bio-techne®

ACD™
a bio-techne® brand

A Guide for RNAscope® Data Analysis

A wealth of gene expression information from tissue context

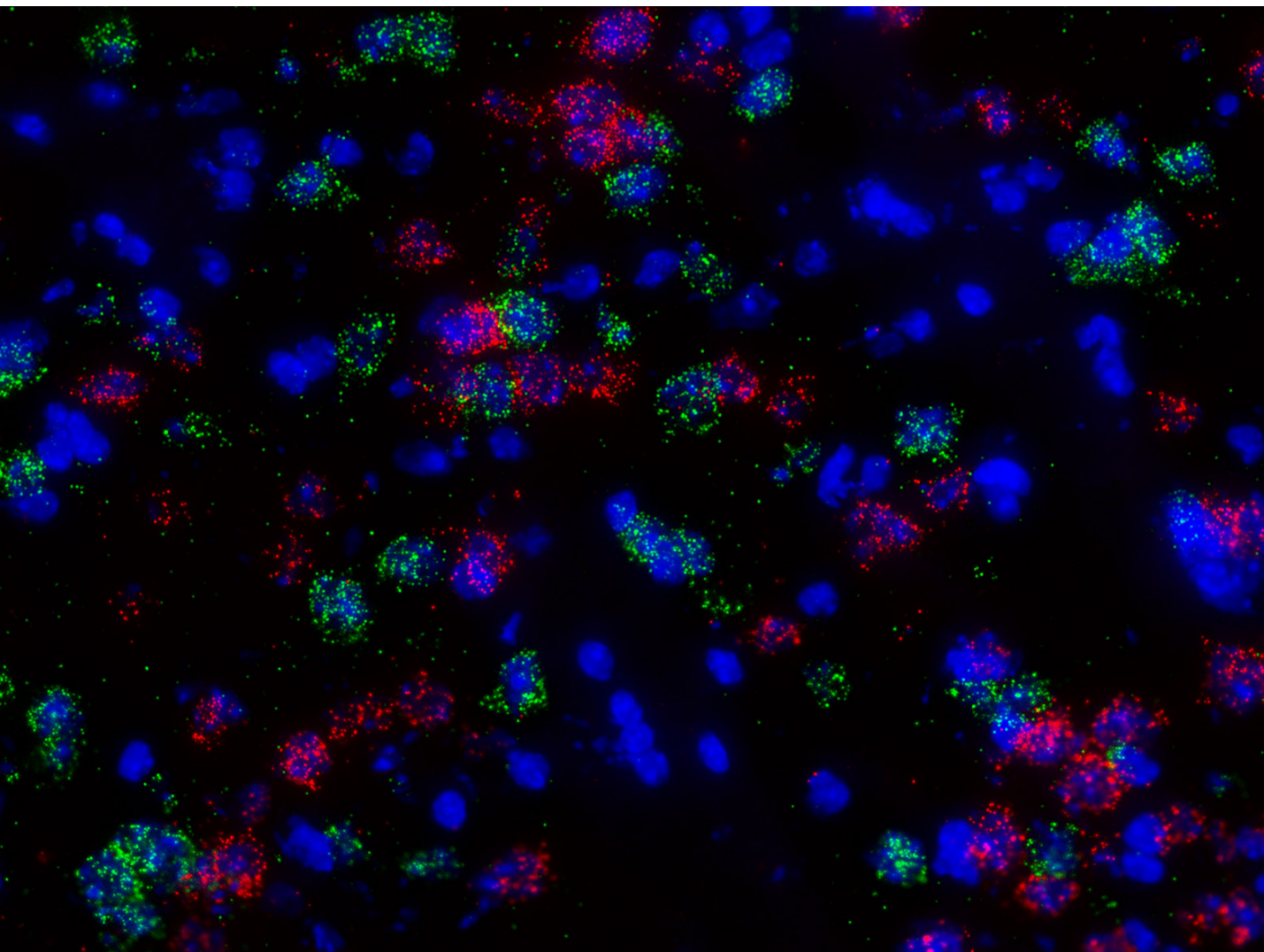


Table of contents

Introduction

RNAscope® technology

The RNAscope® assay provides a powerful method to detect gene expression within the spatial and morphological context of the tissue. The proprietary "double Z" probe design in combination with advanced signal amplification techniques enables highly specific and sensitive detection of target RNA, which can be visualized as a dot, with each dot representing a single RNA transcript. This robust, high signal-to-noise technology allows for the detection of gene transcripts at the single molecule level with single-cell resolution and can further expand our understanding of gene expression in cell lines and tissues samples. The multiplexing capabilities of both the chromogenic and fluorescent RNAscope® assays facilitate the simultaneous visualization of multiple targets.

Here, we provide data analysis guidelines for several types of RNAscope® staining results, including semi-quantitative and quantitative analysis methods for each of the various gene expression scenarios.

Disclaimer 1; Advanced Cell Diagnostics recommends performing quality control checks by running positive (POLR2A, PPIB, or UBC) and negative (dapB) controls alongside samples to assess tissue and RNA quality.
Disclaimer 2; The image analysis examples used in this document were from HALO™ Analysis Software from Indica Labs. Please refer to https://acdbio.com/analysis-software for more information on data analysis software options. Additionally, ImageJ Image Processing and Analysis or CellProfiler can be used.

Singleplex target expression scenarios

1. **Homogeneous target expression in a particular cell type**
2. **Heterogeneous target expression in a particular cell type**
3. **Target expression in ≥2 different cell types**
4. **Subpopulation or region-specific target expression**
5. **Subcellular localization of target expression**
6. **Qualitative assessment for presence or absence of target**

1: Homogeneous target expression in a particular cell type

Scenario description

In the homogeneous target expression scenario cells display relatively uniform staining for the target RNA, indicating homogeneous gene expression among the same cell type (Figure 1). The overall homogeneous target expression level of the entire cell population throughout a tissue section can be assessed by measuring the average number of dots per cell.

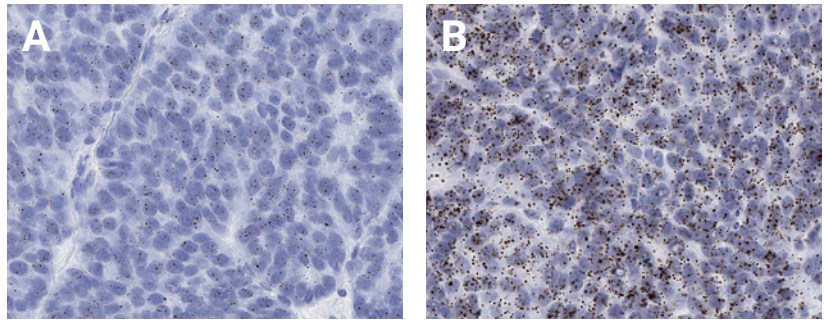


FIGURE 1. Homogeneous expression of *MICA* (A) and *MICB* (B) in human ovarian cancer tissue using the RNAscope® 2.5 HD BROWN assay.

Analysis guidelines

The average number of dots per cell can be measured by using either a semi-quantitative histological scoring methodology (called methodology #1) based on the ACD scoring criteria shown in Table 1 or image-based quantitative software analysis tools (called methodology #2) illustrated in Figure 2. According to methodology #1 guidelines, *MICA* and *MICB* expression are scored as 1 and 2, respectively.

Methodology #1: Semi-quantitative histological scoring methodology based on ACD scoring criteria.

Table 1 outlines the semi-quantitative ACD scoring system for the RNAscope® assay.

ACD Score	Scoring Criteria
0	No staining or <1 dot/ 10 cells
1	1–3 dots/cell
2	4–9 dots/cell and none or very few dot clusters
3	10–15 dots/cell and/or <10% dots are in clusters
4	>15 dots/cell and/or >10% dots are in clusters

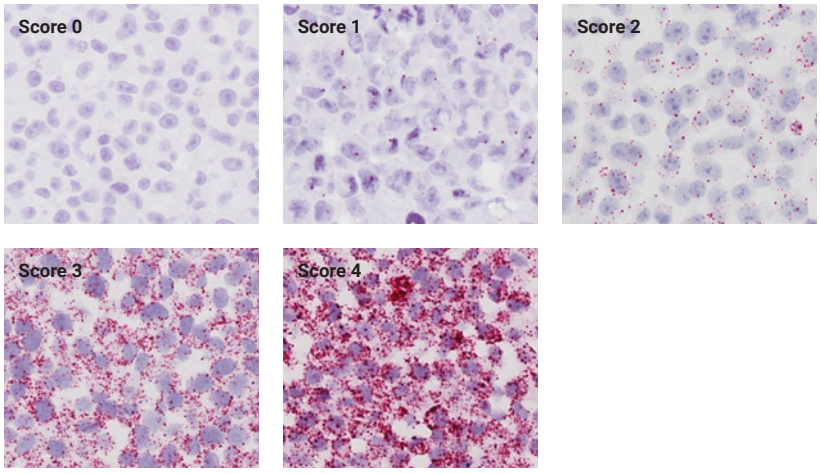


TABLE 1. The semi-quantitative ACD scoring system for the RNAscope® assay and representative images for each score. A score of 0 has no staining or less than 1 dot for every 10 cells, whereas a score of 4 has greater than 15 dots per cell. Note: Scoring categories can be condensed to 0–3, similar to the more common method for IHC, or expanded to capture a wider range of expression.

Methodology #2: Image-based quantitative software analysis

Figure 2 illustrates the image-based quantification of the average number of dots or transcripts per cell exemplified by the HALO™ software system from Indica Labs.

