RNAscope® image analysis using HALO® brightfield and fluorescent ISH modules

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INTRODUCTION

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

Introduced in 2012, RNAscope® (Advanced Cell Diagnostics Inc., Hayward, CA, USA) 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 and FISH modules available with the HALO® platform can be employed to quantitatively assess chromogenic and fluorescent RNAscope assays, respectively.

CHROMOGENIC ISH QUANTIFICATION

The HALO ISH module is used to quantify up to two chromogenically-labeled ISH probe 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 the user's 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. The ratio of probe 1 to probe 2 is reported, particularly important if a control probe is being employed to normalize signal counts. Finally, 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 Bio for RNAscope signal quantification.[2]

In-situ RNA detection provides the capability to explore the complex interactions between immune and tumour cells, an important application in cancer research. An example of this is shown in Figure 1 where an RNAscope® assay has been used to investigate immune checkpoint markers, PD-L1 and CTLA4, in tumor and stromal cells in non-small cell lung cancer. Here, we
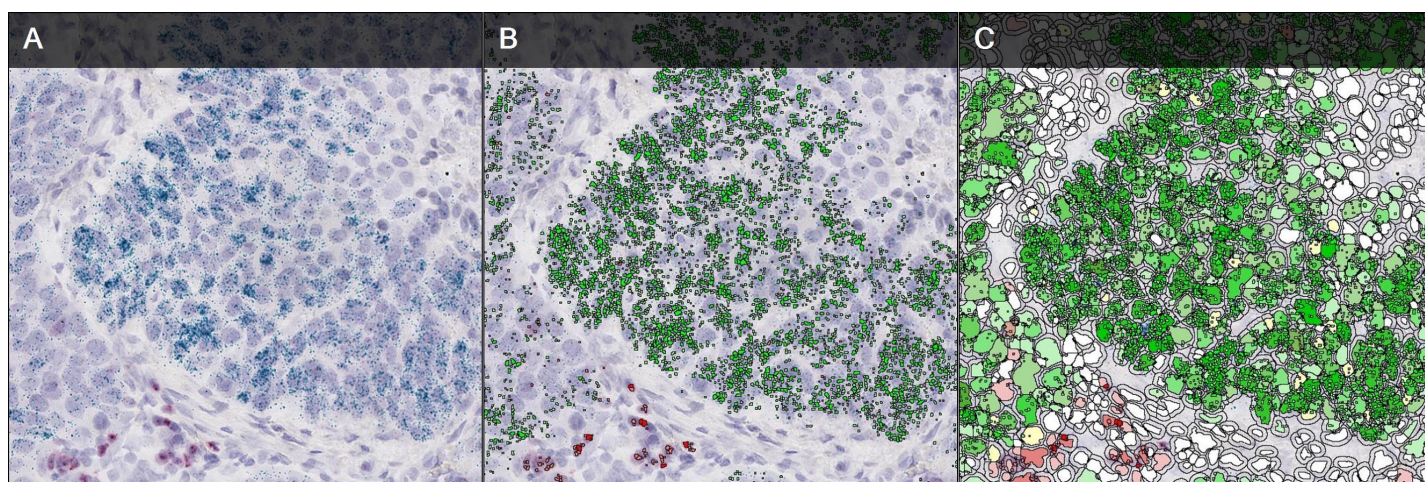
Fig. 1 An *RNAscope® assay of a non-small-cell lung carcinoma, probed for the immune checkpoint markers PD-L1 (green) and CTLA4 (red) (A).* HALO mark-up images are shown in (B) and (C) which visualize where the ISH module has detected probes (B) and where cells have been detected (C). Orange and red dots shown in (B) represent single and clusters of red probes, with the two shades of green dots representing the

Expression of PDL-1 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 CTLA4-positive immune cells in the tissue. If desired, HALO annotation tools or the tissue classifier module can be used to automatically generate separate outputs for tumor and stromal compartments.

