# Quantitative evaluation of tumor tissues using a combined fluorescence in situ hybridization and immunofluorescence assay

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## INTRODUCTION

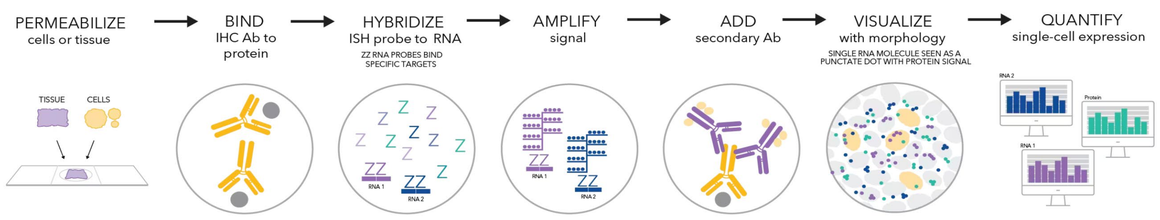
Understanding the complex interactions between tumor, immune, and stromal cells in the tumor microenvironment (TME) is essential for understanding how patients respond to treatments. Understanding the spatial interactions between various cell types in the TME is also crucial for implementing successful immunotherapy strategies against various cancer types, however, patient samples are very precious and often need to be conserved. To enable researchers to acquire more data with a single sample, ACD has developed an RNA and Protein Co-Detection workflow to enable the simultaneous interrogation of cell-type specific expression of multiple genes and the identification of protein biomarkers using immunofluorescence (IF).

Using ACD's multi-omic Co-Detection workflow, a section of a tissue microarray containing breast and lung cancer tissue was stained with target probes for *PanCK, PD1*, and *CTLA4*, as well as for the protein biomarker PD-L1. A second section was stained with target probes for *TNFA, CCR5*, and *IFNG*, as well as for the protein biomarker CD4. Following image acquisition, the FISH-IF module of HALO® was used in combination with HALO AI to quantify RNA and IF signal in the tumor and stroma compartments, as well as exclude artifacts e.g., auto-fluorescent red blood cells. The infiltration analysis using the HALO® Spatial Analysis module was performed on cell phenotypes of interest, for example, Th1 cells expressing CD4, *TNFA*, and *IFNG*.

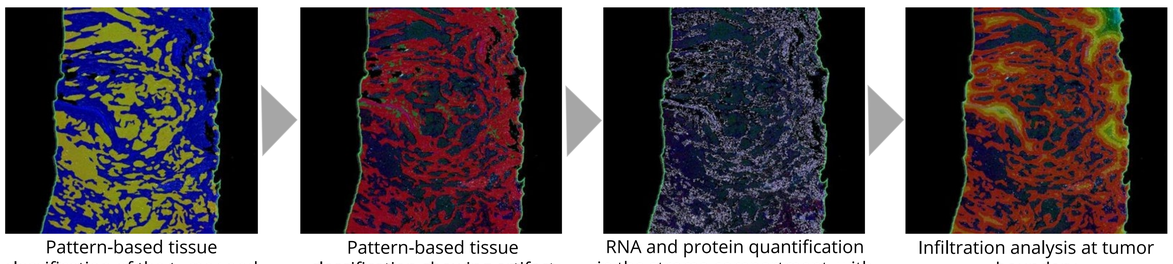
This study demonstrates a powerful workflow that enables the single-slide characterization and quantification of both RNA and protein expression in a single assay.

## METHODS

### RNA Protein Co-Detection




### Example Image Analysis Workflow



Pattern-based tissue classification of the tumor and stroma compartments in yellow and blue

Pattern-based tissue classification showing artifact (green) exclusion in the stroma (red) compartment.

RNA and protein quantification in the stroma compartment, with artifact exclusion

