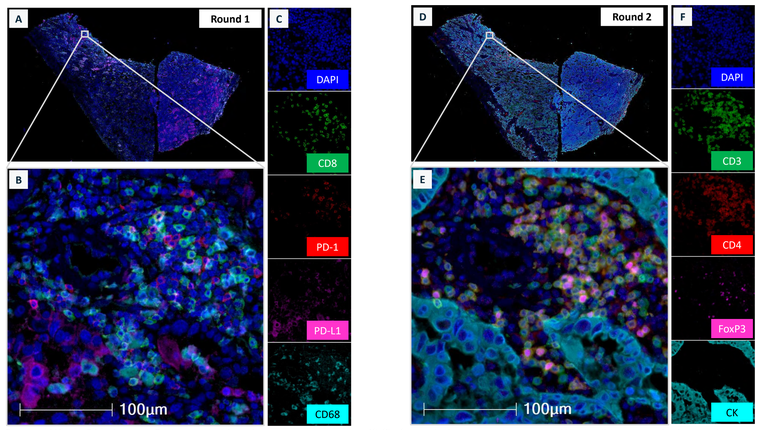
Methods

Stained with UltiMapper™ I/O mIHC Assays and Imaged with CyteFinder® II HT Instrument

The UltiMapper I/O Immuno8 assay was developed to stain FFPE tissue, including the colorectal cancer, non-small cell lung cancer, and human tonsil tissue used in this experiment. The Immuno8 kit staining is all done in 1 run on a Leica Bond Automated Stainer. All 8 antibodies are stained at the same time, and all antibody barcodes are amplified at the same time. Complementary imaging probes are introduced and hybridize with their respective DNA barcodes (set of 4) and imaged with the RareCyte CyteFinder® II HT slide scanner.

After round 1 of imaging, the complementary imaging probes are dehybridized and replaced with a new set of imaging probes that hybridize with DNA barcodes to antibodies 5-8 and imaged with the RareCyte CyteFinder II HT slide scanner. A visual diagram of the staining and imaging procedure is shown below.



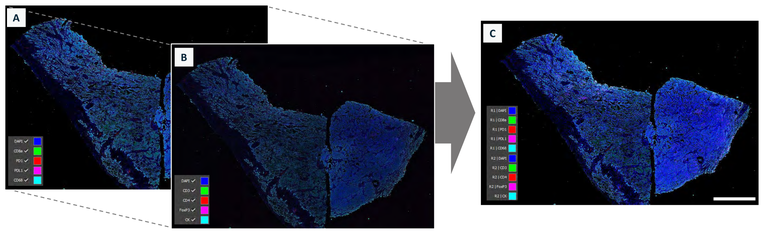


Scanned Whole Slide Images: Each round of imaging generated an ome.tiff file for each tissue sample scanned. Whole slide composite images for one of the non-small cell lung cancer tissue sections are shown above (A,D). A a single region of interest is also shown (B,E) to highlight the staining of each of the 8 antibody assay targets and the corresponding DAPI staining (C,F).

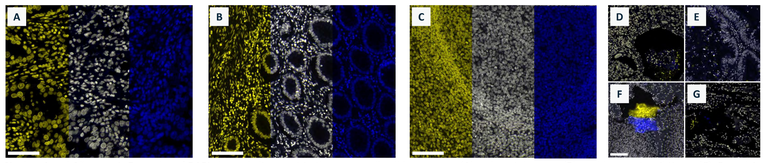
Analyzed with HALO®

Image analysis was conducted with HALO 3.1 software.

Serial Stain Registration: The two ome.tiff images from rounds 1 and 2 of staining were aligned using the Serial Stain Registration tool to align the slides on a cellular level. Once registration was completed, the aligned slides were fused into a single 10-channel slide with the Serial Stain/Section module for further analysis.

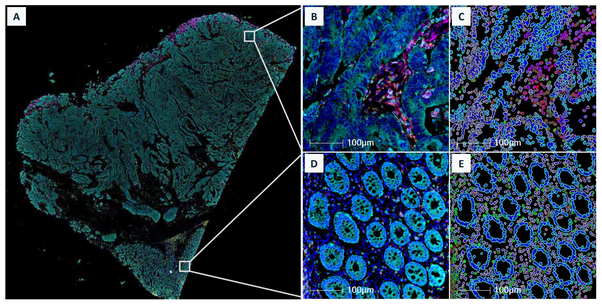


Fusing Serial Stained Images: After image registration was performed to account for any tissue deformations, the image from the first round of staining (A) and the image from the second round of staining (B) were fused into a single image file with 10 channels (C). Scale bar is 2mm.

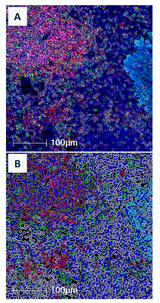


Nuclear Level Alignment: Examples of DAPI channel alignment in the non-small cell lung cancer (A), colorectal cancer (B), and human tonsil tissue (C). Round 1 DAPI channel shown in blue and Round 2 DAPI channel shown in yellow. Well aligned nuclei display as white. Misaligned nuclei show as blue or yellow and were mainly seen in areas of tissue that was disturbed between rounds of imaging resulting in rinsed off cellular debris in necrotic areas (D, E) and small movements or fold in the tissue (F,G). Scale bars are 100µm.

