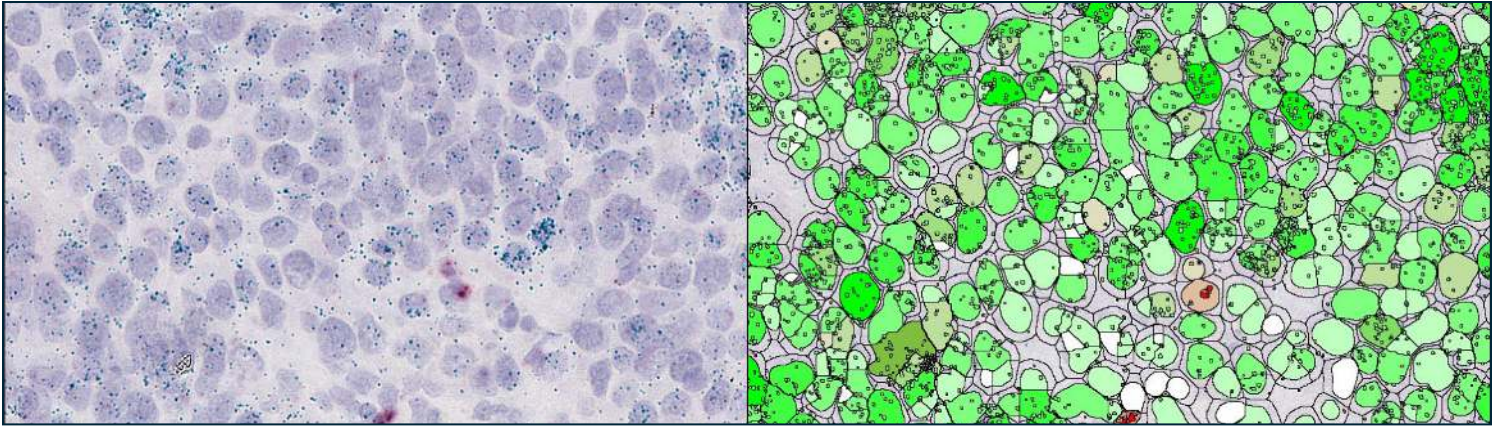# QUANTITATIVE RNAscope™ IMAGE ANALYSIS GUIDE

RNAscope™ technology from Advanced Cell Diagnostics (ACD), a Bio-Techne brand, enables RNA target expression analysis within intact cells and tissue with high sensitivity and specificity in both chromogenic and fluorescence assays. RNAscope assays allows scientists to evaluate gene expression in a specific region of tissue, in a cell subpopulation, or in a subcellular compartment. The RNAscope Duplex and Multiplex assays enable simultaneous detection of more than one target RNA thus facilitating the evaluation of multiple cell phenotypes and spatial relationships within a complex tissue microenvironment. In addition, with the new RNA-protein co-detection assay, RNAscope chromogenic *in situ* hybridization (ISH) can be combined with immunohistochemistry (IHC), and RNAscope fluorescence *in situ* hybridization (FISH) assays can be combined with immunofluorescence (IF) for simultaneous detection of RNA and protein targets on the same tissue. BaseScope™ assays enable detection of splice variants, point mutations, and highly homologous sequences while miRNAscope™ assays detect anti-sense oligonucleotides (ASOs), micro RNAs (miRNAs), and small interfering RNAs (siRNAs). The recently launched DNAscope™ assays identifies copy number variations (CNVs), translocations, and other chromosomal anomalies. Whether you are just starting out in your RNAscope analysis or are an experienced user, this guide will enable you to advance your biomarker research. Here, we provide examples of exemplary assays, discuss experimental considerations, address common challenges, introduce **HALO® image analysis solutions**, and answer common quantitative image analysis questions.

RNASCOPE QUANTIFICATION WITH THE HALO® IMAGE ANALYSIS PLATFORM

HALO® is the gold standard image analysis platform for quantitative tissue analysis in digital pathology. HALO is a modular platform that is configured based on application areas and workflows and has flexibility to change over time. Below, we discuss the RNAscope modules from Indica Labs, which are suitable for analysis of single-plex, duplex, and multiplex RNAscope assays.

In addition to the RNAscope modules (ISH, ISH-IHC, FISH, and FISH-IF), Indica Labs provides the Area Quantification and Area Quantification FL modules free with every HALO purchase. For addressing questions where cell segmentation is not required, these area-based modules provide a simple means to quantify RNAscope assays. Analyze up to five stains including hematoxylin with the Area Quantification module, or an unlimited number of dyes in fluorescence with the Area Quantification FL module.

indica labs

The ISH module of HALO analyzes a diverse array of assays including chromogenic RNAscope assays, chromogenic BaseScope assays, DNAscope, and miRNAscope assays **(Figure 1 and Table 1)**.

INTEGRATING RNASCOPE ASSAYS WITH IMAGE ANALYSIS SOLUTIONS

The RNAscope ISH assays are available in both chromogenic and fluorescent formats and can be used with the new RNA-protein co-detection workflow and reagents from ACD. See **Table 1** to identify your assay of interest, learn about the detection capabilities and learn which ACD RNAscope products are used with which HALO modules. In addition to the HALO modules shown, HALO AI, the Tissue Microarray module, and the Tissue Classifier module may be used with any RNAscope assay. HALO image analysis software supports all configurations listed. See **Figure 1** for an example of a BaseScope assay analyzed with the ISH module and the Tissue Microarray module.

