RNASCOPE IMAGE ANALYSIS USING HALO® AND HALO AI™

INTRODUCTION

Investigating RNA at a single cell level can give important information about a cell's dynamic gene expression. When analyzed *in situ* and in the context of the whole tissue, researchers can identify differences in gene expression associated with disease states, such as cancer and neurological disease.

RNAscope® from ACD™, a Bio-techne brand, has revolutionized the capability of studying RNA *in situ*.[1] Utilizing a unique probe design to ensure target-specific signal amplification, the RNAscope *in situ* hybridization (ISH) assay generates punctate dots of signal within cells. Following an RNAscope assay, the question of quantification and data interpretation needs to be addressed. Manually counting RNA probe signals by eye is time-consuming, laborious, and open to subjective interpretation, especially in cases where signal clustering occurs. Digital image analysis provides an automated, consistent method to generate quantitative data from RNAscope assays.

In this application note, we describe how the **ISH module** and **FISH module** available with the **HALO® image analysis platform** can be employed with **HALO AI™** to quantitatively assess chromogenic and fluorescence RNAscope assays, respectively.

CHROMOGENIC ISH QUANTIFICATION

The HALO ISH module is used to quantify up to three chromogenically-labeled ISH probes and a nuclear counterstain within tissue sections imaged with a brightfield scanner or microscope. The ISH module can be configured to report probe copies per region of interest or probe copies per cell, depending on user preference and the quality of the nuclear staining. The module counts single probe copies and automatically segments probe clusters to get an accurate signal count. Cells are classified as 0, +1, +2, +3, and +4 based on the number of probe copies they contain, and an H-Score is calculated from this information. Importantly, the default classification settings in the ISH module are those recommended by ACD for RNAscope signal quantification.[2]

In-situ RNA detection provides the capability to explore the complex interactions between immune and tumor cells, an important application in cancer research. An example of this is shown in **Figure 1** where an RNAscope assay is used to investigate immune checkpoint markers, *PD-L1* and *CTLA4*, in tumor and