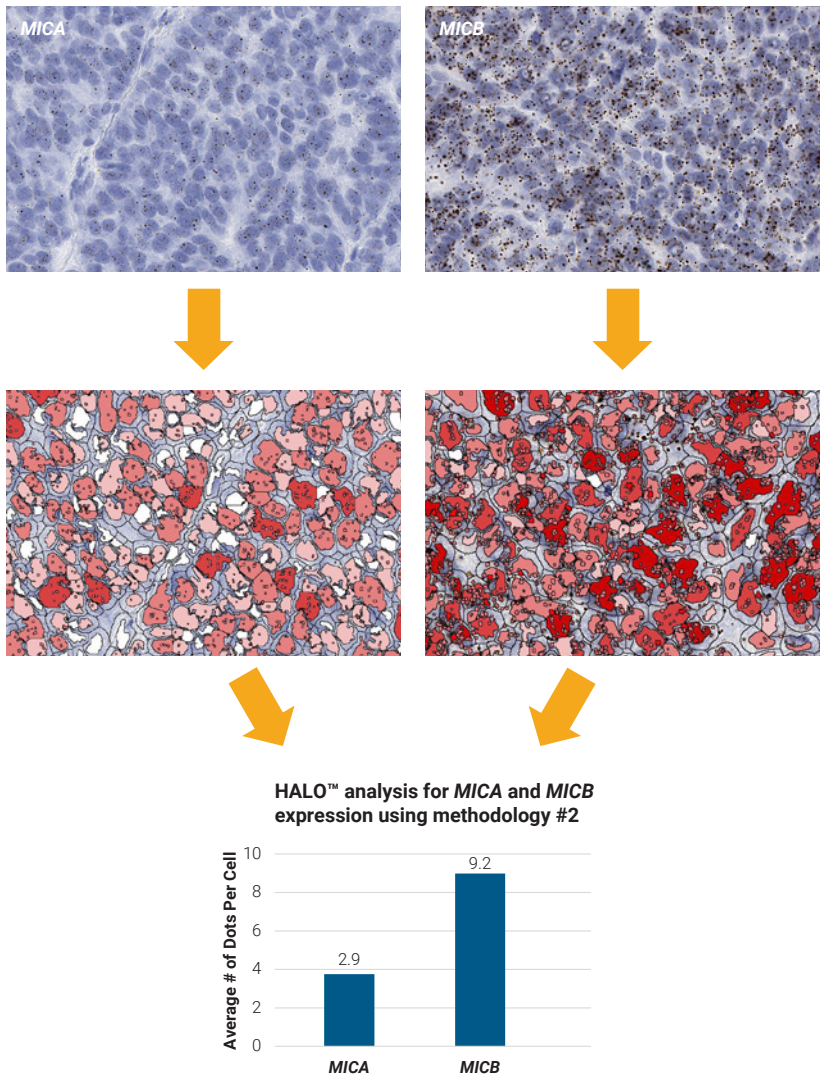


FIGURE 2. Quantitative image-based data analysis by HALO™ software from Indica Labs for *MICA* and *MICB* expression shown in Figure 1.

References

The following is a list of publications in which the authors have quantified their RNAscope® results and therefore can be used as a reference to see examples of how users have represented their target expression data:

Barriga, F. M., *et al.* (2017). "Mex3a Marks a Slowly Dividing Subpopulation of Lgr5+ Intestinal Stem Cells." *Cell Stem Cell* 20(6): 801–816.

Cartwright, E. K., *et al.* (2016). "CD8(+) Lymphocytes Are Required for Maintaining Viral Suppression in SIV-Infected Macaques Treated with Short-Term Antiretroviral Therapy." *Immunity* 45(3): 656–668.

Greer, P. L., *et al.* (2016). "A Family of non-GPCR Chemosensors Defines an Alternative Logic for Mammalian Olfaction." *Cell* 165(7): 1734–1748.

Huang, W.-C., *et al.* (2017). "Diverse Non-genetic, Allele-Specific Expression Effects Shape Genetic Architecture at the Cellular Level in the Mammalian Brain." *Neuron* 93(5): 1094–1109.

Keeler, A. M., *et al.* (2016). "Cellular Analysis of Silencing the Huntington's Disease Gene Using AAV9 Mediated Delivery of Artificial Micro RNA into the Striatum of Q140/Q140 Mice." *J Huntingtons Dis* 5(3): 239–248.

Lake, B. B., *et al.* (2016). "Neuronal subtypes and diversity revealed by single-nucleus RNA sequencing of the human brain." *Science* 352(6293): 1586–1590.

Li, X., *et al.* (2016). "Epithelia-derived wingless regulates dendrite directional growth of *drosophila* ddaE neuron through the Fz-Fmi-Dsh-Rac1 pathway." *Mol Brain* 9(1): 46.

Ouwendijk, W. J., *et al.* (2013). "T-cell infiltration correlates with CXCL10 expression in ganglia of cynomolgus macaques with reactivated simian varicella virus." *J Virol* 87(5): 2979–2982.

Seidemann, E., *et al.* (2016). "Calcium imaging with genetically encoded indicators in behaving primates." *Elife* 5.

Silberstein, L., *et al.* (2016). "Proximity-Based Differential Single-Cell Analysis of the Niche to Identify Stem/Progenitor Cell Regulators." *Cell Stem Cell* 19(4): 530–543.

References, *continued*

Singh, V. B., *et al.* (2017). "Blocked transcription through KvDMR1 results in absence of methylation and gene silencing resembling Beckwith-Wiedemann syndrome." *Development* 144(10): 1820–1830.

Wei, L., *et al.* (2016). "Inhibition of CDK4/6 protects against radiation-induced intestinal injury in mice." *J Clin Invest* 126(11): 4076–4087.

2: Heterogeneous target expression in a particular cell type

Scenario description

In the heterogeneous target expression scenario cells display different levels of staining for the target RNA, indicating heterogeneous gene expression among the same cell type (Figures 3 and 4). In this scenario both the expression level and the percent of cells expressing the target at different levels could be of importance.

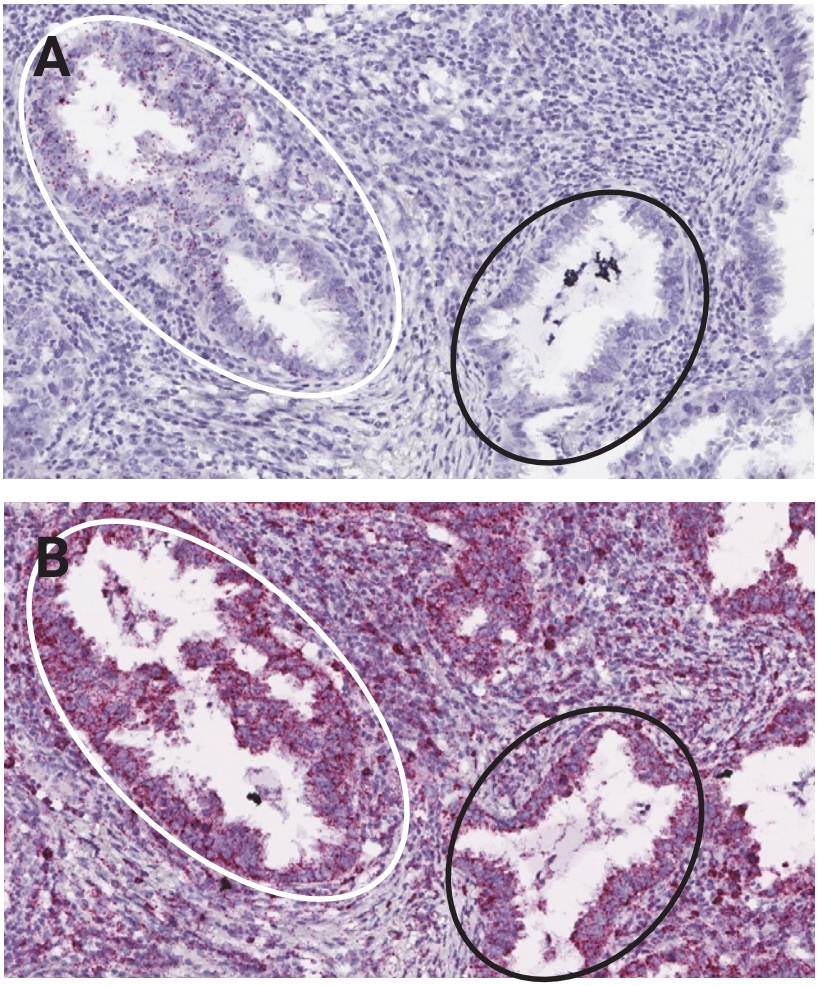


FIGURE 3. Heterogeneous expression for *AFAP1-AS1* (A) and positive control staining (B) in human lung cancer tissue using the RNAscope® 2.5 HD RED assay. Shown are two foci, one that expresses *AFAP1-AS1* (white circle) and one that does not express *AFAP1-AS1* (black circle)(A). This demonstrates heterogeneous *AFAP1-AS1* expression between two tumor foci and suggests the presence of two clonal tumor populations. The expression of the *PPIB* housekeeping gene is uniform between the tumor cells of each foci, indicating good quality RNA expression in both foci (B).

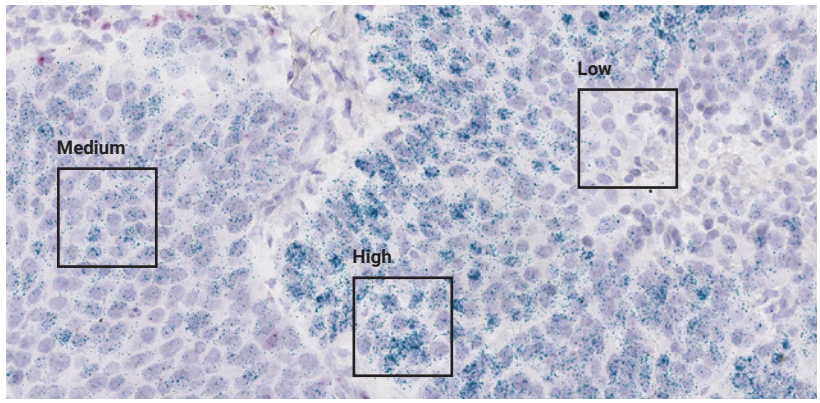


FIGURE 4. Heterogeneous expression of *PD-L1* in green showing high, medium and low expression in human lung cancer tissue using the RNAscope® 2.5 HD Duplex assay. *CTLA4* is shown in red.