BEFORE YOU START: EXPERIMENTAL DESIGN AND ASSAY VALIDATION CONSIDERATIONS

As you design your RNAscope experiment, the first decision to make is whether to use a brightfield or fluorescence imaging modality. If you have equal access to both technologies, consider choosing a fluorescence workflow, particularly when utilizing multiple probes or stains. **(Figure 2-3)** Brightfield workflows add a layer of complexity to image analysis as each stain in the image must be deconvolved using RGB values so that each target can be individually quantified. Image analysis is simplified in fluorescence since each channel is acquired separately at the point of image acquisition, thus eliminating the need for a color deconvolution step.

Fluorescence is recommended particularly for probe targets that are either very high copy number or very low copy number. Fluorophores that emit in the visible region of the spectrum have a dynamic range that is two to three-fold higher than commonly used chromogens. Therefore, if you are working with a probe target with low to very low expression, fluorescence assays offer a wider dynamic range for detection. For high copy number probes, color deconvolution can be problematic as brightfield images with high expression can saturate to black making it impossible for the algorithm to accurately determine the source color.

As you are developing your assay, consider whether you need to output probe quantification for individual cells or if summary data for the entire tissue will suffice. If cellular data is required for answering your biological question, take care to preserve the nuclear morphology during sample preparation. If the nuclei are over digested, image analysi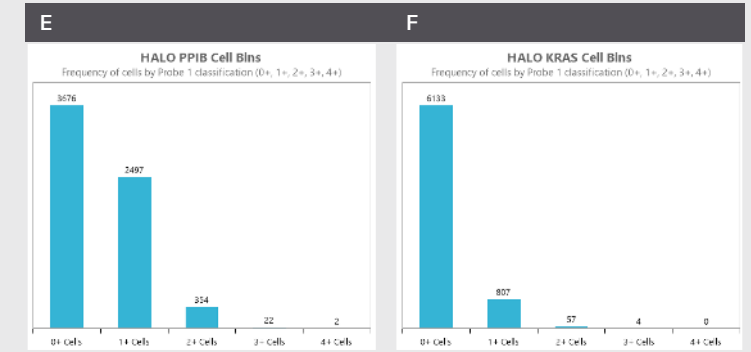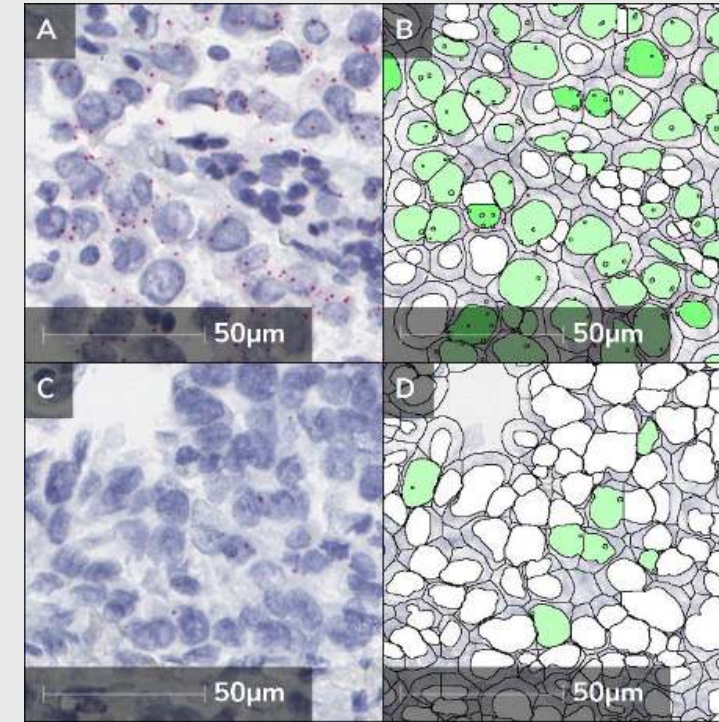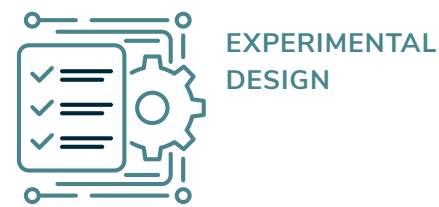s software will not accurately segment cells, leaving only an area-based quantitation as an option. Also, if you wish to spatially analyze expression data, you need to output **Object Data** in HALO, which means distinct nuclear morphology is required for cell detection. Learn more about Object Data in **Figure 4**.

For a successful RNAscope, BaseScope or miRNAscope assay, we recommend that you first confirm RNA quality in your samples using negative and positive control probes only. This will also help to optimize your pretreatment conditions and assay workflow. By running positive and negative control probes on your samples, you will confirm that the samples have been optimally prepared, and that RNA quality is preserved. After a successful run with control probes, you can then move on to adding your target probes. Using quality samples along with optimized experimental conditions will ensure good staining and contribute to higher quality images.


Figure 1. BaseScope analysis of KRAS and PPIB positive control using the HALO ISH module. (A) PPIB positive control shown with HALO mark-up image in (B). (C) KRAS assay shown with mark-up in (D). (E) Histogram of cell counts binned according to PPIB probe score. (F) Histogram of cell counts binned according to KRAS probe score. In the mark-up images, cells with probes are shown in varying shades of green with darker cells having more probes. White cells have no probes or a number below the minimum number defined by the user.

RNASCOPE   WORKFLOW

EXPERIMENTAL DESIGN
- Decide between fluorescence and brightfield modalities.
- Select ACD assay that meets your probe and protein detection needs.
- Select positive and negative controls suitable to your assay.
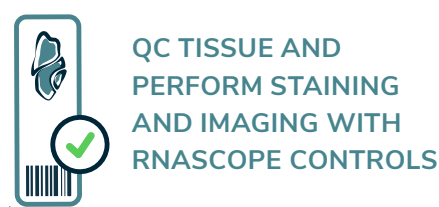
QC TISSUE AND PERFORM STAINING AND IMAGING WITH RNASCOPE CONTROLS
- Optimize pretreatment conditions and assay workflow.
- Ensure your assay conditions maintain nuclear integrity if you require cell-based measurements and/or spatial analysis.