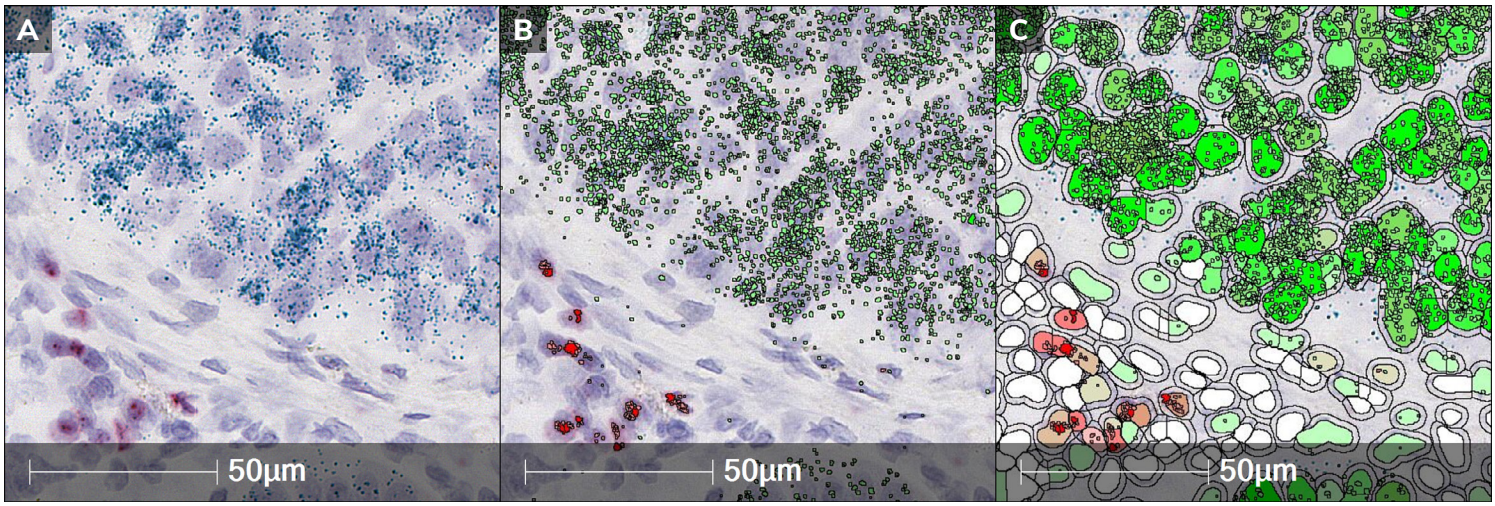

Figure 1. An RNAscope assay of a non-small-cell lung carcinoma, probed for the immune checkpoint markers *PD-L1* and *CTLA4*. **A.** RNAscope image with *PD-L1* probe in blue and *CTLA-4* probe in red. **B.** HALO mark-up images for ISH analysis with cell detection turned off where green spots represent *PD-L1* and red spots represent *CTLA4*. **C.** HALO mark-up image for ISH analysis with cell detection enabled. The cell mark-up color is dependent on the number of probes detected where cells in darker shades of red or green indicate cells with more probe copies, lighter shades contain fewer probe copies, and white cells contain no probe copies. A custom HALO AI network was used for nuclear segmentation.

stromal cells in non-small cell lung cancer. Here, we identify three cell populations of interest: tumor cells with high expression of *PD-L1* (green probe), tumor cells with low expression of *PD-L1*, and T-lymphocytes identified by high levels of *CTLA4* expression (red probe). For this application, the cell classification feature and resulting H-Score reported by the ISH module are used to quantify the number of *PD-L1* high and low tumor cells and the density of *CTLA4+* immune cells in the tissue.

In addition to generating summary data for all cells, HALO can be configured to report information for each individual cell. This object level or "cell-by-cell" data can be used to evaluate expression heterogeneity and the spatial distribution of different cell populations relative to one another using the **Spatial Analysis module** of HALO. For example, it might be of interest to analyze the distance between *CTLA4+* immune cells and *PD-L1* high and low cells or the density of *CTLA4+* cells around the margin of the tumor. When performing Proximity Analysis with the Spatial Analysis module, two populations of interest are defined by the user and a proximity distance is defined. In **Figure 2**, Proximity Analysis results are shown for examining the proximity of *CTLA4+* cells within 30 μm of *PD-L1* high cells (as defined by 3+ or 4+).

A common challenge in RNAscope sample preparation is maintaining nuclear integrity. When nuclear integrity is compromised during the sample preparation, or when a sample has densely packed nuclei, HALO AI can provide improved nuclear segmentation, as shown in **Figure 3**. HALO AI has two pre-trained networks for nuclear segmentation in addition to the option to train your own network.

For RNAscope applications where specific tissue classes need to segmented and analyzed, for example analyzing tumor and stroma separately in oncology research, tissue classification can be a powerful method to automate region of interest selection without the need for manual annotations. There are two options for tissue classification, the HALO **Tissue Classifier module** and HALO AI.

For applications where the tissue classes can be easily identified by eye based on staining or morphology, the HALO Tissue Classifier can be an option. The HALO Tissue Classifier uses a train-by-example random forest algorithm to segment tissues into classes on a pixel-by-pixel basis. The algorithm can be trained to classify within seconds, and once trained, the user can choose what tissue classes will be analyzed by the RNAscope module (e.g. tumor class, stroma class or both).