In addition to generating summary data for all cells, HALO can be configured to report information for each individual cell. This "cell-by-cell" data can be used to evaluate expression heterogeneity and the spatial distribution of different cell populations relative to one another using HALO's spatial analysis module. For example, it might be of interest to analyze the distance between CTLA4-positive immune cells and PD-L1 high and low cells or the density of CTLA4-positive cells around the margin of the tumor.

FLUORESCENCE ISH QUANTIFICATION

Fluorescent RNAscope assays are preferred over chromogenic assays when the simultaneous evaluation of multiple targets is required. HALO's **FISH module** can quantify probe signals and co-expression of up to four 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 ACD Bio's recommended RNAscope scoring guidelines.

RNAscope assays are being used increasingly in neuroscience. An example is shown in Figure 2. Here, the HALO FISH module is used to quantify two RNAscope probes in mouse brain, Dopamine Recepter D1 (Drd1) and Dopamine Receptor D2 (Drd2). A representative histogram produced by the FISH module is shown in Figure 2B.
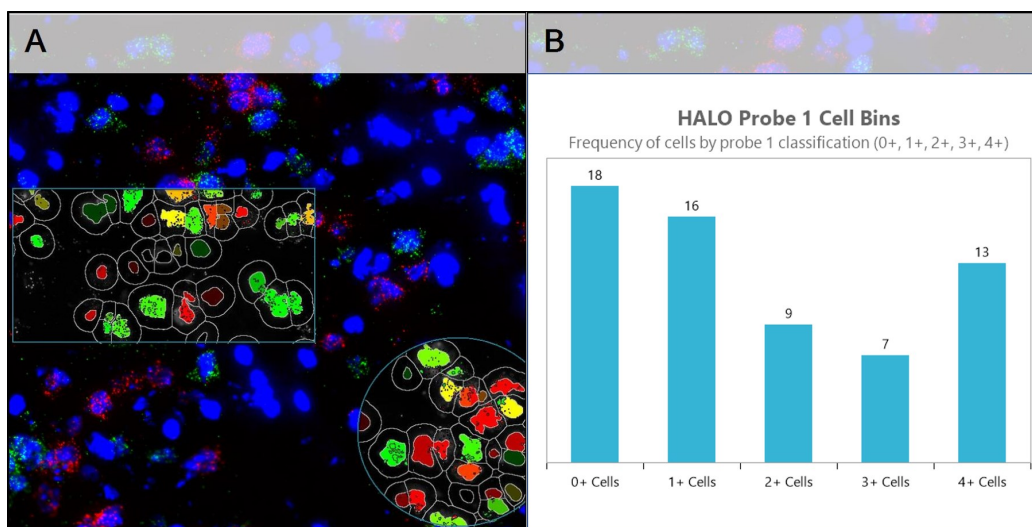
Fig.2 *A fluorescent RNAscope assay for Drd1 (red) and Drd2 (green) in the striatum of healthy mouse brain was analysed using the* **FISH module** *in HALO (A).* The HALO mark-up image shows probe and cell detection in two different regions of the tissue. The color of the cell mark-up is dependent on the number of probes it contains, e.g. a cell containing large number of green probes will be dark green, and a cell with similar numbers of each probe will be yellow. One of the histograms that is automatically generated for this analysis is shown in (B).

Other modules for FISH analysis available in HALO include the **FISH Amplification & Deletion Analysis module**, used to quantify up to two fluorescently labeled DNA probes to measure amplification or deletion, and the **FISH Break apart & Fusion Analysis module**, used to measure gene rearrangements detected using break-apart fusion probes.

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 both chromogenic and fluorescent RNAscope assays. While the images shown in Figure 1 and 2 are representative fields of view, it is important to note that optimized ISH and FISH analysis settings can be applied to whole tissue sections and across batches of images using the HALO platform thus offering a means to generate quantitative data in a high throughput manner.

REFERENCES

1. Wang. F*., et al.* RNAscope: A novel in situ RNA analysis platform for formalin- fixed, paraffin-embedded tissues. J. Mol. Diagn. 2012. 14:22–29.
2. A Guide for RNAScope® Data Analysis, Advanced Cell Diagnostics, Inc, 2017.

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