Combining the RNAscope ISH technology with IHC to spatially resolve RNA and protein targets simultaneously

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Highlights

The combined RNAscope ISH-IHC/IF workflow can detect RNA and protein targets on the same sample with high sensitivity and specificity at the single cell level while preserving the tissue morphology. This report presents the applications of the dual ISH-IHC/IF workflow in neuroscience, immuno-oncology, and beyond. This technique can be used to:

- Characterize immune cells
- Identify the cellular source of secreted proteins
- Visualize neuronal cell type markers
- Confirm trafficking and activation of CAR T cells

Spatially resolved gene expression has emerged as a crucial technique to understand complex multicellular interactions within the tissue. Traditional tissue-based assays such as RNA *in situ* hybridization (ISH) and immunohistochemistry (IHC) or immunofluorescence (IF) allow gene expression analysis with spatial resolution at the RNA and protein level, respectively. Traditional RNA ISH has been challenging due to low sensitivity and specificity, as well as time-consuming and cumbersome to perform. The highly sensitive and specific RNAscope™ technology has been developed to overcome the limitations of a traditional RNA ISH while also providing robust single molecule RNA detection with single cell resolution.[1]

To interrogate complex cellular interactions within a tissue, researches require a multi-omics approach where multiple RNA and protein targets can be visualized within the same tissue sample. Simultaneous detection of RNA and proteins can reveal cellular sources of secreted proteins, identify specific cell types, and visualize the spatial organization of cells within the tissue.[2] Given the similarities in workflow, the RNAscope ISH assay can be combined with IHC/IF to achieve simultaneous visualization of RNA and protein on the same sample. Referred to as dual ISH-IHC/IF, this workflow has been used extensively in neurobiology, immuno-oncology[3], developmental biology[4], cell and gene therapy and inflammation[5], among other research areas.

This report gives recommendations for selecting antibodies and performing the dual ISH-IHC/IF technique (Figure 1, 2) and explores the natural complementarity of RNAscope ISH with IHC/IF in three major application areas: neurobiology, immuno-oncology, and cell and gene therapy.