DEVELOP AND VALIDATE IMAGE ANALYSIS
- Select analysis modules that match your experimental objectives.
- Validate results against ground truth and leverage object data in validation.

PERFORM ASSAY WITH EXPERIMENTAL PROBE(S) AND CONTROLS
- Perform experiment using conditions optimized in Step 2.
- Remember to include appropriate positive and negative controls.
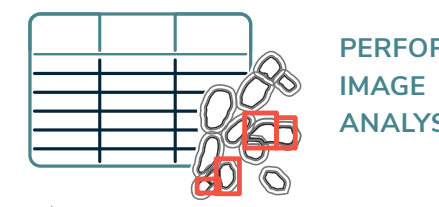
PERFORM IMAGE ANALYSIS
- QC test slides before performing image analysis. Where possible, use annotations or tissue classifiers to eliminate slide artefacts.
- Reach out to support@indicalab.com for assistance optimizing your image analysis.

ANALYZE, PLOT, AND INTERPRET RESULTS
- Create histograms, perform spatial analysis such as Proximity Analysis, Nearest Neighbor Analysis, Infiltration Analysis, or create a Density Heatmap.

ACD Offering			Indica Labs Offering		Detection			
ACD Assay	ACD Product Family	ACD	HALO RNAscope Modules	HALO Area-Based Analyses Modules	Detects	Probes	Proteins	Nuclear Counterstain
RNAscope ISH	RNAscope Chromogenic	NA	ISH	Area Quantification	mRNA or lncRNA target (>300nt)	2	0	1
RNAscope ISH-IHC	RNAscope Chromogenic	RNA-Protein Co-detection Ancillary Kit	ISH-IHC	Area Quantification	mRNA or lncRNA target (>300nt) and protein	2	1	1
RNAscope FISH	RNAscope Fluorescent Multiplex	NA	FISH	Area Quantification FL	mRNA or lncRNA target (>300nt)	4	0	1
RNAscope FISH-IF	RNAscope Fluorescent Multiplex	RNA-Protein Co-detection Ancillary Kit	FISH-IF	Area Quantification FL	mRNA or lncRNA target (>300nt) and protein	4	1	1
BaseScope ISH	BaseScope Chromogenic	NA	ISH	Area Quantification	Splice variants, short/ highly homologous sequences, and point mutations (50-300nt)	2	0	1
BaseScope ISH-IHC	BaseScope Chromogenic	RNA-Protein Co-detection Ancillary Kit	ISH-IHC	Area Quantification	Splice variants, short/ highly homologous sequences, and point mutations (50-300nt) and protein	2	1	1
DNAscope	DNAscope Chromogenic	NA	ISH	Area Quantification	Copy number variations (CNVs), translocations, and other chromosomal anomalies	2	0	1
miRNAscope	miRNAscope Chromogenic	NA	ISH	Area Quantification	ASOs, miRNAs, siRNAs, and other smaller RNA sequences (17-50nt)	1	0	1
miRNAscope -IHC	miRNAscope Chromogenic	RNA-Protein Co-detection Ancillary Kit	ISH-IHC	Area Quantification	ASOs, miRNAs, siRNAs, and other smaller RNA sequences (17-50nt) and protein	1	1	1

Table 1. Capabilities and products of the ACD RNAscope assays and image analysis solutions from Indica Labs. HALO AI, the Tissue Microarray module, and the Tissue Classifier module may be used with any RNAscope assay. HALO image analysis software supports all configurations. *Antibodies are not included in Co-detection Ancillary Kits from ACD.

It is critical to verify and validate the assay on a small set of validation slides. When running the control sample experiments, perform the experiment workflow end-to-end if possible, including tissue fixation, mounting, staining, image acquisition, and image analysis. This assay validation will ensure your assay is optimized sufficiently to provide you with consistent experimental outputs to address your biological questions and ensures that you have the right biomarkers, probes, and software to achieve your desired endpoint(s). Failure to confirm a suitable image analysis pipeline is a common mistake – and one that is easily avoided.

Finally, if you have additional questions on assay development, reach out to the ACD and Indica Lab Applications teams. Contact information for both teams is found at the end of this document.
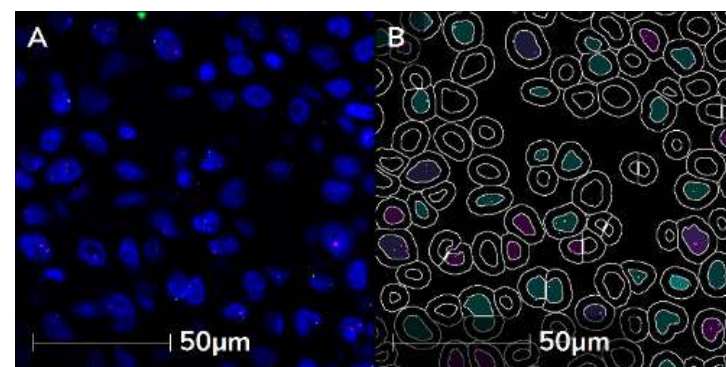
Figure 2. Duplex FISH assay analyzed with the FISH module of HALO. (A) Duplex FISH assay with nuclei in blue, probe 1 in green, and probe 2 in magenta. (B) HALO FISH analysis mark-up image, where cyan nuclei represent cyan probe positivity and cyan spots represent probes. Similarly, green nuclei represent green probe positivity and green spots represent the green probe. Purple nuclei represent dual positivity.