For more challenging applications, we recommend employing HALO AI for more robust, deep learning-based tissue classification. HALO AI includes several neural networks which vary in terms of network depth and training time. MiniNet, for example, is a shallow neural network that trains quickly and is recommended for applications with more complexity than can be managed by the HALO Tissue Classifier. DenseNet, on the other hand, is a deeper network which is ideal for the most complex applications. In the example shown in **Figure 4**, a kidney tissue section is probed with a single chromogenic ISH probe with hematoxylin counterstain. In the absence of eosin or other special stains, the glomeruli boundaries are difficult to identify by eye in these tissue sections; therefore, DenseNet was used to

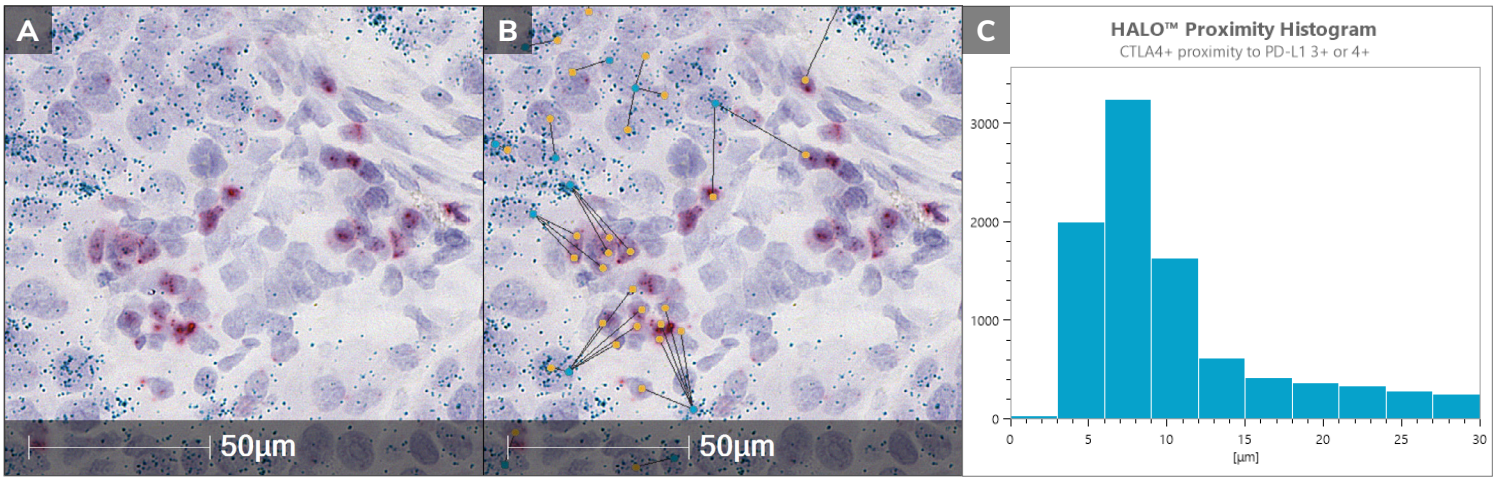

Figure 2. Proximity analysis of *CTLA4+* cells to *PD-L1* high cells (3+ or 4+) from RNAscope image analysis performed in Figure 1. **A.** RNAscope ISH image with *PD-L1* probe in blue and *CTLA-4* probe in red. **B.** Proximity Analysis mark-up image where blue dots represent *PD-L1* high cells and yellow dots indicate *CTLA4+* cells. **C.** Proximity Histogram shows the distribution of distances across the tissue core analyzed.

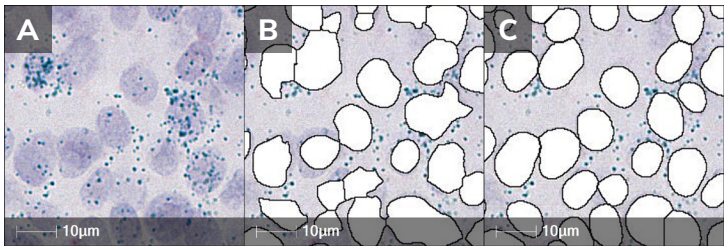

Figure 3. HALO AI enables accurate nuclear segmentation in cases where nuclei are densely packed and/or when nuclear integrity is compromised. **A.** RNAscope image with nuclei that are overlapping in areas and densely packed in others. **B.** HALO markup image using Traditional nuclear segmentation in the ISH module. **C.** HALO markup image where a custom HALO AI network was used for nuclear segmentation in the ISH module.

train a classifier to identify glomeruli for analysis with the ISH module.