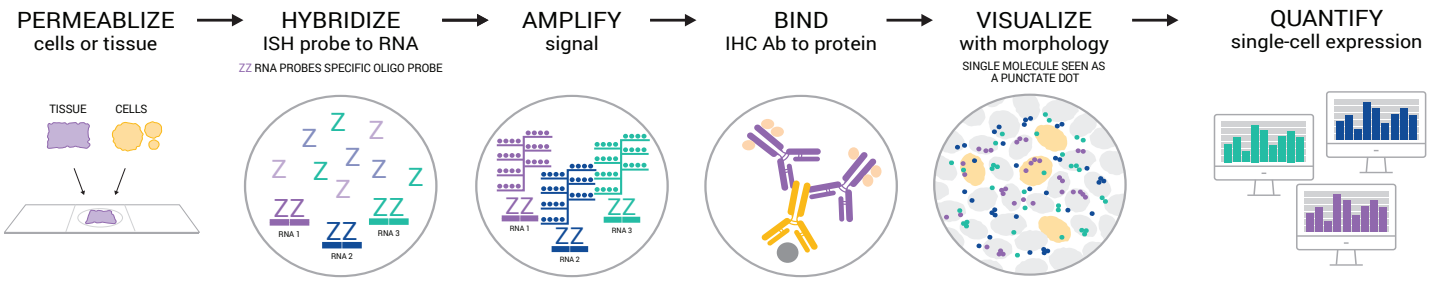
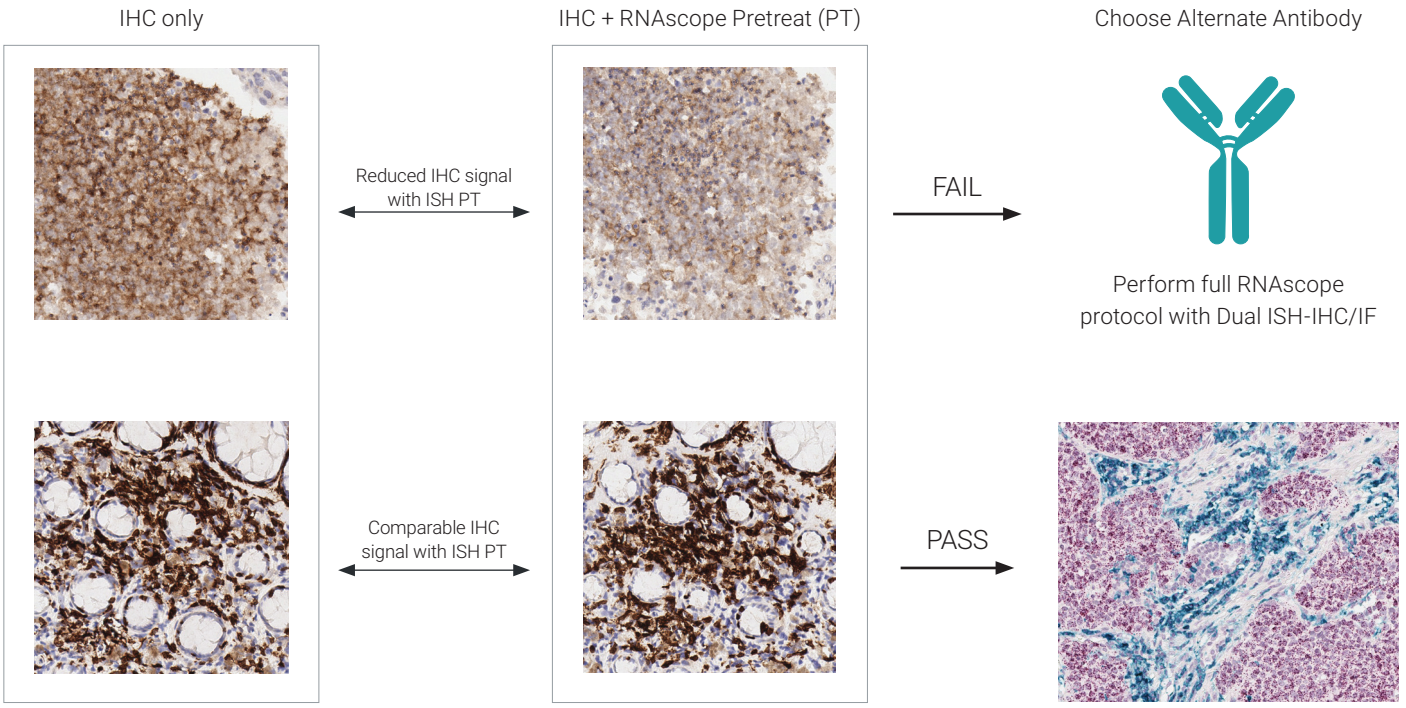


FIGURE 1. Dual ISH-IHC/IF workflow for visualizing RNA and protein targets using the RNAscope ISH technology followed by IHC/IF.

A



B

Immuno-oncology

ISH targets
- Checkpoint markers
- Cytokines and chemokines
- Tumor markers

IHC/IF targets
- Immune cell markers

Neurobiology

ISH targets
- Specific neuronal genes
- Circular RNAs
- Splice variants

IHC/IF targets
- Neuronal cell markers

Cell & Gene Therapy

ISH targets
- CAR 5' or 3' UTR
- T cell activation markers
- AAV vector
- Transgene

IHC/IF targets
- Immune cell markers
- Cell type-specific markers

IHC Validation
- Validate antibody specificity and protein localization

FIGURE 2. (A) Antibody validation for dual ISH-IHC/IF with the RNAscope assay. (B) Key applications of the dual ISH-IHC/IF workflow.