Analysis guidelines

The expression level and the percent of cells expressing the target at different levels can be analyzed in the following ways:

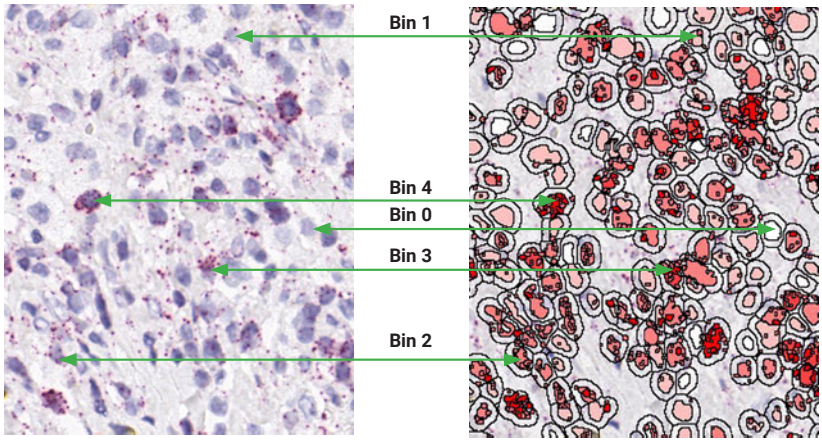
1) Assess the overall expression level of the entire sample (average number of dots per cell) using methodology #1 or methodology #2.

2) The dynamic range of expression (cell-by-cell expression profiles) can be quantified for the entire tissue section or selected regions of interest by binning cells with different levels of expression into separate bins as shown in Figure 5. The data can be presented as a histogram to represent the expression level distribution or can be calculated as a Histo score (H score). This H score can be achieved by semi-quantitative analysis or quantitative image-based software analysis where the percentage of cells within each bin characterized by a certain expression level or number of dots (ACD scores ranging from 0 to 4) is estimated and the overall H score (range of 0 to 400) is calculated as follows:

$$\text{H-score} = \sum_{\text{Bin } 0 \rightarrow 4} (\text{ACD score or bin number} \times \text{percentage of cells per bin})$$

For further reference, the H score method will be referred to as methodology #3.

Methodology #3: H-score



	% of Cells	Weighted Formula
Bin 0 (0 Dots/Cell)	8	0 * 8
Bin 1 (1–3 Dots/Cell)	46	+ 1 * 46
Bin 2 (4–9 Dots/Cell)	39	+ 2 * 39
Bin 3 (10–15 Dots/Cell)	5	+ 3 * 5
Bin 4 (>15 Dots/Cell)	2	+ 4 * 2
H-Score		147

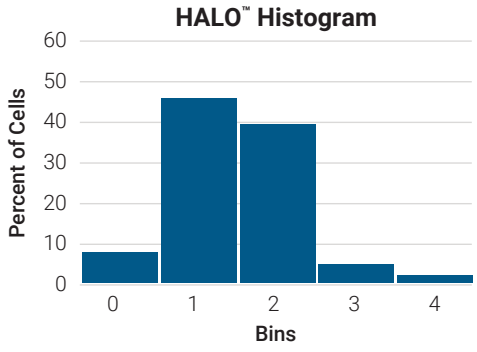
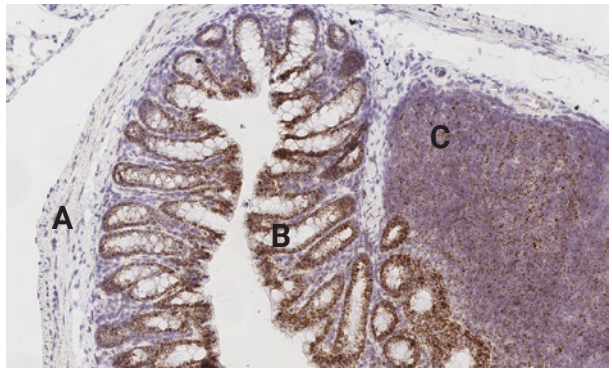


FIGURE 5. Heterogeneous expression quantification and H score with HALO™ analysis. H-score is assigned based on the number of cells with the same range of number of dots per cell. A similar analysis can be done manually by a pathologist or trained scientist.

3: Target expression in ≥2 different cell types

Scenario description

In this scenario the target of interest is expressed in two or more distinct cell types (Figure 6). In this scenario both the expression level and the percent of cells expressing the target at different levels could be of importance.



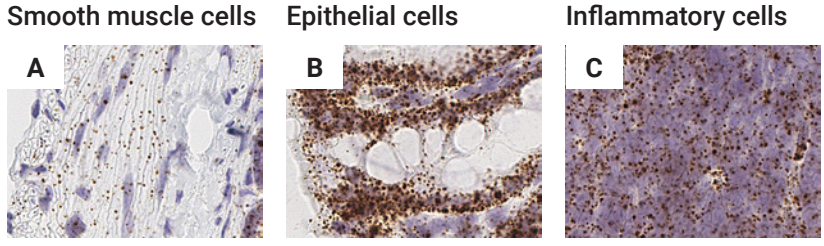


FIGURE 6. Detection of *Ctnnb1* (*β-catenin*) expression in mouse colon with TNBS-induced colitis. Here we can observe *Ctnnb1* expression in smooth muscle cells (muscularis propria) (A), colonic epithelial cells (B), and inflammatory cells (lymphoid aggregate) (C).

Analysis guidelines

In the scenario that a biomarker or target is expressed in two or more different cell types, one can analyze both cell types independently according to methodologies #1 and #2. H score (methodology #3) can also be derived, but it is recommended to use software analysis. The analyses can also be combined to calculate the percentage positive for each cell type of interest (Table 2).

Sample ID	Score for Cell Type 1	Cell Type 1 Percentage Positive	Score for Cell Type 2	Cell Type 2 Percentage Positive
Sample ID #1	0	4%	2	22%
Sample ID #2	3	33%	3	15%
Sample ID #3	3	67%	1	80%

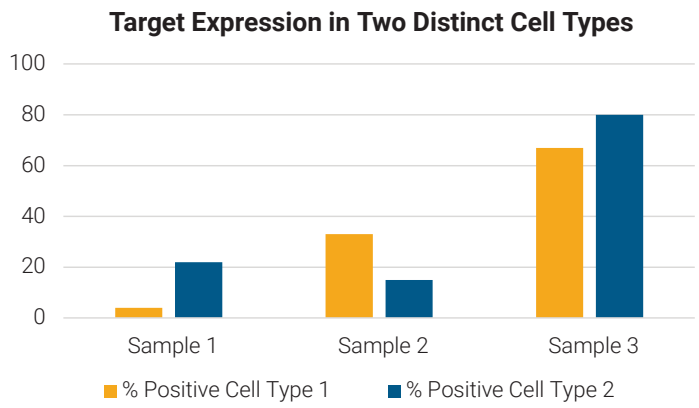


TABLE 2. Example output showing methodology #1 with percentage positive.

Disclaimer: Numerical values listed in tables are example outputs for illustration purposes only and are not a reflection of the images presented.

4: Subpopulation or region-specific target expression

Scenario description

In this scenario the target of interest is specifically expressed in a (sub)population of cells or a particular region (Figures 8 and 9). In this scenario both the expression level and the percent of cells expressing the target at different levels could be of importance.

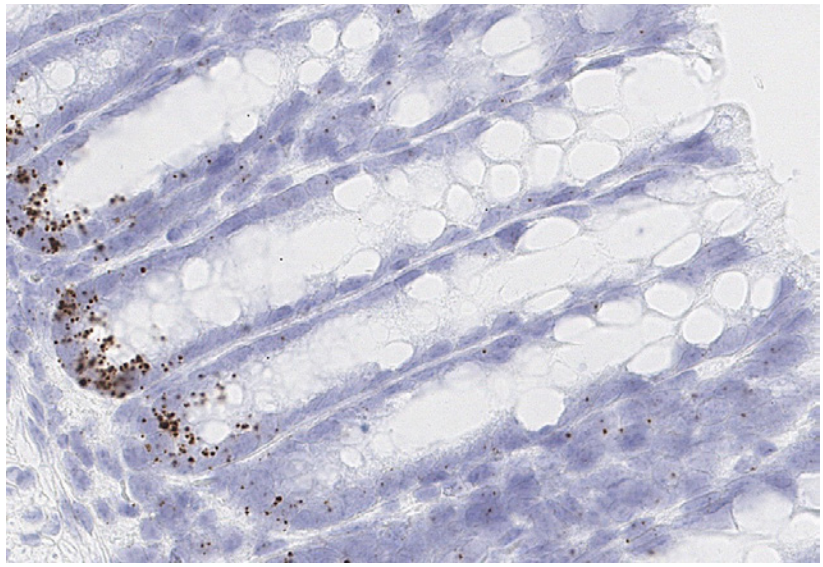


FIGURE 7. Region-specific expression pattern for *Lgr5* in mouse colon using the RNAscope® 2.5 HD BROWN assay.

Disclaimer: Numerical values listed in tables are example outputs for illustration purposes only and are not a reflection of the images presented.

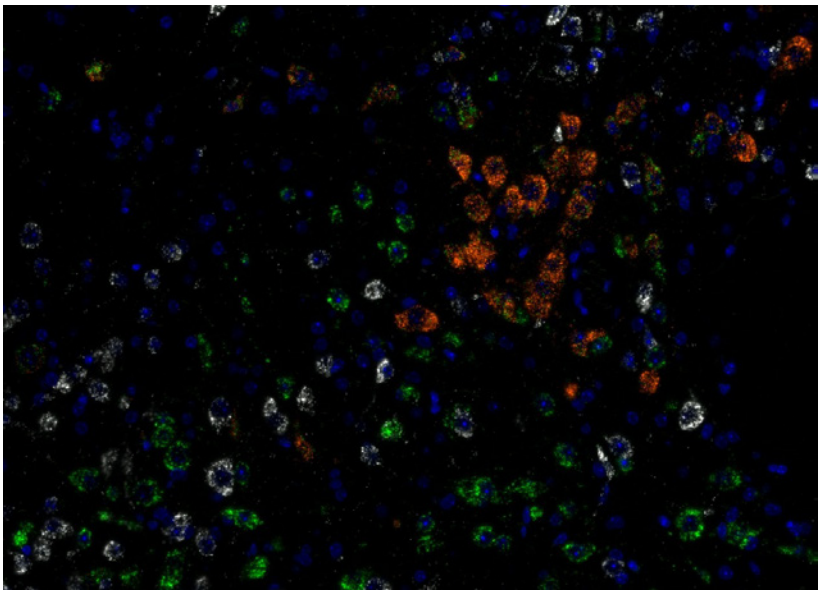


FIGURE 8. Subpopulation-specific expression for *Vglut1* (red) and *Vglut2* (green) on glutamatergic neuronal populations and *Vgat* (white) on GABAergic neurons in normal mouse brain using the RNAscope® LS Multiplex Fluorescent Assay. Cells are counterstained with DAPI (blue).