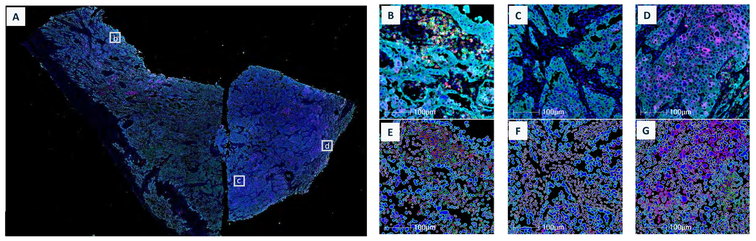
Cellular Analysis: The HighPlex FL module was used to analyze the nuclear, cytoplasm, or membrane biomarkers. Nuclear detection was based on the DAPI signal from round 2 to ensure only cells that made it through both rounds of staining were quantified. This module segments individual cells and outputs summary (per image) and object (per cell) data.



Colorectal Cancer Segmentation: 8Plex colorectal tissue slide at lowest magnification (A) and 10X for an area of colorectal cancer (B) and normal colorectal tissue (D). The corresponding cell segmentation masks (C, E) with the markup colors of the cell nucleus and cytoplasm indicating positivity.



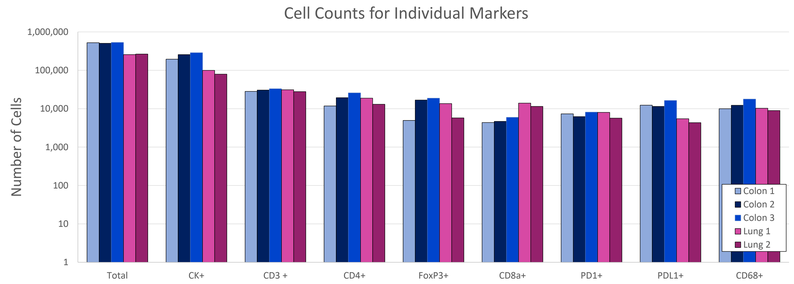
Tonsil Segmentation: Human tonsil control tissue slide at 10X (A) with a corresponding cell segmentation masks (B).



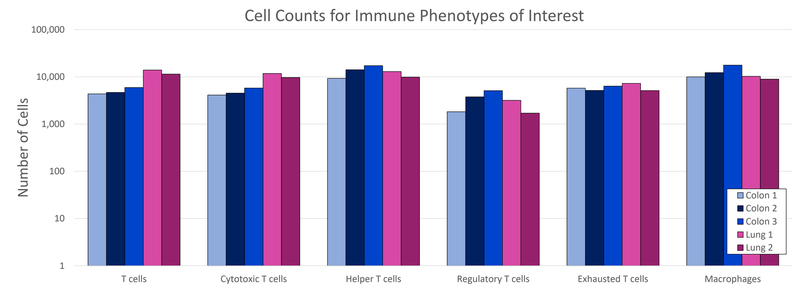
Non-small Cell Lung Cancer Segmentation: 8Plex colorectal tissue slide at lowest magnification (A) and multiple 10X areas of colorectal cancer (B-D) to illustrate the heterogeneity of staining patterns across this single piece of tissue. The corresponding cell segmentation masks (E-G) with the markup colors of the cell nucleus and cytoplasm indicating positivity. Scale bars are 100µm.

Results

Co-registration of two 4-plex imaging rounds and fusing into a single image file allowed for the seamless cell phenotyping of markers across imaging rounds. The 8-plex assay and workflow is compatible across multiple tumor sample types. The samples were characterized based on binned expression and co-expression of biomarkers. The following major cell phenotypes were detected: PD-L1 checkpoint expression, T cells, cytotoxic T cells, T-helper cells, regulatory T-cells, exhausted T cells, macrophages, and tumor cells.



Positive cell counts: The number of cells positive for each individual maker is displayed for each of the three samples of non-small cell lung cancer and human tonsil tissue. Sample Lung 3 was excluded from the results due to low antigen retrieval during the automated staining process.



Positive Immune Phenotypes: The number of cells positive for each individual marker is displayed for each kit and tissue type in the graphs on the left. Sample Lung 3 was excluded from the results due to low antigen retrieval during the automated staining process.

Phenotype Definitions: T cells: CD3+; Cytotoxic T cells: CD3+ & CD8+; Helper T cells: CD3+ & CD4+; Regulatory T cells: CD3+, CD4+, & FoxP3+; Exhausted T cells: CD3+ & PD-1+; Macrophages: CD68+

Conclusions

- Here we demonstrate a workflow to generate an 8-marker fluorescence image from a slide that is cyclically stained and imaged.
- The UltiMapper I/O Immuno8 kit produced reproducible staining across multiple serial sections in multiple tissue types.
- HALO allows for cellular level alignment and fusing of serially stained images to generate a single fluorescence image composed of 10 channels.
- HALO HighPlex FL module can be used to identify phenotypic cell populations and expression levels.
- The 8-plex assay workflow involving InSituPlex mIF, CyteFinder® II HT scanner, and HALO analysis supports the ability to measure biomarkers in situ in streamlined and scalable approach.

Contact Information

- To learn more about UltiMapper 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

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