Guidelines for Performing Dual ISH-IHC/IF

There are three general guidelines when performing the dual ISH-IHC/IF workflow. First, it is highly recommended that the RNAscope ISH assay be performed before the IHC/IF assay (Figure 1). Second, when selecting an antibody for the dual ISH-IHC/IF workflow, it is important to use an antibody that has been demonstrated to work well when IHC/IF is performed independently using conditions recommended by the manufacturer. Third, since the RNAscope pretreatment protocol includes protease treatment, verify that the protein of interest can withstand the pretreatment conditions by first performing the RNAscope pretreatment steps followed by the IHC/IF protocol. If there is no loss of IHC/IF signal and no increase in non-specific IHC/IF signal with the pretreatment, the antibody can be used for the dual ISH-IHC/IF workflow with target probes (Figure 2A). For more specific guidelines, including technical notes, please visit: www.acdbio.com/science/applications/research-solutions/dual-ish-and-ihc.

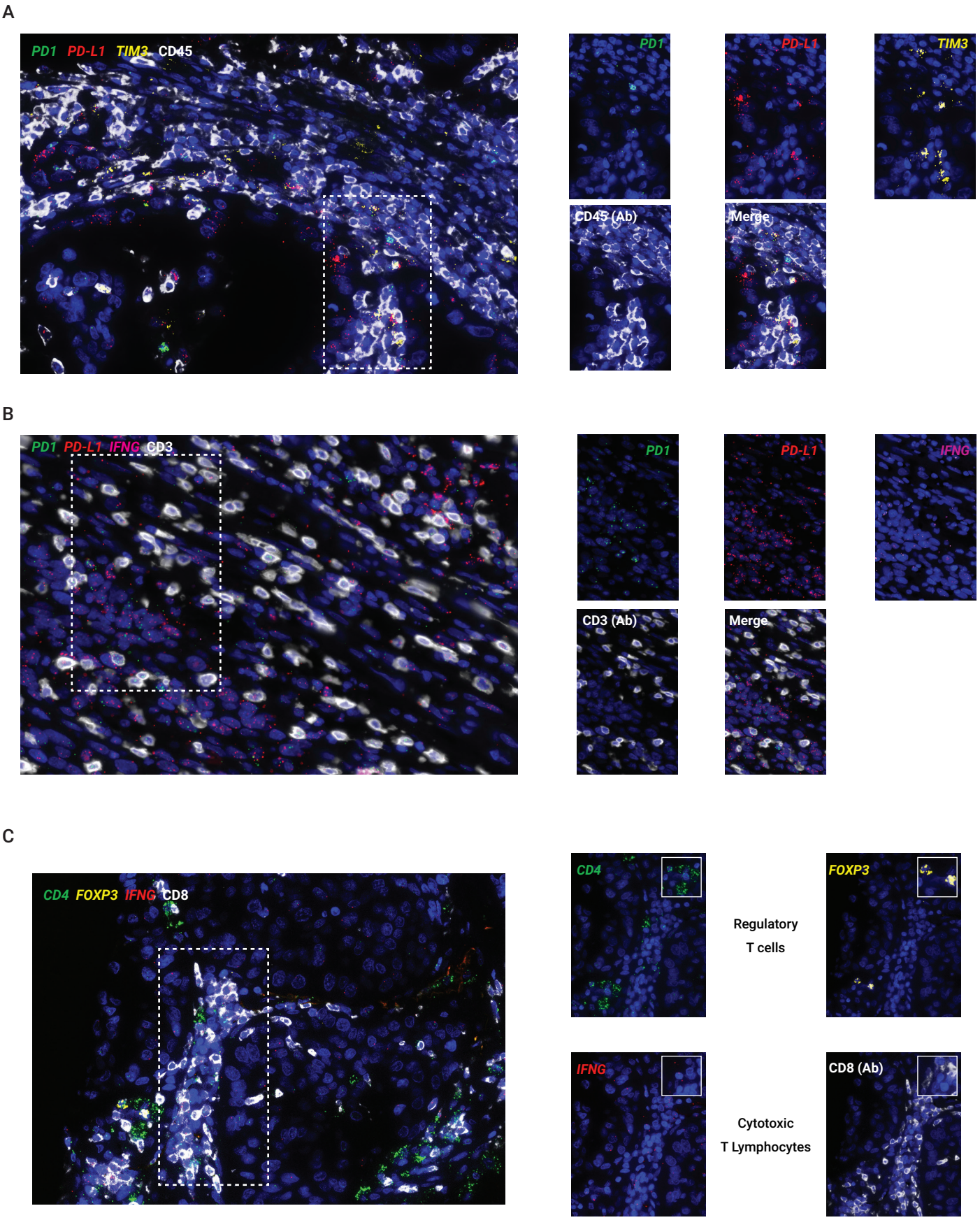
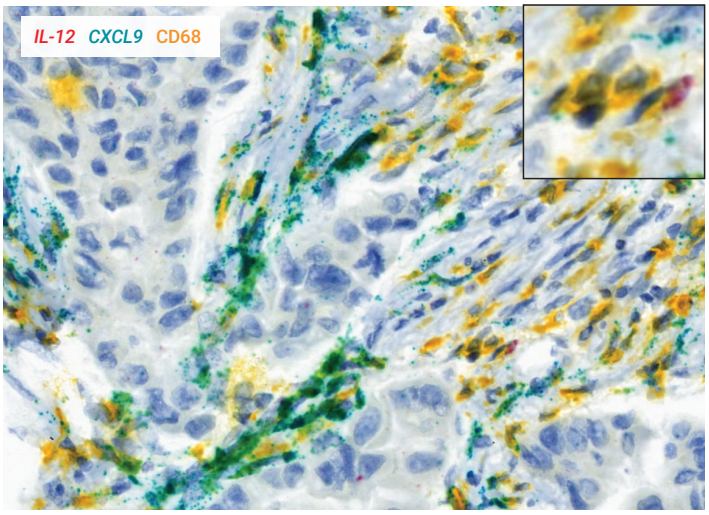