RNAscope QUANTIFICATION SCORING PROTOCOL IN HALO

RNAscope quantification for ISH and FISH assays is based on enumerating the number of chromogenic or fluorescence probe spots. RNAscope assay staining is assessed using a 0, 1+, 2+, 3+, and 4+ scoring system that is integrated into the HALO RNAscope modules. In addition, it is important to consistently score clusters of spots. HALO image analysis software defines the **Minimum Copies/Cell** according to ACD guidelines.

KEY PARAMETERS FOR ISH ANALYSIS:

In this section, we present a summary of key parameters for each assay. For a detailed step-by-step protocol, see the User Guides in the Resources section.

Exclusion Stain:

- **Definition**: Red/Green/Blue optical densities define the **Exclusion Stain.** Use when carbon deposits in tissue, chromogen aggregates, ink, melanin, or imaging artifacts from dust are present.
- **Use**: Remove additional stains from analysis by taking advantage of the color deconvolution step.
- **How to set:** Use the **Real-time Tuning Window** to select an edge of an artifact, zoom in to 9-36 pixels, selecting a variety of pixels spanning the artifact color in order to obtain a representative average value.

Probe SPOT Size:

- **Definition:** Minimum, Maximum Probe size in µm².
- **Use**: Exclude objects too small or large to be spots.
- **How to set:** Set empirically with **Real-time Tuning Window** adjusting sliders such that all spots have appropriate masks. Test a field of view analysis and compare the cell-by-cell level data with a visual inspection.

Probe Size:

- **Definition:** Area of a typical probe in µm². Determines the probe counts within probe clusters. If a cluster is 50 µm², the number of counts would be = 50 / **Probe Size.**
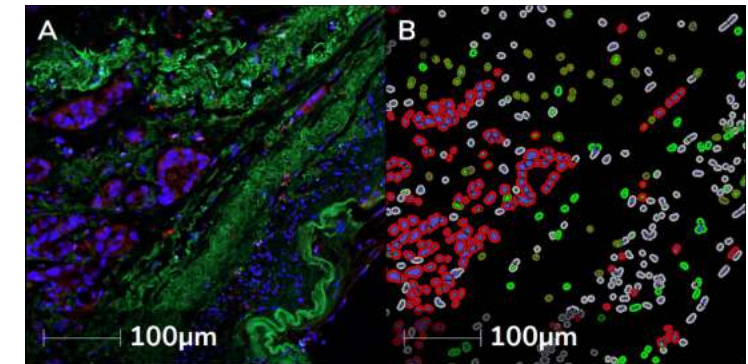
Figure 3. FISH-IF analysis with the FISH-IF module of HALO. (A) FISH-IF assay with nuclei in blue, protein biomarkers in red and green and a FISH probe in cyan. (B) HALO FISH-IF mark-up image, where blue nuclei with light gray cytoplasmic rings are negative for both proteins and probe. Green nuclei or cytoplasm represent positivity for the green IF marker, red cytoplasm represents positivity for the red IF marker, cyan spots represent the cyan probe, and olive-green cytoplasm represent triple positivity for the green and red IF markers and the cyan FISH probe.

- **Use:** Break large clusters of probes into an approximate probe count.
- **How to set:** Use the **Ruler** annotation tool to measure diameter of spots, calculate Approximate probe size ≈ π d²/4.

Store Object (Cell) Data

- **Definition:** This parameter controls whether cell-by-cell level data is included in results **Object Data.**
- **Use:** Gather more than summary data by including quantitative information about every cell. Use in conjunction with spatial analysis.
- **How to set:** Set to **True** when nuclear segmentation is possible and cell-by-cell level data is desired. This parameter must be set to **True** for use with Spatial Analysis module.

KEY PARAMETERS FOR FISH

Probe Signal Minimum Intensity

- **Definition:** Threshold at which probe is detected. Decrease to detect more, increase for less.
- **Use:** Exclude objects too dim to be spots (like autofluorescence signals).
- **How to set:** Set empirically with **Real-time Tuning Window** adjusting sliders such that all spots have appropriate masks.

Probe Spot Size

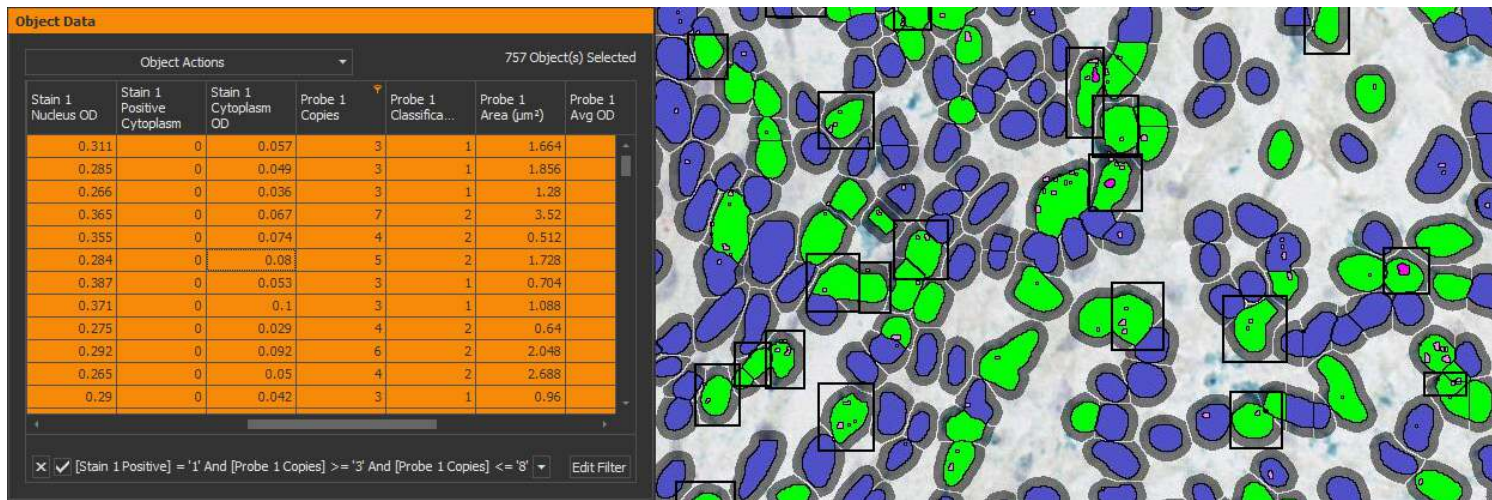- **Definition:** Minimum, Maximum Probe size