Analysis guidelines

In the scenario that a biomarker or target is expressed in a specific (sub)population of cells or a specific region, one can specifically analyze this relevant cell (sub)population or region according to methodologies #1 and #2. H score (methodology #3) can also be derived, but it is recommended to use software analysis. Furthermore, percentage positive cells can be assessed based on number of cells with ≥1 dot/cell.

Sample ID	Avg Dots/ Cell	% Cells in Bin 0	% Cells in Bin 1	% Cells in Bin 2	% Cells in Bin 3	% Cells in Bin 4	H-Score	% Positive Cells
Region #1	0.55	69.68	29.86	0.45	0.00	0.00	30.77	30.3
Region #2	0.74	77.23	21.28	1.47	0.02	0.00	24.27	25.5
Region #3	1.55	54.88	40.24	4.88	0.00	0.00	50.00	42.7

TABLE 3. Example output showing methodologies #2 and #3 with percentage positive.

5: Subcellular localization of target expression

Scenario description

In this scenario RNA can be expressed in a particular subcellular compartment, eg. nuclei or cytoplasm, and the expression level within that particular subcellular compartment could be of importance (Figures 9 and 10).

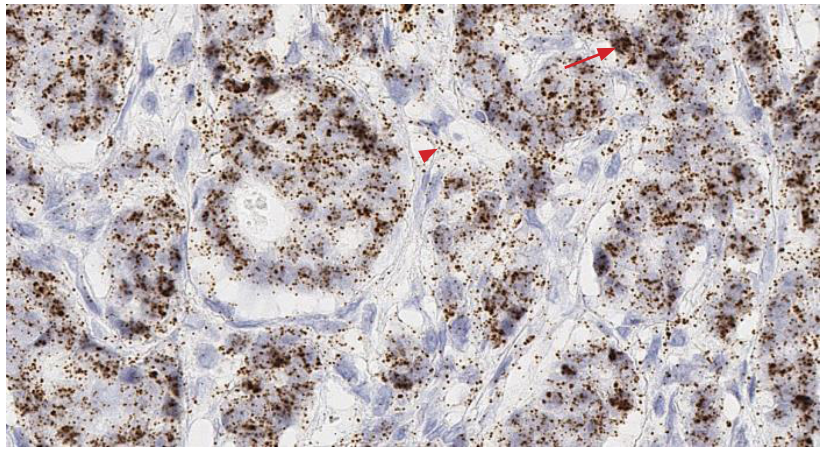


FIGURE 9. Subcellular expression of *GAS5* in breast cancer using the RNAscope® 2.5 HD BROWN assay. *GAS5* is detected in both the nucleus (arrow) and cytoplasm (arrow head).

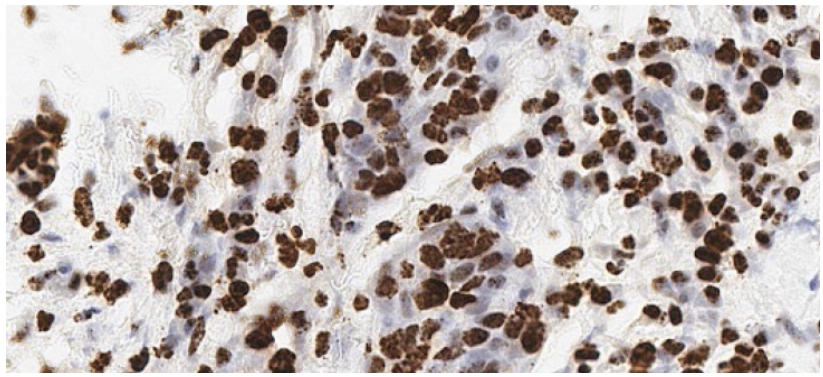


FIGURE 10. Subcellular expression of *MALAT1* in lung cancer using the RNAscope® 2.5 HD BROWN assay. *MALAT1* is detected primarily in the nucleus.

Analysis guidelines

In the scenario that a biomarker or target is expressed in a specific subcellular compartment data analysis can be challenging due to 2D representation (the image) of a 3 dimensional structure (the tissue). However, a qualitative analysis of the relative expression within each compartment could be informative and methodologies #1 and #2 can be applied for quantification. Furthermore, percentage positive cells can be assessed based on number of cells with ≥1 dot/cell.

6: Qualitative assessment for presence or absence of a target

Scenario description

For certain disease assessments, a qualitative analysis with simply a positive or negative for the presence of a marker could be more important than quantifying the expression of the marker (Figure 11).

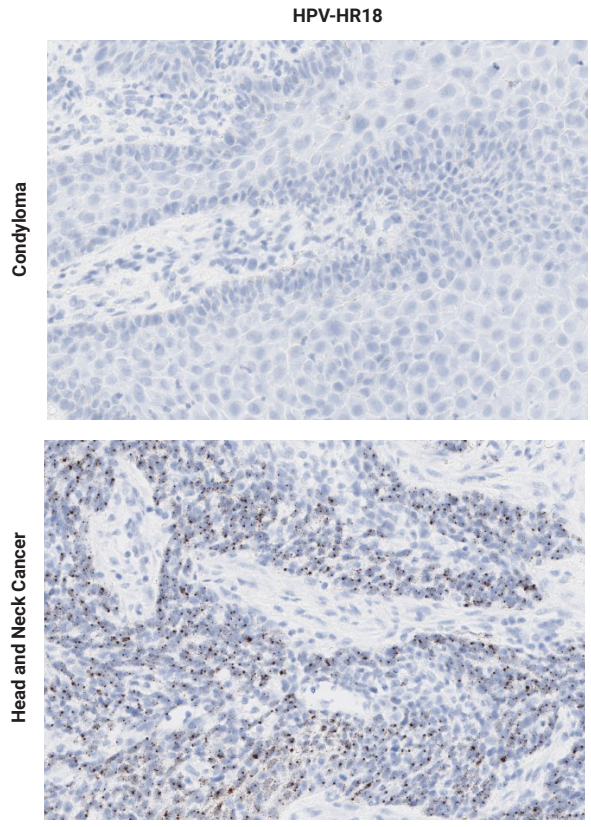


FIGURE 11. Detection of HPV infection in human samples using the RNAscope® 2.5 HD BROWN assay. The probe HPV-HR18 detects 18 high risk subtypes of the HPV E6/E7 gene. The condyloma tissue sample is negative for high risk HPV, whereas the Head and Neck tumor sample is positive for high risk HPV.

Analysis guidelines

Within the cell population of interest, the assessment of marker expression needs to be reviewed in conjunction with the positive and negative control slides. If the positive and negative control slides have stained appropriately, and depending on the threshold previously set to determine positivity for that particular marker, the cells of interest could be called positive or negative for the marker.

Duplex/multiplex target expression scenarios

1. **Expression of 2 independent targets in different cell types**
2. **Target co-expression in a particular cell type**
3. **Target expression in a rare cell type**
4. **Cell population interactions**
5. **Multiplex target expression**

1: Expression of 2 independent targets in different cell types

Scenario description

For this scenario the assay simultaneously detects the expression pattern of two genes of interest. In this scenario both the expression level and the percent of cells expressing each of the targets could be of importance and therefore can be analyzed according to the singleplex scenarios applicable for each of the targets (Figures 12 and 13).

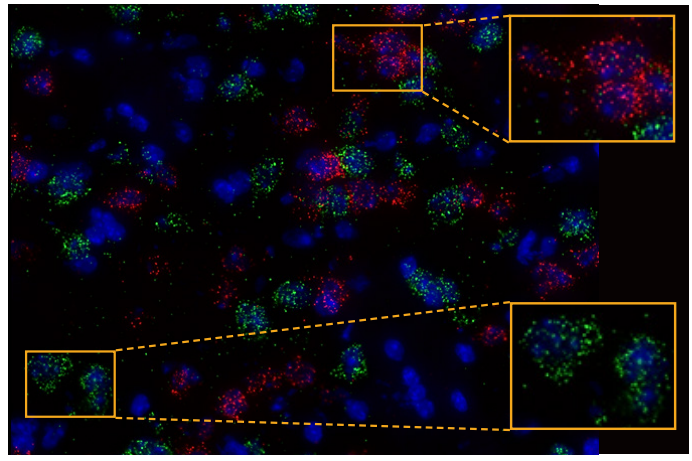


FIGURE 12. *Drd1* (red) and *Drd2* (green) expression on distinct medium spiny neuronal cell populations in the striatum of normal mouse brain using the RNAscope® Multiplex Fluorescent Assay. Cells are counterstained with DAPI (blue).