FIGURE 3. Dual ISH-IF to visualize immune cells and checkpoint markers in the tumor microenvironment. The RNAscope Multiplex Fluorescent V2 assay was combined with IF to detect: (A) *PD1* (green), *TIM3* (yellow), and *PDL1* (red) mRNA expression and CD45 (white) protein expression in lung tumor; (B) *IFNG* (pink), *PD1* (green), and *PD-L1* (red) mRNA expression and CD3 protein (white) expression in a cervical cancer tumor; (C) *CD4* (green)/*FOXP3* (yellow) dual positive regulatory T cells and *IFNG* (red)/CD8 (white) dual positive cytotoxic T lymphocytes in lung tumors.

A



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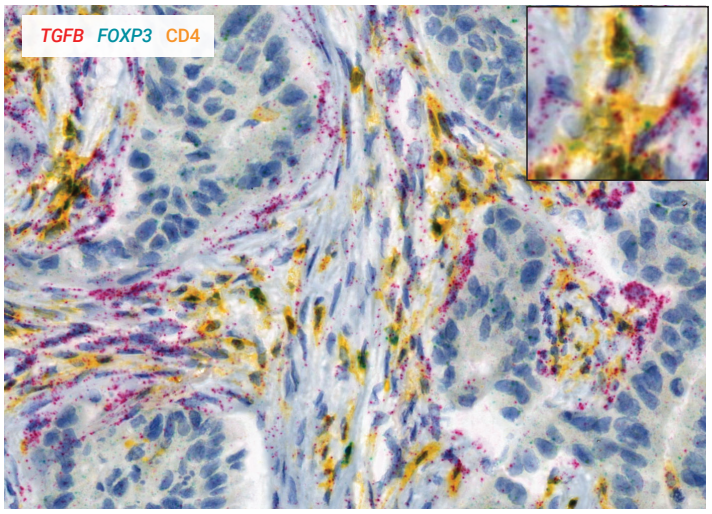

FIGURE 4. Visualization of immune cells, chemokines, and cytokines in human lung tumors using the RNAscope VS Duplex Assay combined with IHC. (A) RNA transcripts for secreted factors *IL-12* (red) and *CXCL9* (teal) were detected in combination with the macrophage marker protein CD68 (yellow). (B) RNA transcripts for the secreted factor *TGFB* (red) and the transcription factor *FOXP3* (teal) were detected in combination with the T cell marker protein CD4 (yellow).