FLUORESCENCE ISH QUANTIFICATION

Fluorescence RNAscope assays are preferred over chromogenic assays when the simultaneous evaluation of multiple targets is required. The FISH module of HALO can quantify probe signals and co-expression of an unlimited number of fluorescent probes on a per cell or per area basis with the same analysis options and outputs as described for the brightfield ISH module, including cell classification (0, 1+, 2+, 3+ and 4+) based on the recommended RNAscope scoring guidelines from ACD.

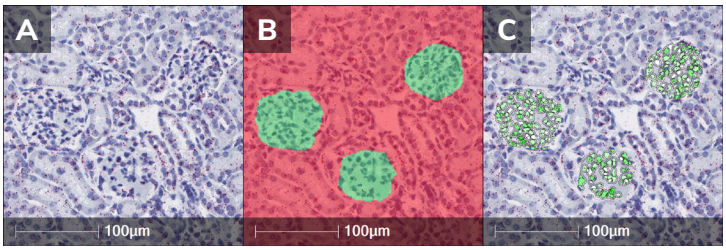

Figure 4. HALO AI tissue classification networks can be embedded into RNAscope modules. **A.** RNAscope image of *POLR2A* positive control probe on kidney tissue. **B.** HALO AI Tissue Classifier markup image where glomeruli are shown in green and tubules are shown in red. **C.** HALO markup image where the ISH module performs analysis on tissue identified by HALO AI as glomeruli. A custom HALO AI tissue classification network developed on kidney tissue was used in this analysis.

FISH image analysis can be easier to optimize as each fluorescence channel is captured individually, which eliminates the need for a color deconvolution step in the analysis. The FISH module has the same capabilities as the ISH module, including scoring according to ACD guidelines, a method to count spots in clusters, automatic H-Scores, and the user can choose whether cell segmentation is performed. In addition, all HALO RNAscope modules have the option to quantify cellular phenotypes of interest. **Figure 5** shows an immuno-oncology FISH assay with *CXCL9*, *CD8*, *IFNγ*, and DAPI. In this assay, a Phenotype was defined as *CD8*+, *IFNγ*+, and *CXCL9*+ to investigate effector T cells actively producing the *IFNγ* cytokine that induces an