Infiltration analysis at tumor boundary

## RESULTS

### RNA Probe Quantification

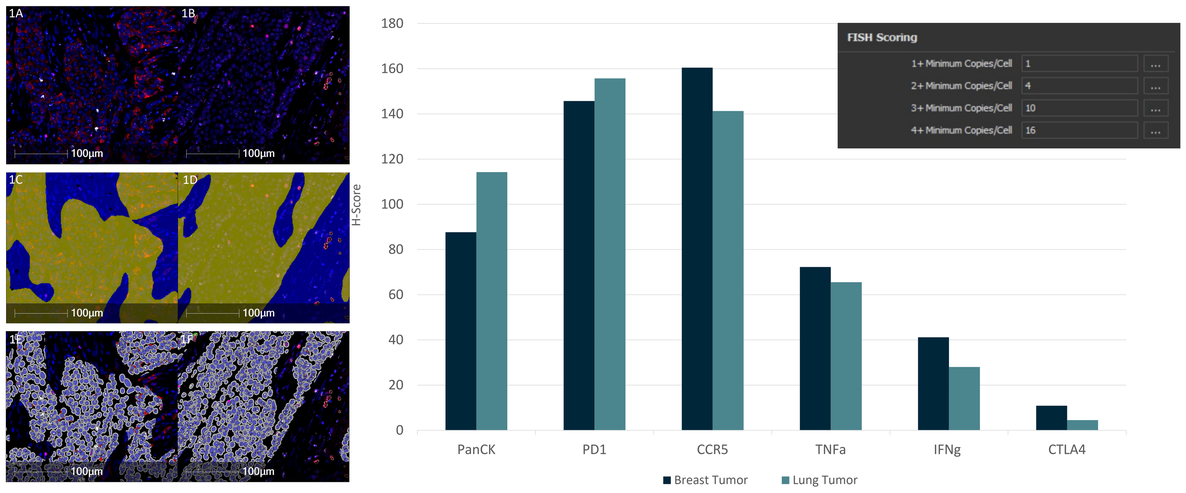



*Figure 1:* Probes for *Pan-CK* in red, *PD-1* in white and *CTLA4* in pink and probes for *TNFA* in red, *CCR5* in white and *IFNG* in pink are shown in breast cancer tissue (1A,1B). Tumor and stroma AI segmentation is shown in yellow and blue respectively (1C, 1D). FISH analysis shows positivity for probes on a per cell basis in the tumor compartment only (1E, 1F). The H-score value falls between 0 and 400 and indicates the amount of expression for each probe based on minimum intensity thresholds and copy counts per cell. This score was automatically calculated in HALO by the following equation: H-Score = (1×% $Probe\ 1{+}Cells$ ) + (2×% $Probe\ 2{+}Cells$ ) + (3×% $Probe\ 3{+}Cells$ ) + (4×% $Probe\ 4{+}Cells$ ).

### RNA & Protein Positive Phenotype Quantification

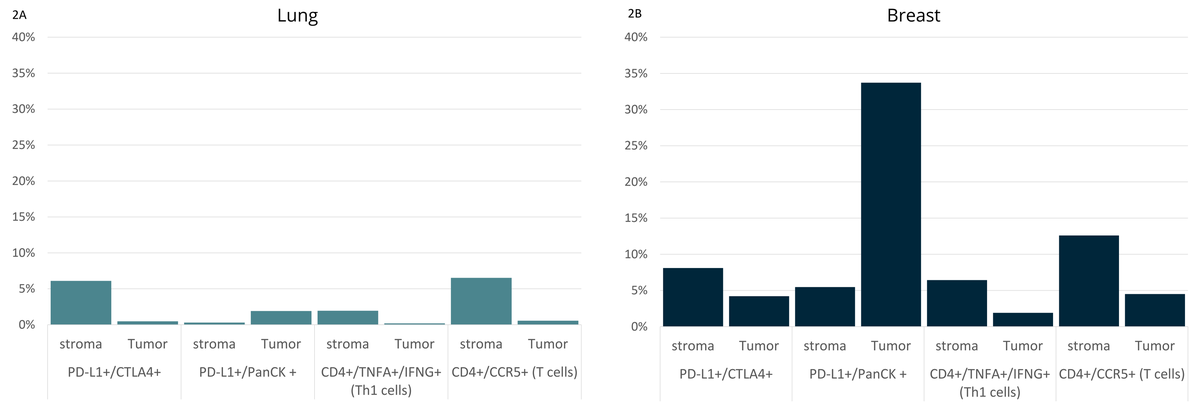



*Figure 2:* Quantification of RNA probes and protein biomarkers using the FISH-IF module of HALO. Phenotypes of interest were specified based off both RNA and protein expression in lung cancer (2A) and breast cancer (2B). Expression of these phenotypes was further stratified across tumor and stroma tissue compartments identified automatically using HALO AI.