Applications of the Dual ISH-IHC/IF Workflow

IDENTIFY CELLULAR SOURCE OF SECRETED PROTEINS

- By strategically selecting the ISH and IHC/IF markers, the RNAscope ISH assay can target the transcript for secreted proteins such as cytokines or chemokines and IHC/IF can target cell markers in order to identify the cellular source of secreted proteins (Figure 3B, 3C, 4, and 6).

CHARACTERIZE IMMUNE CELLS IN THE TME

- Immune cell infiltration into the tumor, their activation state, and checkpoint expression pattern can all be fully characterized with the dual ISH-IHC/IF method by using the RNAscope ISH assay to detect activation markers and IHC/IF to detect immune cell markers such as CD3, CD4, CD8, CD68, and CD45. (Figure 3, 4, and 6).

SPATIAL MAPPING OF NEURONAL SUBTYPES

- For spatial mapping of different neuronal subtypes within specific areas of the brain, the RNAscope ISH assay can detect specific target genes in neuronal subtypes and IHC/IF can be used to label neuronal cells (Figure 5A).
- Comprehensive spatial transcriptomic analysis can also be achieved by using the RNAscope HiPlex ISH assay for up to 12 targets in combination with IF (Figure 5B).
- The BaseScope ISH assay can also be combined with IHC/IF to identify cell type-specific expression of splice variants and circRNAs (Figure 5C).

DETECTION OF ACTIVATED CAR T CELLS

- Dual ISH-IF/IHC can also be applied to study the trafficking and activation of engineered immune cells such as CAR T cells in tissues.
- Cells co-expressing the CAR vector and activation markers such as GZMB or IFNG can be identified by the RNAscope assay and a T cell marker identified by IHC/IF can be used to detect activated CAR+ T cells in tissues (Figure 6).

VALIDATION OF ANTIBODIES

Antibodies are widely used for the detection of protein targets in discovery, diagnostic, and prognostic applications. However, there can be discordant results between IHC/IF antibodies[6-8]. Dual RNAscope ISH-IHC/IF can be used to validate the specificity of the antibody by simultaneously comparing the localization of the RNA and protein signal for the same target.