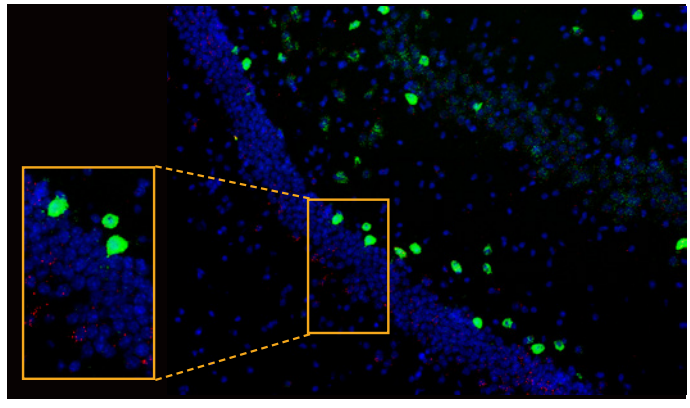
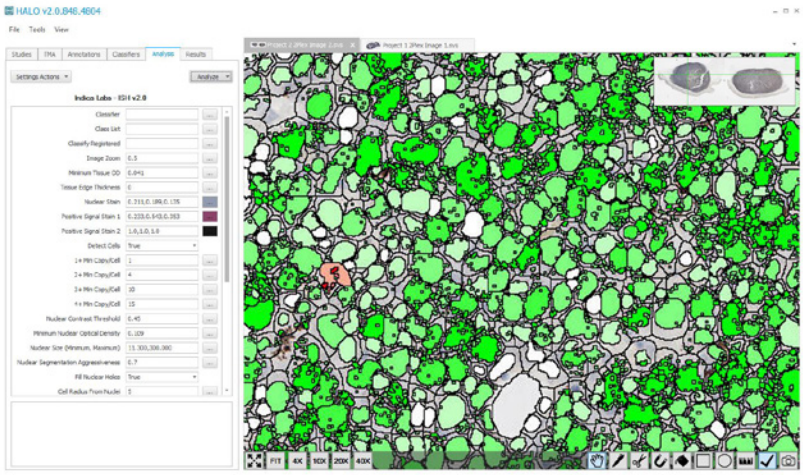
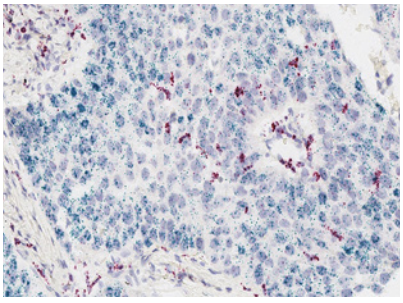


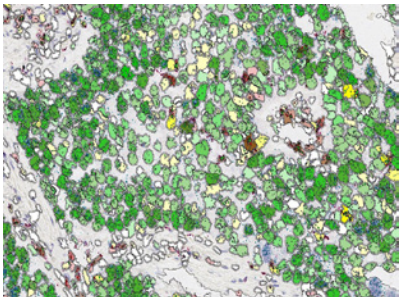
FIGURE 13. *Cnr1* (green) and *Drd1* (red) expression in the hippocampus of normal mouse brain using the RNAscope® Multiplex Fluorescent Assay. Cells are counterstained with DAPI (blue).



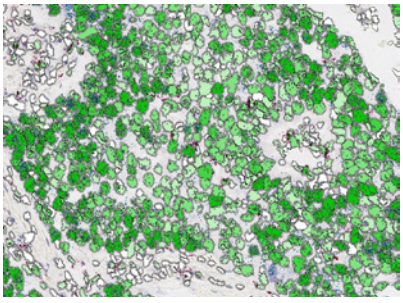
RNAscope® Assay



Dual Probe Markup



Green Probe Markup



Red Probe Markup

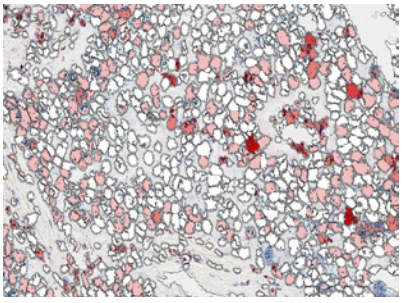


FIGURE 14. Example of a HALO™ analysis on lung cancer tissue stained with the RNAscope® 2.5 HD Duplex assay for *CD45* (red) and *PD-L1* (green).

Analysis guidelines

To determine the target expression for each of the genes within a particular cell type, one can qualitatively and quantitatively assess the expression pattern by using methodologies #1, #2 and #3. These methodologies can be combined to create percentage of cells positive. Cells can be scored visually at 40x based on number of cells with ≥1 dot/cell across the entire tissue section. Percent dual positive can be calculated in two ways (Table 4):

$$1)\ \frac{A+B}{\text{Total Number of Cells}} \qquad 2)\ \frac{A+B}{\text{Total Cells A}} \quad \text{or} \quad \frac{A+B}{\text{Total Cells B}}$$

		Sample #1	Sample #2
Target A (green)	Avg Dots/Cell	0.65	2.26
	% Cells in Bin 0 (0 Dots/Cell)	64.03	27.67
	% Cells in Bin 1 (1–3 Dots/Cell)	32.92	49.01
	% Cells in Bin 2 (4–9 Dots/Cell)	2.93	21.57
	% Cells in Bin 3 (10–15 Dots/Cell)	0.10	1.50
	% Cells in Bin 4 (>15 Dots/Cell)	0.02	0.25
	% Positive Cells	35.97	72.33
	H–Score	39.16	97.64
Target B (red)	Avg Dots/Cell	0.04	0.35
	% Cells in Bin 0 (0 Dots/Cell)	97.08	77.40
	% Cells in Bin 1 (1–3 Dots/Cell)	2.79	21.61
	% Cells in Bin 2 (4–9 Dots/Cell)	0.13	0.97
	% Cells in Bin 3 (10–15 Dots/Cell)	0.00	0.00
	% Cells in Bin 4 (>15 Dots/Cell)	0.00	0.01
	% Positive Cells	2.92	22.60
	H-Score	3.07	23.61
	% Dual Positive Cells	2.40	19.14
	% Dual Negative Cells	63.50	24.22

TABLE 4. Example output showing methodologies # 2 and # 3 with percentage positive and negative.

Disclaimer: Numerical values listed in tables are example outputs for illustration purposes only and are not a reflection of the images presented.

2: Target co-expression in a particular cell type

Scenario description

In this scenario cells co-express two genes simultaneously, enabling identification of the target of interest along with the cell type that expresses that target of interest or the co-expression of two particular targets (Figures 15–18).

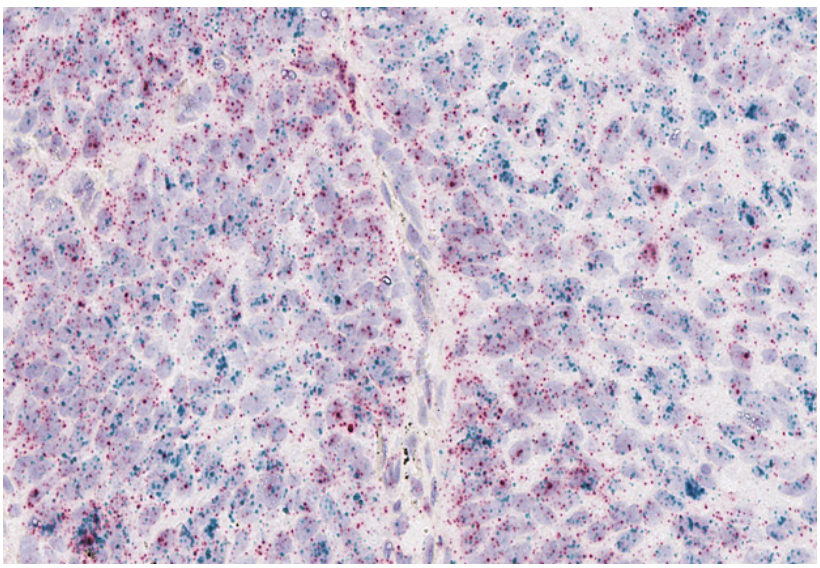


FIGURE 15. Co-expression of *PD-L1* (green) and *TGFβ1* (red) in lung tumor cells using the RNAscope® 2.5 HD Duplex assay.

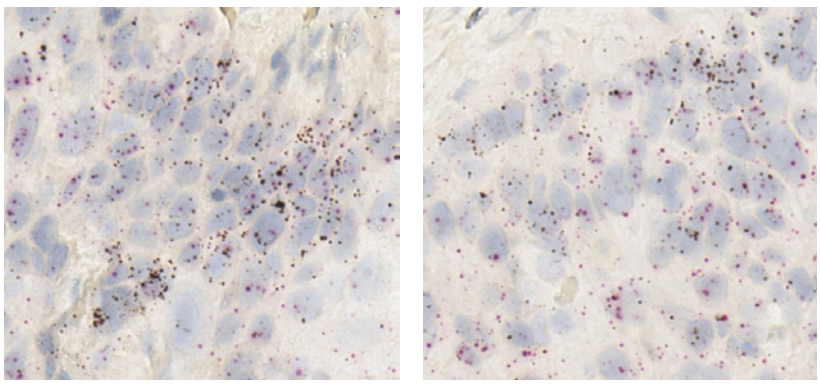


FIGURE 16. Co-expression of the *NRG1* ligand (brown) and the *ERBB3* receptor tyrosine kinase (red) in esophageal tumor cells using the RNAscope® 2.5 LS Duplex assay.