### PDL1+ Cell Density Heatmap

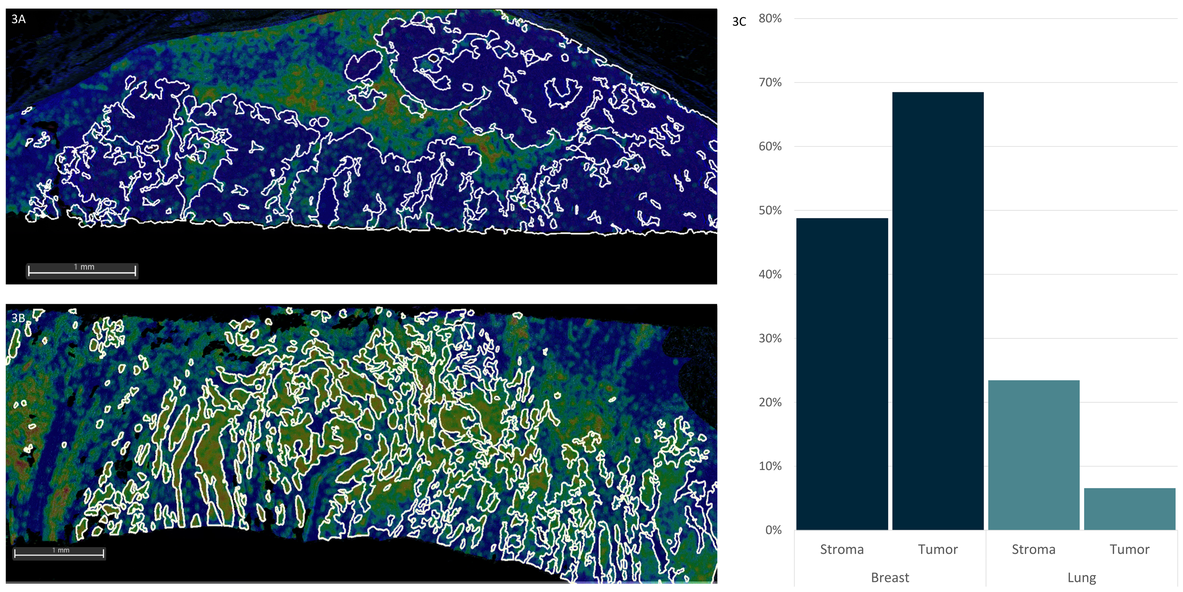



*Figure 3:* Using cell data generated from the FISH-IF analysis, density heatmap analysis was performed within a 25 µm radius of every pixel within the images. Here we investigated the quantification and visualization of PDL1+ cells across the tumor and stroma compartments to evaluate prognosis. Hotspots of PD-L1+ cells localized to different tissue compartments for lung cancer (3A) and breast cancer (3B), with stroma having an increased percentage of PD-L1+ cells in lung cancer when compared to breast cancer (3C).

### Infiltration Analysis of Th1+ Cells

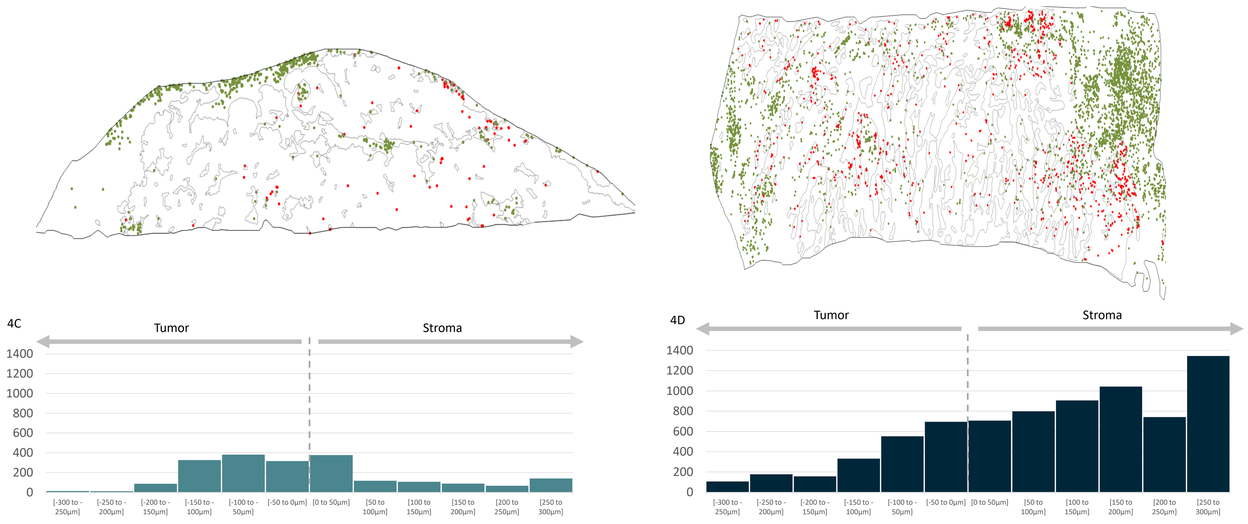



*Figure 4:* The spatial plots show the outline of the analysis regions in black annotations, and the tumor boundaries of lung cancer (4A) and breast cancer (4B), automatically generated by a HALO AI tissue classifier, in grey. Th1+ cells are shown within the infiltration margin in red (inside margin e.g., tumor) and green (outside margin e.g., stroma). Th1+ cell counts are also shown inside and outside of the tumor boundary in lung cancer (4C) and breast cancer (4D), in increments of 50 µm. The tumor boundary is denoted by the dashed line on the bar graphs.

## CONCLUSIONS

PD-L1 showed a distinct pattern of expression between lung and breast tumors. In breast tissue, a higher level of PD-L1 expression was seen in the tumor cells while in lung tissue, PD-L1 expression was higher in immune cells. Infiltration analysis also indicated a higher infiltration of the Th1 cells in breast cancer tissue compared to lung cancer. In addition to conserving sample tissue, co-detection of protein and RNA on the same slide allows characterizing and correlating cell identity and gene expression in a single workflow. ACD's RNA-protein co-detection assay followed by Indica Lab's HALO image analysis software, represents a powerful and streamlined workflow for quantitative analysis of both RNA and protein expression.



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