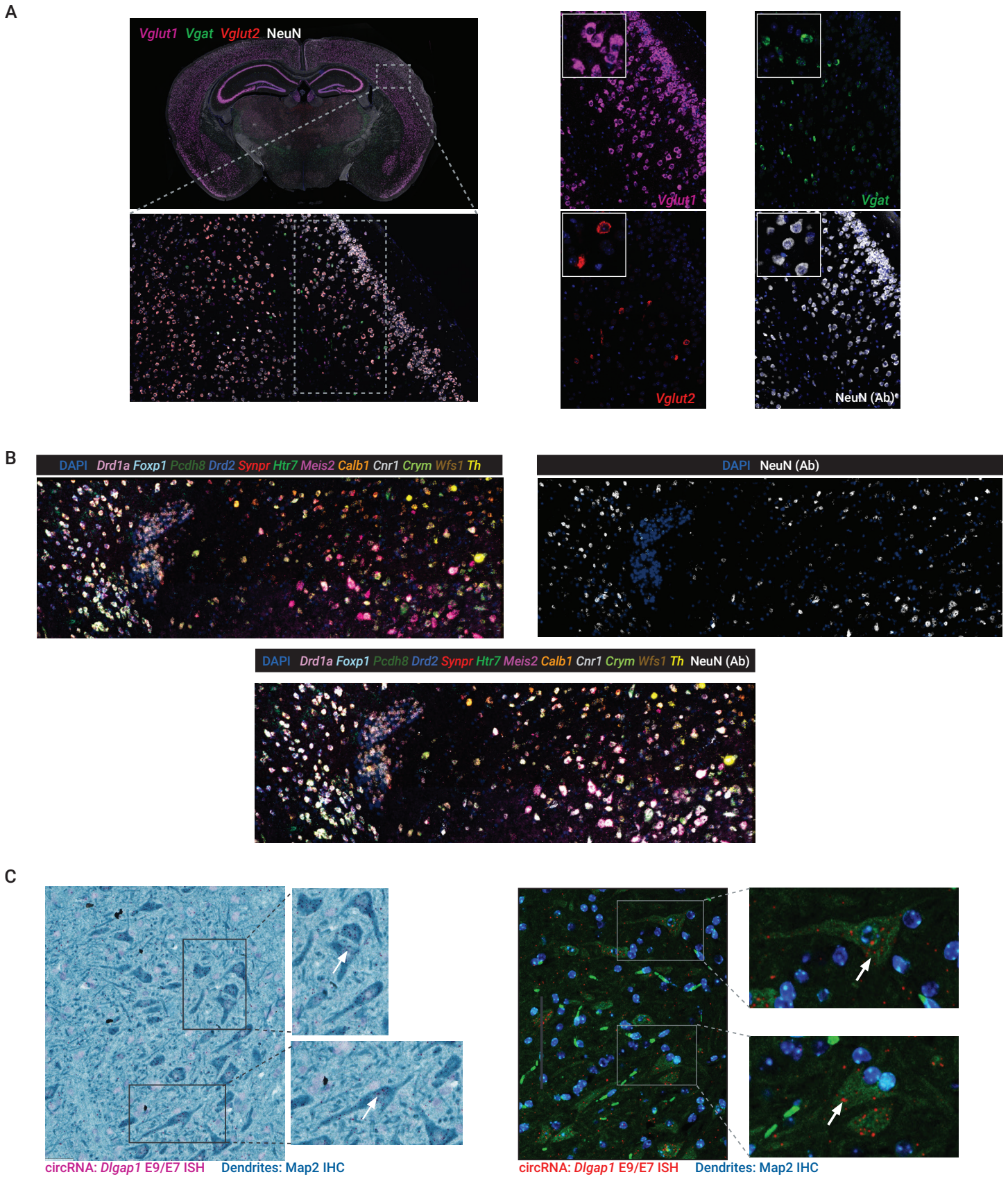

FIGURE 5. Dual ISH-IHC/IF to spatially map different neuronal subtypes and identify cell type-specific expression of circRNA splice variants. (A)The RNAscope Multiplex Fluorescent V2 assay was combined with IF to visualize the spatial expression of *Vglut1* (pink), *Vglut2* (red) and *Vgat* (green) mRNA among neurons marked by the neuronal marker protein NeuN (white) in a coronal section of the mouse brain. (B) The RNAscope HiPlex assay, which can identify up to 12 targets, provides comprehensive spatial mapping of the *Drd1+* and *Drd2+* striatal neuronal subtypes in combination with IF for the neuronal marker protein NeuN (white). (C) The BaseScope ISH assay for the circRNA *Dlgap1* (red) was combined with IHC for the dendritic cell marker protein Map2 (green) in the mouse brain. Given the ability of the Fast Red chromogen to fluoresce, the image was visualized under brightfield (left) and fluorescent (right) microscopy.