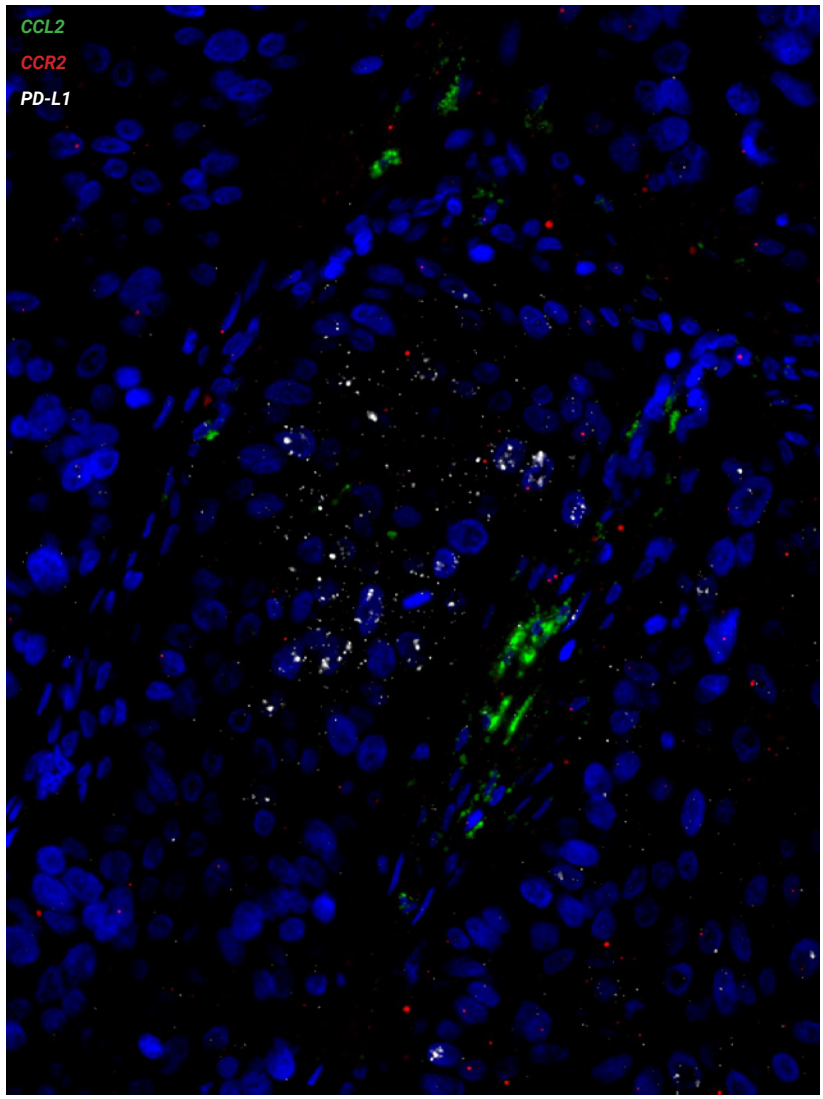


FIGURE 17. Co-expression of the *CCL2* chemokine (green) and the *CCR2* chemokine receptor (red) in lung cancer using the RNAscope® 2.5 LS Multiplex Fluorescent Assay. Cells are counterstained with DAPI (blue). Also shown is *PD-L1* in white.

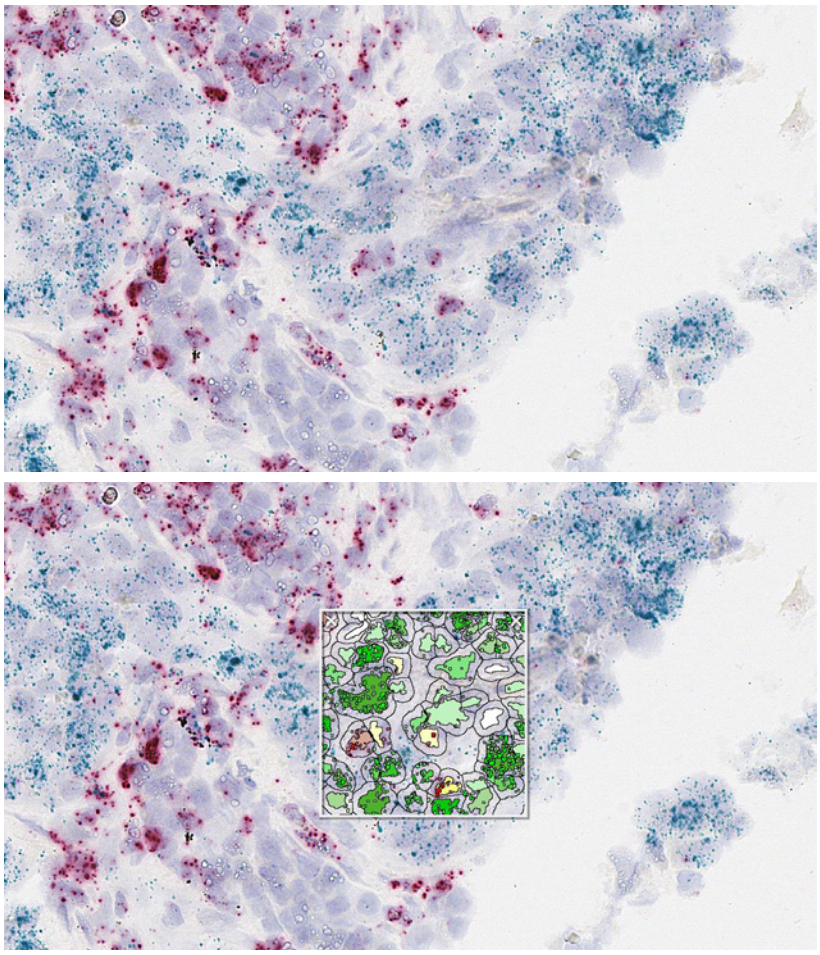


FIGURE 18. Co-expression analysis of *TIM3* (red) and *PD-L1* (green) mRNA in lung cancer using the RNAscope® 2.5 HD Duplex assay. Here we can observe cells singly expressing each marker (red and green shaded output) as well as cells co-expressing both markers (yellow shaded cell output) in a tumor compartment.

Analysis guidelines

To determine the target co-expression, one can qualitatively assess the degree of simultaneous co-expression of the target with the cell type marker or both targets of interest or perform image-based quantitative software analysis to obtain this information. Hereto, methodologies #1, #2 and #3 can be applied.

To determine co-expression across markers and positivity in specific cell populations, these methodologies can be combined to calculate percentage of cells positive. Cells can be scored visually at 40x based on number of cells with ≥1 dot/cell across the entire tissue section. Percent dual positive is defined as number of cells positive for both Target 1 and Target 2 / total number of cells (see Table 4).

3: Target expression in a rare cell type

Scenario description

In this scenario a small number of cells show expression for a particular target and therefore identifying the number of cells that express the target of interest could be more relevant than the average expression level per cell (Figure 19).

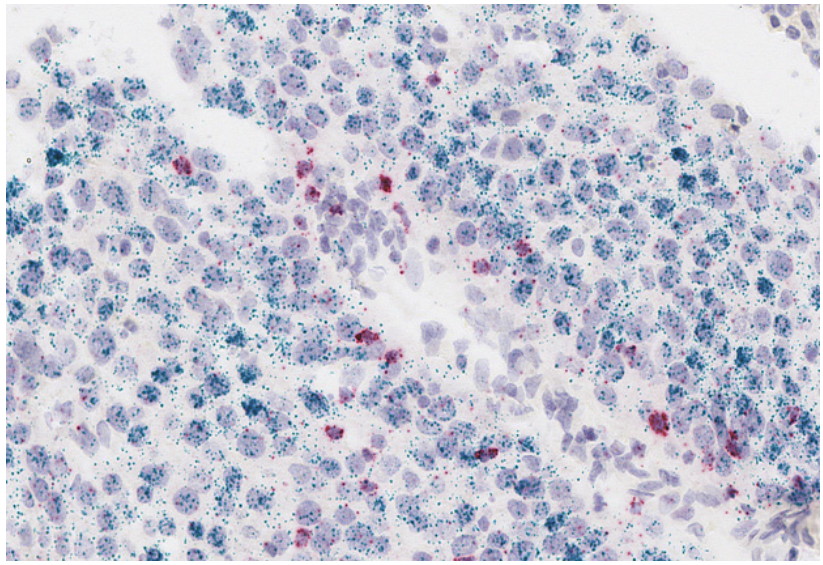


FIGURE 19. *CD8*-positive T cells (red) infiltrating a *PD-L1*-positive (green) tumor region in lung cancer using the RNAscope® 2.5 HD Duplex assay.

Analysis guidelines

To determine the rare cell expression, one can qualitatively and quantitatively assess the expression pattern by using methodologies #1 and #2.

4: Cell population interactions

Scenario description

In this scenario specific cell type populations and their spatial relationship to one another are being investigated (Figure 20). Hereto, one can assess the number of cells expressing the targets of interest and their proximity to one another within the tissue environment.

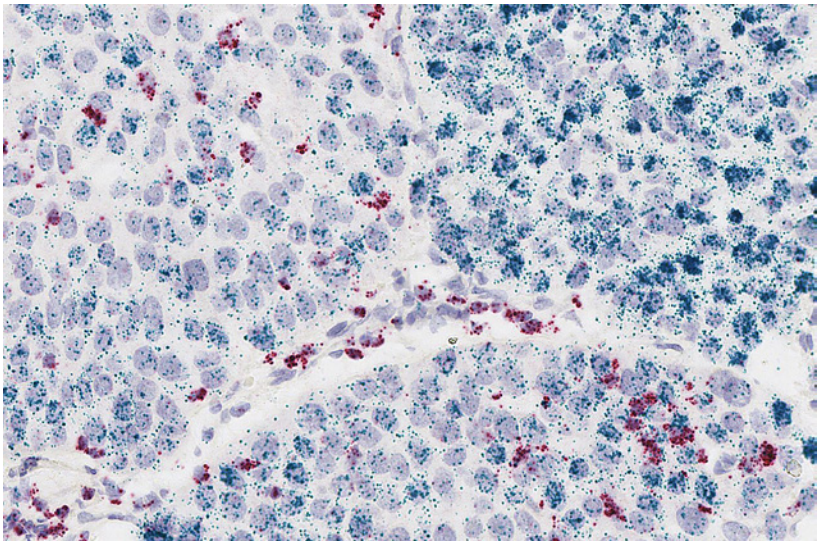


FIGURE 20. The spatial relationship of *CD45*-positive cells (red) with *PD-L1*-positive (green) tumor cells in lung cancer using the RNAscope® 2.5 HD Duplex assay.

Analysis guidelines

One can qualitatively and quantitatively assess the expression pattern by using methodologies #1 and #2.

5: Multiplex target expression

Scenario description

In this scenario, multiple target expression patterns in one or more cell types are being investigated (Figures 21, 22 and 23).