Figure 4. Object Data facilitates complex filtering of cell and object populations for visualization and assay validation. An ISH-IHC assay run with Object Data enabled was filtered for cells positive for the protein biomarker (green) and that had 3 to 8 probes (pink). Selecting the filtered cells in the Object Data table shown on the left side (orange rows indicate cells selected) highlights these cells with black rectangles in the image markup to the right.

in μm². Copies smaller than this will not be counted.

- **Use:** Exclude objects too small or large to be spots.
- **How to set:** Set empirically with **Real-time Tuning Window** adjusting sliders such that all spots have appropriate masks.

Probe Copy Intensity

- **Definition:** Typical probe copy integrated intensity. Determines the number of copies within a cluster.
- **Use:** Break large clusters of probes into an approximate probe count.
- **How to set:** Using the default value of 0.15, optimize other parameters, and run analysis on an area of low expression with **Store Object (Cell) Data** set to **True**. In the **Object Data**, set the **Probe Copies** filter to 1, in order to isolate cells with single probes. Take an average of **Probe Cell Intensity** values and update the **Probe Copy Intensity** parameter. For further optimization, evaluate low to moderate expressing cells or ROIs and compare copy number and cell scoring in the **Object Data** output to a visual assessment.

Store Object (Cell) Data

- **Definition:** This parameter controls whether cell-by-cell level data is included in results **Object Data.**
- **Use:** Gather more than summary data by

including quantitative information about every cell. Use in conjunction with spatial analysis.
- **How to set:** Set to **True** when nuclear segmentation is possible and cell-by-cell level data is desired. This parameter must be set to **True** for use with Spatial Analysis module.

TOP 3 CHALLENGES WITH QUANTITATIVE RNASCOPE IMAGE ANALYSIS

The most common challenges with quantification of RNAscope images are nuclear segmentation in brightfield assays, color deconvolution in brightfield assays, and managing spot clusters.

NUCLEAR SEGMENTATION

Poor nuclear structure due to over-digestion is one of the most common challenges with RNAscope and wreaks havoc on quantification **(Figure 5).** First, optimize your sample preparation and see the **RNAscope TroubleShooting Guide** to prevent over-digestion of nuclei during sample preparation. During image analysis, determine whether nuclear segmentation can be achieved with **Traditional** nuclear segmentation or whether **HALO AI™** is required. With HALO AI, you have three options in brightfield, and analogous options exist in fluorescence assays: **AI Default, AI Custom**, and your AI Custom network. First try **AI Default,** and if the segmentation looks good, proceed to optimize it. If AI Default segmentation is poor, switch to **AI Custom** with **Nuc Seg - BF or FL**. If **AI Custom**

cannot be tuned sufficiently, then proceed to training your own AI Custom network on top of **AI Custom**. The value of HALO AI in challenging nuclear segmentation applications is illustrated in **Figures 5 and 6**. Finally, if you cannot accurately segment nuclei with available tools, proceed to use Area Quantification and Area Quantification FL modules **(Figure 7)**.

A second challenge in brightfield nuclear segmentation arises when the presence of a saturated chromogen obscures the hematoxylin signal underneath. Ideally, this issue should be addressed during sample preparation. If it must be addressed during image analysis, apply **Nuclear Stain Weight** to the IHC chromogen(s) in an ISH/IHC assay or **Nucleus Weight** to the IF dye(s) in a FISH/IF assay to detect the presence of a nucleus.
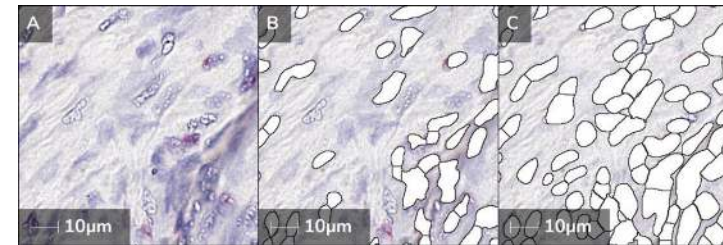

Figure 5. Impact of HALO AI on nuclear segmentation in diffuse and lightly stained nuclei. (A) RNAscope assay with magenta probe. **(B)** HALO mark-up image with traditional nuclear segmentation analysis. **(C)** Mark-up image with HALO AI analysis. In both mark-up images, nuclear masks are shown in white with black lines.