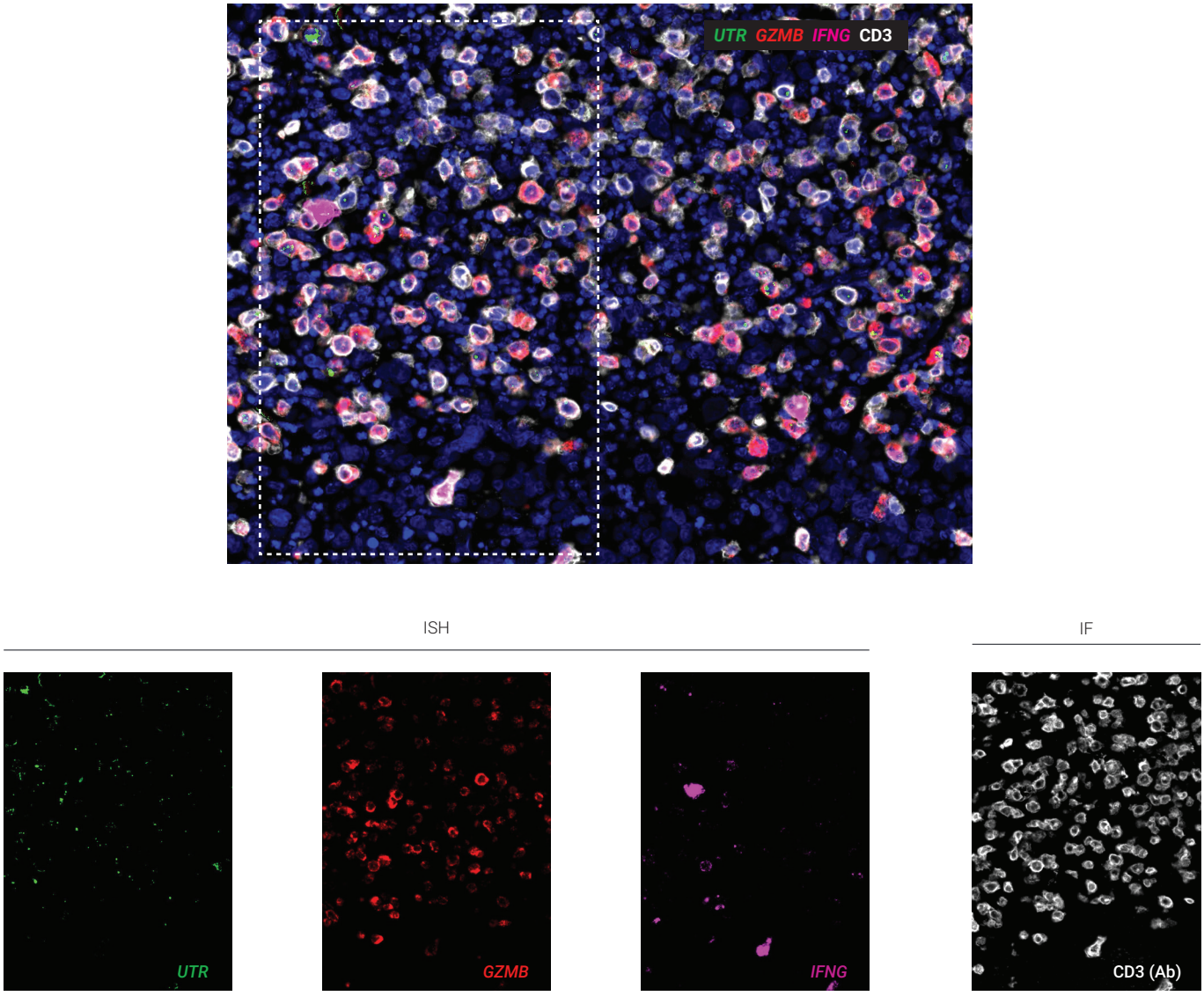

FIGURE 6. The RNAscope Multiplex Fluorescent V2 assay was combined with IF to visualize tumor infiltration of activated anti-BCMA CAR-T cells. The RNAscope ISH assay for the *3' UTR* of the CAR vector (green), *GZMB* (red), and *IFNG* (pink) was followed by IF for CD3 (white) in xenograft tumors from anti-BCMA CAR-T cell treated RPMI-8226 mice.

Summary

The dual RNAscope ISH-IHC/IF workflow is a powerful technique that can be used to study gene expression signatures at the RNA and protein level with spatial and single cell resolution. This report provides guidelines for performing the dual ISH-IHC/IF protocol and elaborates on the application of the methodology in immuno-oncology, neuroscience, and cell and gene therapy research, as well as utility in antibody validation.

By leveraging the strength of the similar workflows of RNAscope ISH and IHC/IF assays, the dual ISH-IHC/IF methodology combines transcriptomics and proteomics in the same tissue section and provides a multi-omics approach for characterizing complex tissues and revealing cell type specific gene expression with spatial and single cell resolution.

References

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