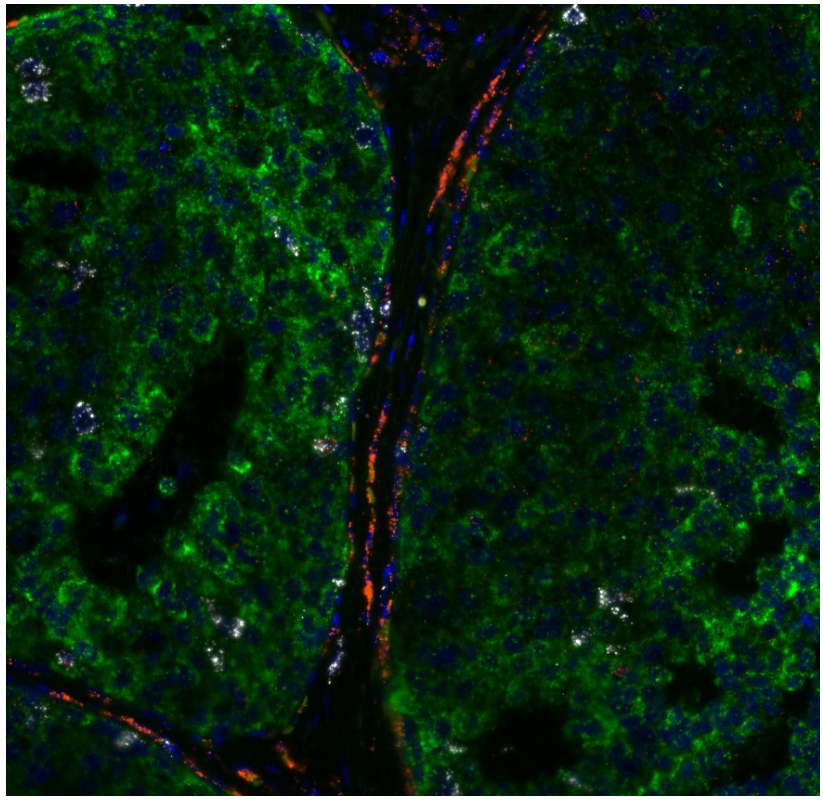


FIGURE 21. Multiplex expression of *KRT19* (green), *PECAM1* (red) and *MKI67* (white) in human breast cancer using the RNAscope® 2.5 LS Multiplex Fluorescent Assay. Cells are counterstained with DAPI (blue).

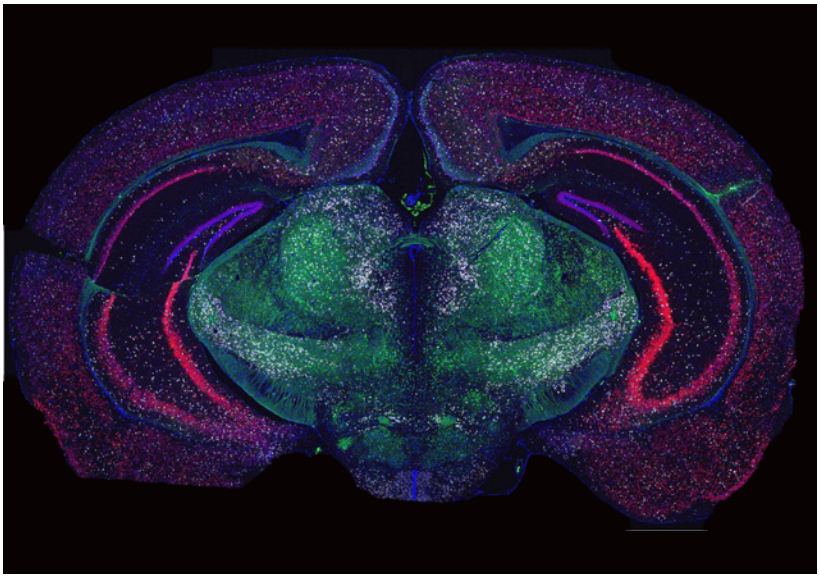


FIGURE 22. Multiplex expression pattern for *Vglut1* (red), *Vglut2* (green) and *Vgat* (white) in mouse brain using the RNAscope® 2.5 LS Multiplex Fluorescent Assay. Cells are counterstained with DAPI (blue).

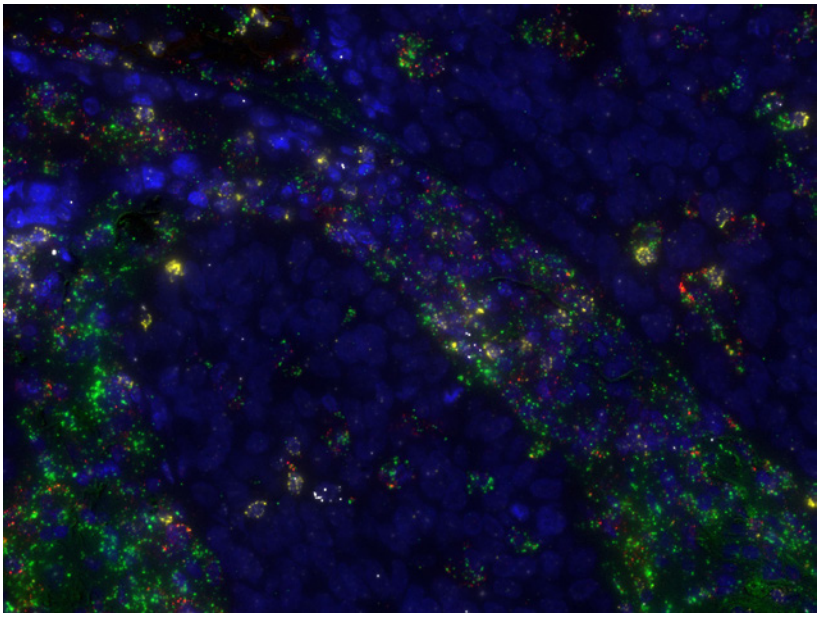


FIGURE 23. Multiplex expression of *TGFβ1* (green), *CD8A* (yellow), *CD4* (red) and *TNFα* (white) in human lung cancer using the RNAscope® 2.5 LS Multiplex Fluorescent Assay. Cells are counterstained with DAPI (blue).

Analysis guidelines

One can qualitatively and quantitatively assess the expression pattern by using methodologies #1, #2 and #3. For further guidelines on how to quantify RNAscope® fluorescent assay results see Tech Note SOP 45-006.

To understand co-localization across markers and positivity in specific cell populations, percentage of cells positive can be scored visually at 40x based on number of cells with ≥1 dot/cell.

		Sample #1	Sample #2	Sample #3
Target A	Avg Dots/ Cell	0.68	0.08	0.26
	% Positive Cells	5.36	14.94	11.30
	H–Score	2.17	4.00	17.59
Target B	Avg Dots/Cell	1.10	2.27	1.05
	% Positive Cells	20.12	36.64	27.64
	H-Score	164.27	67.78	111.34
Target C	Avg Dots/Cell	2.09	0.81	8.62
	% Positive Cells	76.66	49.02	60.50
	H-Score	79.97	60.39	3.22
A+B Dual Positive Cells		74.75	91.96	23.08
B+C Dual Positive Cells		30.00	34.82	39.57
A+C Dual Positive Cells		39.57	35.56	47.57
A+B+C Triple Positive Cells		9.00	47.39	66.94

TABLE 5. Example output showing methodologies #2 and #3 with percentage positive and negative.

Disclaimer: Numerical values listed in tables are example outputs for illustration purposes only and are not a reflection of the images presented.

References

The following is a list of publications in which the authors have used the RNAscope® multiplex fluorescent assay and therefore can be used as reference to see examples of how users have represented their target multiplex expression data:

1. **Borel, F., *et al.* (2016).** "Therapeutic rAAVrh10 Mediated SOD1 Silencing in Adult SOD1(G93A) Mice and Nonhuman Primates." *Hum Gene Ther* 27(1): 19–31.

2. **Branco, T., *et al.* (2016).** "Near-Perfect Synaptic Integration by Nav1.7 in Hypothalamic Neurons Regulates Body Weight." *Cell* 165(7): 1749–1761.

3. **Cosker, K. E., *et al.* (2016).** "The RNA-binding protein SFPQ orchestrates an RNA regulon to promote axon viability." *Nat Neurosci* 19(5): 690–696.

4. **Greer, P. L., *et al.* (2016).** "A Family of non-GPCR Chemosensors Defines an Alternative Logic for Mammalian Olfaction." *Cell* 165(7): 1734–1748.

5. **Keeler, A. M., *et al.* (2016).** "Cellular Analysis of Silencing the Huntington's Disease Gene Using AAV9 Mediated Delivery of Artificial Micro RNA into the Striatum of Q140/Q140 Mice." *J Huntingtons Dis* 5(3): 239–248.

6. **Tan, S. H., *et al.* (2014).** "Wnts produced by Osterix-expressing osteolineage cells regulate their proliferation and differentiation." *Proc Natl Acad Sci USA* 111(49): E5262–5271.

7. **Ziskin, J. L., *et al.* (2013).** "*In situ* validation of an intestinal stem cell signature in colorectal cancer." *Gut* 62(7): 1012–1023.

Appendix

Validation of NGS and RT-PCR with RNAscope®

Transcriptomics validation by RNAscope® expression analysis

High-throughput transcriptome analyses generate a wealth of gene expression data but are often in need of validation within the tissue environment. The RNAscope® technology offers single-cell resolution for gene expression profiling with spatial information as exemplified by Lake *et al.* 2016. "Neuronal subtypes and diversity revealed by single-nucleus RNA sequencing of the human brain." *Science* 352: 1586–90).

This paper demonstrates a robust and scalable method for identifying and categorizing single nuclear transcriptomes, revealing shared genes sufficient to distinguish previously unknown and orthologous neuronal subtypes as well as regional identity and transcriptomic heterogeneity within the human brain. RNAscope® ISH for a set of selected markers confirmed subtype- and layer-specific expression patterns in the cortex as revealed by single-nucleus RNA sequencing of the human brain.