COLOR DECONVOLUTION

If you are struggling to identify image analysis settings that accurately reflect the data, confirm you have high quality color deconvolution. Optimizing color deconvolution in chromogenic spot assays is both essential and challenging. There is a strong correlation between good color deconvolution and quality image analysis. When performing color deconvolution, aim to acquire pure chromogen color. This can be challenging as with small spots there are few pixels to work with and there may also be DNA stain bleeding through. While we usually recommend sampling 9-36 pixels in the **Stain Selection** step of algorithm development, with spot color deconvolution, try selecting 4 pixels. Be sure to sample 4 pixels of different shades so that you acquire an average. Evaluate your color deconvolution with the **Real-time Tuning Window**. If there is significant signal falsely assigned to a channel, try selecting colors

again and re-evaluate. Ideally, the **Real-time Tuning Window** should reveal discreet structures of the pure chromogen color where signals are correctly assigned to their respective channels. To appreciate the impact of color deconvolution on image analysis, see **Figure 8**.

It is critical to optimize your chromogenic staining so that the color does not saturate to black. When in HALO, use *ctrl* and the mouse wheel to zoom in to the image pixels to evaluate how commonly you see black or nearly black pixels.

MANAGING SPOT CLUSTERS

Managing segmentation of spot clusters in your analysis is an important component of quantification. In the ISH and ISH-IHC modules, segmentation of spot clusters is based on area. Any spot that is larger than the area set by the **Probe Size** parameter is counted as multiple spots.

In the FISH and FISH-IF modules, probe clusters are controlled by the **Probe Copy Intensity** parameter which is defined as the spot intensity divided by the number of copies. To define the **Probe Copy Intensity**, leave this metric at the default, optimize the other parameters, run analysis on a field-of-view with **Store Object (Cell) Data** set to **True**, navigate to the **Object Data** to filter the data for cells with 1 spot. Inspect the **Probe Avg Intensity** in this population, acquire an average of 10 spots and update the **Probe Copy Intensity** parameter in the Analysis tab, then re-run analysis.
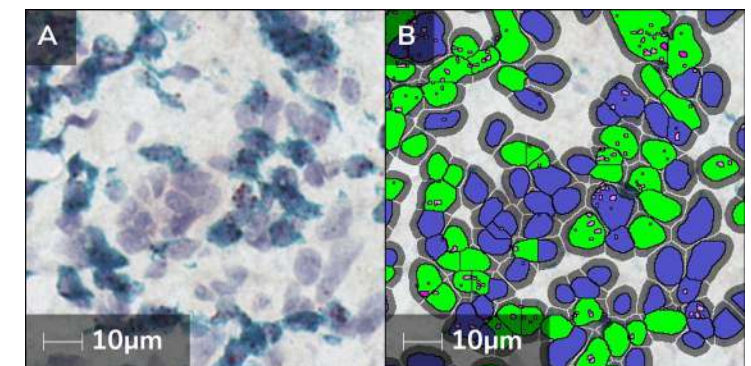

Figure 6. RNAscope analysis of ISH-IHC assay using the ISH-IHC module of HALO and HALO AI for nuclear segmentation. (A) ISH-IHC assay with a magenta probe and a teal protein biomarker. **(B)** HALO mark-up image where blue represents nuclei without probe and lime green represents positivity for the teal IHC biomarker. The pink spots represent the magenta probe, and the gray rings represent a user defined maximum cytoplasmic radius.
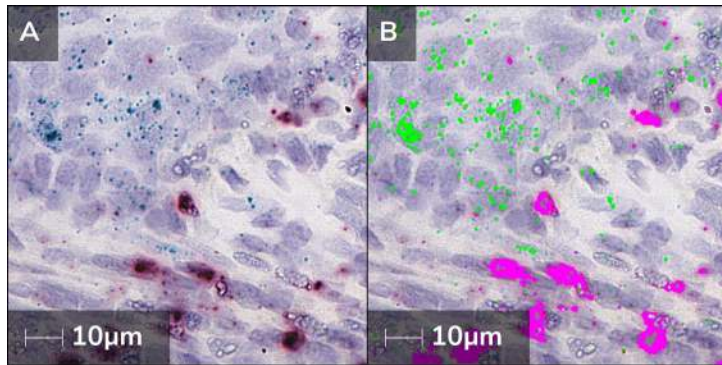
Figure 7. Area Quantification analysis of duplex ISH assay. (A) ISH-IHC assay with blue probe and red protein biomarker. (B) HALO mark-up image where lime green represents blue probe positive pixels and pink represents red protein positive pixels.

CONTROLS AND VALIDATION

Always run positive and negative control probes included in the RNAscope Intro Pack on your sample at the same time as your experimental sample to assess sample RNA quality and permeabilization. When selecting a positive control for your RNAscope assay, PPIB (Cyclophilin B) is the most flexible option. UBC (Ubiquitin C) is good for high expression and POLR2A (DNA-directed RNA polymerase II subunit RPB1) is good for low expression targets **(Figure 9)**. Before analyzing your experimental samples, confirm that the UBC control has a score of 3+/4+ (or 1+ for POLR2A) and the DapB negative control has a score of 0/1+.

In addition to validating permeability and proper probe hybridization, you may choose to leverage controls to normalize your results in HALO. For example, compare PPIB/POLR2A spots per unit area vs experimental spots per unit area.

To validate your quantitative image analysis in HALO, carefully evaluate the **Real-time Tuning**
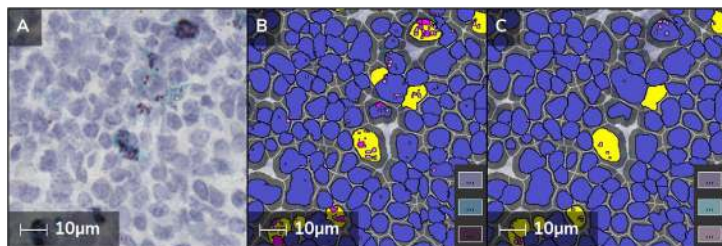

Figure 8. Color deconvolution impacts image analysis results. (A) ISH-IHC assay with magenta probe and blue IHC protein. (B) HALO mark-up image with optimized color deconvolution. (C) Mark-up image with non-optimized color deconvolution. All analysis settings from B were held constant with the exception of the color vectors. See inset for colors used in mark-up shown in B and C.