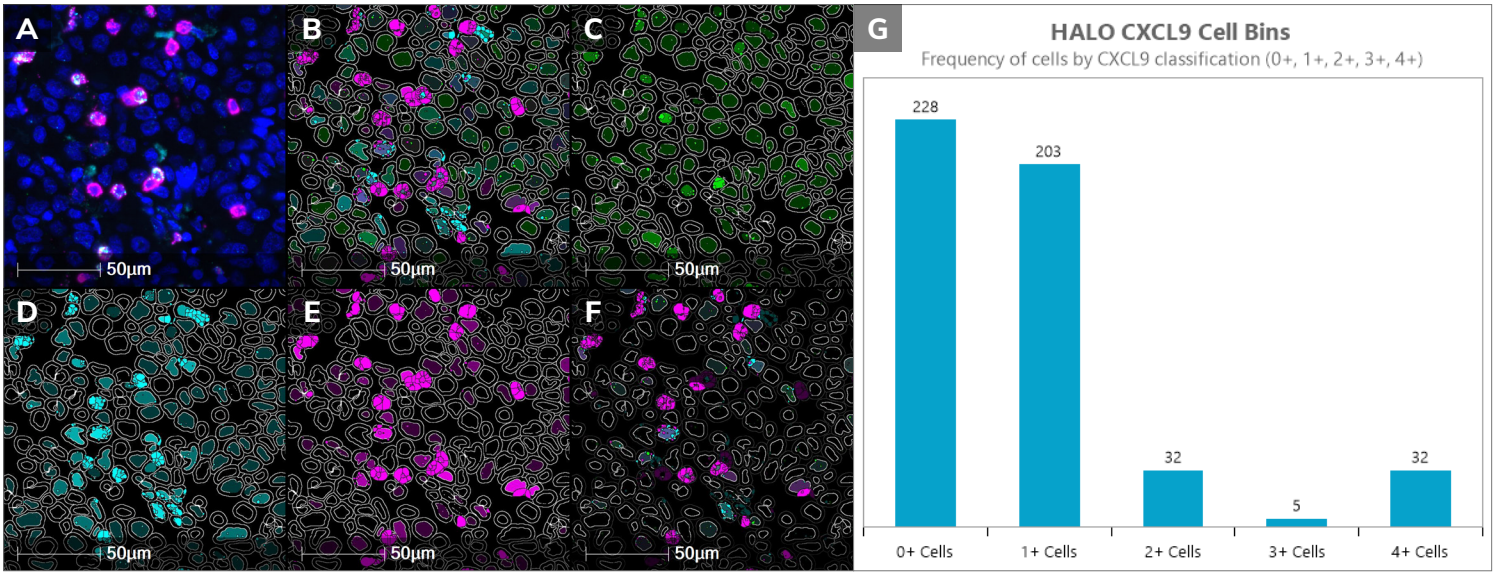

Figure 5. FISH image analysis using the HALO FISH module. **A.** Fluorescence microscopy image with *CXCL9* in cyan, *CD8* in green, *INFγ* in magenta, and DAPI in blue. **B.** HALO Colocalization markup image where all biomarkers are shown in the FISH analysis. The color of the cell markup indicates the relative number of probes present. Cells with higher numbers of probe copies are brightly colored, cells with fewer probe copies are lightly colored and cells with no probe copies are not colored. **C.** *CD8* markup image. **D.** *CXCL9* markup image. **E.** *IFNγ* markup image. **F.** Here, a cell Phenotype was defined with the criteria of *CD8*+, *IFNγ*+, and *CXCL9*+. Only cells that meet the phenotypic criteria are shown with a colored markup. **G.** HALO auto-generates classification histograms for each biomarker. Here, the distribution of *CXCL9* scores is shown.

inflammatory and immune response. In addition, HALO AI was used for nuclear segmentation.

CONCLUSIONS

A standardized method for signal quantification is essential for reliable interpretation of RNAscope assays. The HALO ISH and FISH modules offer quantitative analysis of chromogenic and fluorescent RNAscope assays, respectively. HALO AI advances RNAscope image analysis by providing advanced tissue classification and options to address samples with challenging nuclear morphology. In addition to the ISH and FISH modules discussed here, HALO also supports codetection assays combining ISH with immunohistochemistry with the **ISH-IHC module**, and FISH with immunofluorescence with the **FISH-IF module**. See the Quantitative RNAscope Image Analysis Guide from ACD and Indica Labs for more information on these assays.[2]

While the image analysis shown here are from representative fields of view, it is important to note that optimized ISH and FISH analysis settings can be applied to whole slide images, tissue microarrays, and across batches of images using the HALO platform, thus offering a means to generate quantitative data in a high-throughput manner.

REFERENCES AND RESOURCES

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5. Support for staining optimization:
   Support Help Center
   https://acdbio.com/technical-support/solutions
6. Learn more about RNAscope applications and publications:
   RNAscope Publications with HALO Image Analysis Platform
   https://learn.indicalab.com/rnascope-publications

For specific information about the image analysis methods used in this application note, please email our Applications Scientists at info@indicalab.com.

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