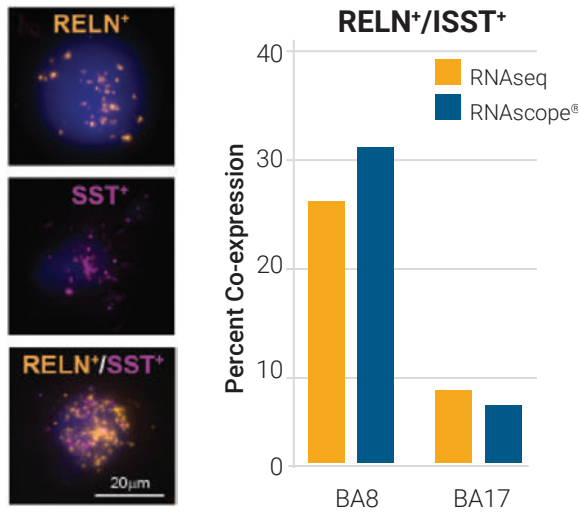
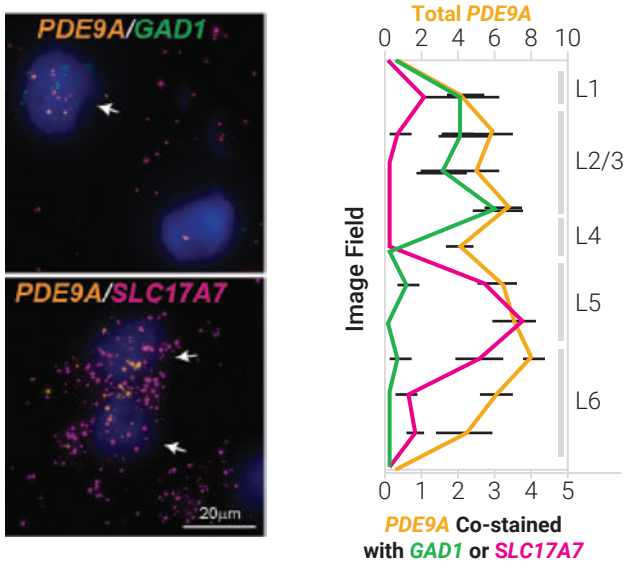


FIGURE 24. RNAscope® co-staining of *RELN* and *SST* in human brain areas BA8 and BA17 shows co-positive cell distributions that are consistent with RNA-seq data.



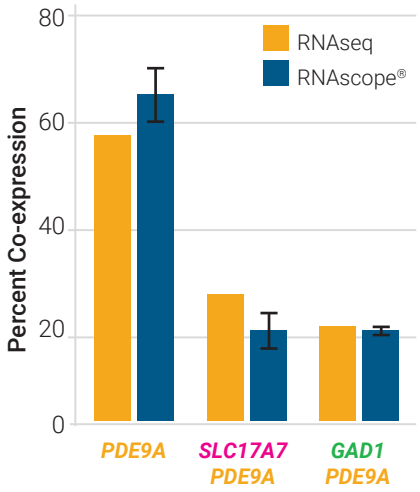


FIGURE 25. *PDE9A*-positive expression in inhibitory and excitatory neuronal subtypes was confirmed by RNAscope® co-staining with *GAD1* (double positive restricted to layers 2/3) and *SLC17A7* (double positive restricted to layers 5/6). RNAscope® data is consistent with RNA-seq data.

RT-PCR validation by RNAscope® expression analysis

Quantitative real-time PCR analyses are often in need of expression validation within the tissue environment. The RNAscope® technology offers single cell resolution for gene expression profiling with spatial information as exemplified by Miner *et al.* 2016. ("Zika Virus Infection in Mice Causes Panuveitis with Shedding of Virus in Tears". *Cell Report*, 16:3208–18).

This paper describes how Zika virus (ZIKV) infection in the eye results in inflammation and injury. ZIKV infected the iris, cornea, retina, and optic nerve and caused conjunctivitis, panuveitis, and neuroretinitis in mice. This manuscript establishes a model for evaluating treatments for ZIKV-induced infections in the eye. RNAscope® ISH for ZIKV RNA was used to confirm qRT-PCR results for viral burden.

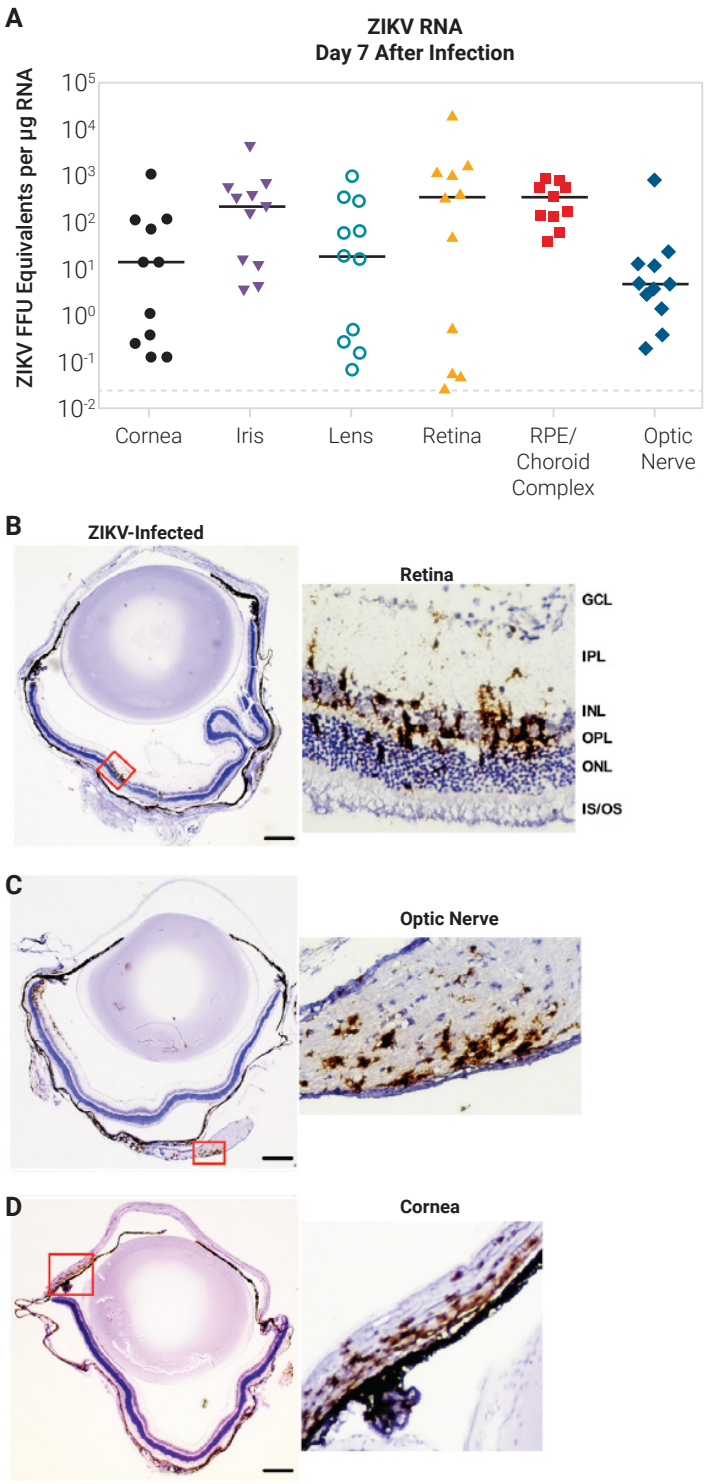


FIGURE 26. The RNAscope® assay confirms RT-PCR findings. ZIKV-infected mouse eyes were assessed for viral RNA by RNAscope® ISH. Abundant ZIKV RNA was apparent in the bipolar and ganglion cell neurons of the neurosensory retina (B), the optic nerve (C), and the cornea of infected animals (D).

Notes

Notes

Notes

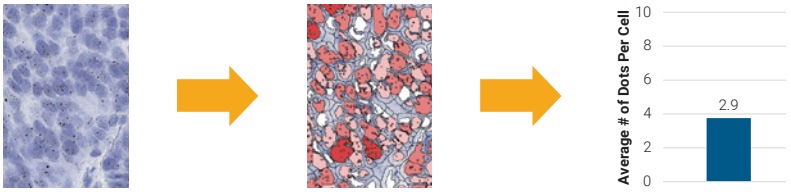
Unfold for a quick reference guide to the three types of analysis methodologies

Methodologies overview

Methodology #1 (see page 5 for more information)
Semi-quantitative histological assessment of the target expression level within a cell population or area of interest using ACD scoring criteria

ACD Score	Scoring Criteria
0	No staining or <1 dot/ 10 cells
1	1–3 dots/cell
2	4–9 dots/cell and none or very few dot clusters
3	10–15 dots/cell and/or <10% dots are in clusters
4	>15 dots/cell and/or >10% dots are in clusters

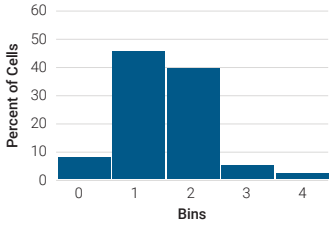
Methodology #2 (see page 6 for more information)
Quantitative image-based assessment of the target expression level within a cell population or area of interest using quantitative digital image analysis software.



Methodology #3 (see pages 10– 11 for more information)
H score: semi-quantitative or quantitative image-based software analysis to visualize the dynamic expression level by binning the percentage of cells with a certain expression level or number of dots in one bin. The number of bins ranges from 0–4 according to the ACD scoring system. The overall H score can range from 0–400 and is calculated as shown:

$$\text{H-score} = \sum_{\text{Bin } 0 \rightarrow 4} (\text{ACD score or bin number} \times \text{percentage of cells per bin})$$

	% of Cells	Weighted Formula
Bin 0 (0 Dots/Cell)	8	0 * 8
Bin 1 (1–3 Dots/Cell)	46	+ 1 * 46
Bin 2 (4–9 Dots/Cell)	39	+ 2 * 39
Bin 3 (10–15 Dots/Cell)	5	+ 3 * 5
Bin 4 (>15 Dots/Cell)	2	+ 4 * 2
H-Score		147



ACD™
a bio-techne brand

California, USA