Window. After the analysis is complete, toggle the markup image on and off to visually validate your image analysis assay against ground truth. In addition, perform the following:

1. Identify a region with few clusters and run analysis on a field of view with **Store Object (Cell) Data** set to **True**.

2. Use the ability to navigate between a single row in the **Object Data** and an object/cell in the image to perform manual counts and compare results.

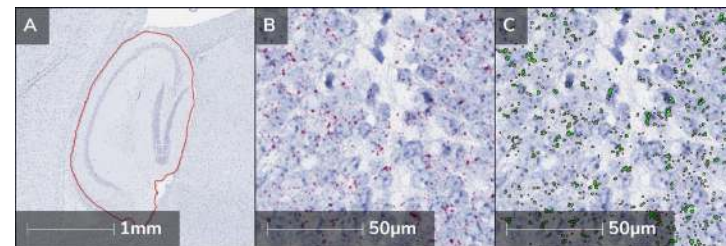3. If they agree, perform whole-slide analysis.

Figure 9. Analysis of POLR2A positive control in ISH assay on mouse brain tissue. (A) POLR2A ISH assay with the hippocampus annotated in red. (B) Single probe RNAscope assay field of view corresponding to box in panel A. (C) Cell Detection is turned off in the ISH mark-up image. Green spots represent the magenta probe.

ADVANCED WORKFLOW SUPPORT FROM INDICA LABS

Indica Labs offers several add-ons and modules to support advanced RNAscope workflows and challenging samples.

TISSUE SEGMENTATION

A common challenge in applications where detecting tissue classes is required, such as tumor and stroma, is determining how to distinguish classes in RNAscope image analysis. The Random-Forest machine learning driven Tissue Classifier module relies on differences in color and texture **(Figure 10).** With limited morphological information present in RNAscope analysis, HALO AI is preferred. HALO AI is a collection of deep learning-based classification and segmentation tools that works with HALO. Leveraging HALO AI is game changing for challenging assays where cell and tissue segmentation are required.
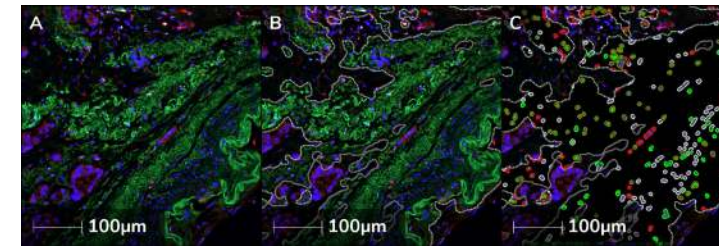

Figure 10. Tissue Classifier module is used on FISH-IF assay in order to analyze a single tissue class. (A) FISH-IF assay with blue nuclei, green and red protein biomarkers, and a cyan probe. (B) Tissue Classifier module was used to create annotations defining green+ and green- tissue. (C) HALO mark-up image where FISH-IF module was used to analyze green+ tissue class. Green nuclei or cytoplasm represent positivity for the green IF marker, red cytoplasm represents positivity for the red IF marker, cyan spots represent the cyan probe, and olive-green cytoplasm represent triple positivity for the green and red IF markers and the cyan FISH probe.

TISSUE MICROARRAY SUPPORT

The Tissue Microarray add-on enables and facilitates tissue microarray workflow by importing and managing TMA metadata, segmenting cores, and enhancing productivity with batch TMA analysis.

SPATIAL ANALYSIS

The Spatial Analysis module in HALO provides a set of tools to quantify the spatial distribution of cells within the tissue. In a single plex assay, spatial analysis can be used to create a **Density Heatmap** to characterize high and low gene expression throughout the tissue **(Figure 11B)**. In a multiplexed assay, one may wish to look at the proximity of cells expressing different genes through a **Proximity Analysis** or a **Nearest Neighbor** analysis **(Figure 11C)**. **Infiltration Analysis** is informative for investigating tumor and immune cell relationships.

Spatial analysis is commonly used in immuno-oncology where researchers are investigating immune cell infiltration into the tumor microenvironment or cell-to-cell interactions between immune and tumor cell populations. Using the RNAscope technology, it is possible to visualize and assess gene expression of multiple cytokines, chemokines, tumor markers and checkpoint markers within the tumor microenvironment. By detecting expression of soluble factors, this assay can determine the activation states of tumor infiltrating immune cells.

COMMON QUESTIONS, TROUBLESHOOTING AND TIPS & TRICKS

Q: Should I capture my RNAscope images at 20x or 40x magnification?
A: Image acquisition for RNAscope images is recommended at 40x magnification.

Q: I'm not sure how to optimize the parameters for my RNAscope module in HALO. Where do I start?
A: Sign up for the Indica Labs **Learning Portal**, navigate to your module of interest, download the User Guide, and watch the training tutorial video. Watch the **RNAscope Masterclass webinar** and reach out to support@indicalab.com for a refresher training on your modules.
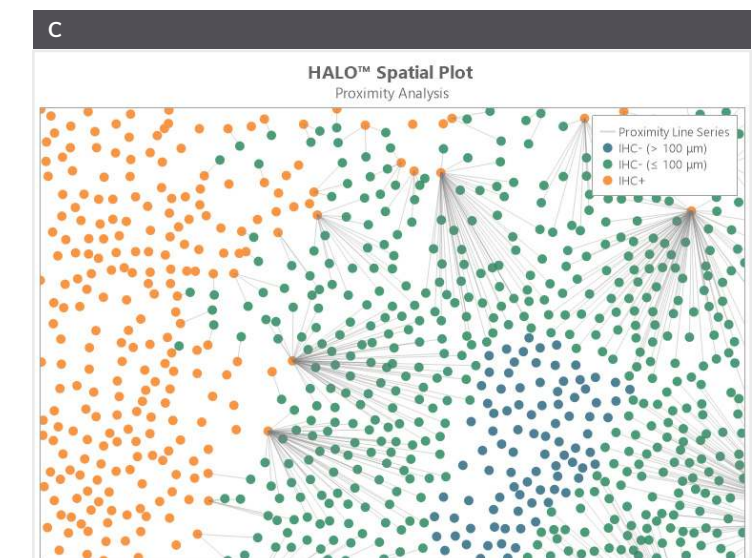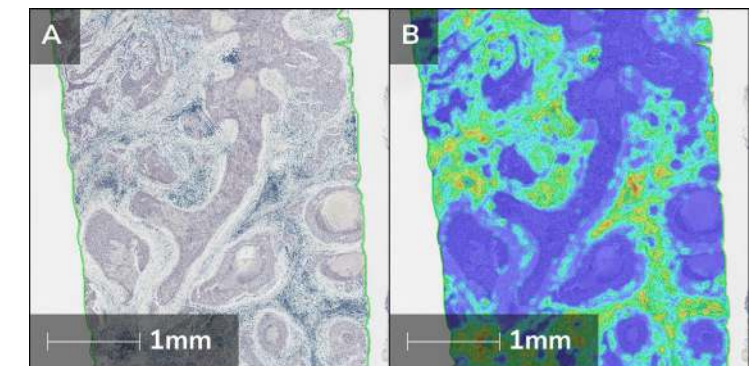

Figure 11. Spatial analysis of RNAscope data. (A) An ISH-IHC assay with magenta ISH probe and blue protein. (B) A Density Heatmap is shown of a single-plex magenta ISH probe where blue represents areas of low expression and red represents areas of highest expression. (C) A Proximity Analysis examining the number of cells expressing a blue IHC marker in the stroma within 100 μm of a single-plex magenta ISH probe in the stroma is shown. See also internal legend.

Q: How can I eliminate tissue artifacts from my image analysis?
A: Manual annotation tools are available to eliminate one off artifacts in image analysis. Use the **Exclusion tool** (scissors) to draw an exclusion layer, or hold *ctrl* down while using the **Magnetic pen, Brush tool**, etc. These tools are also handy to remove areas where tissue has folded back on itself. If you need to remove the tissue edge, use the **Tissue Edge Thickness** parameter in the **Advanced Analysis** menu. Finally, the Tissue Classifier provides another way to remove artifacts, such as tissue folds.

Q: How do you manage artifacts that negatively impact spot counting?
A: Any time a distinct color can be excluded without impacting stains of interest, the **Exclusion Stain** is recommended. If you have two problematic features, such as carbon deposits in lung tissue and red blood cells, you can use HALO AI or the Tissue Classifier module to create an RBC classifier, and you can use the **Exclusion Stain** for the anthracotic pigments.

Q: How do I manage heterogeneous ISH staining patterns, such as PD-L1?
A: If you have morphologically distinct regions, it may be helpful to use the HALO AI and/or Tissue Classifier to isolate tissues of interest for analysis. **Annotations** can also be drawn manually.

Q: What do I do if it is difficult to distinguish my RNAscope signal from background?
A: See the following documentation for staining optimization: **RNAscope TroubleShooting Guide.**

Q: What do I do if my experimental sample has no signal?
A: Confirm that both your positive and negative controls score as expected before making any conclusions on your experimental sample. Confirm that you are using the POLR2A positive control probe for low expression assays.

SUMMARY

Thoughtful RNAscope experimental design combined with rigorous assay execution and validation enables accurate quantification of spots and protein co-detection with the HALO image analysis platform in brightfield and fluorescence assays. HALO is a flexible solution that performs analysis of ISH, ISH-IHC, FISH, and FISH-IF RNAscope assays as well as BaseScope, miRNAscope, and DNAscope assays, and offers solutions for advanced tissue classification, AI-driven nuclear segmentation, tissue microarray workflow support, and spatial analysis. Common preventable challenges in RNAscope analysis include allowing chromogenic staining to saturate to black causing color deconvolution challenges, not taking care to protect the nuclear structure and morphology, and failing to verify and validate an image analysis solution before scaling up.

Reach out with any applications related questions to the applications teams at ACD and at Indica Labs.

RESOURCES

1. Support for staining optimization: **RNAscope TroubleShooting Guide**
2. Support for qualitative image analysis: **A Guide for RNAscope Data Analysis**
3. Current HALO users can sign-up for **Indica Labs Learning Portal** to access additional resources, including module user guides, video tutorials, and masterclass webinars.

Contact

Indica Labs

Reach out to **support@indicalab.com** to reach the global Applications and Technical Support teams at Indica Labs for image analysis support.

Reach out to **info@indicalab.com** to reach Pharma Services at Indica Labs to explore end-to-end pathology research project support.

ACD, a Bio-Techne brand

Reach out to **support.ACD@bio-techne.com** or call **1-888-799-7486** 8:00 am – 6:00 pm to reach the applications team at ACD for assistance optimizing your RNAscope assays. To connect with the Professional Assay Services team at ACD for more information on ISH, Dual ISH, and IHC/IF services, call **1 (877) 613-5844**.