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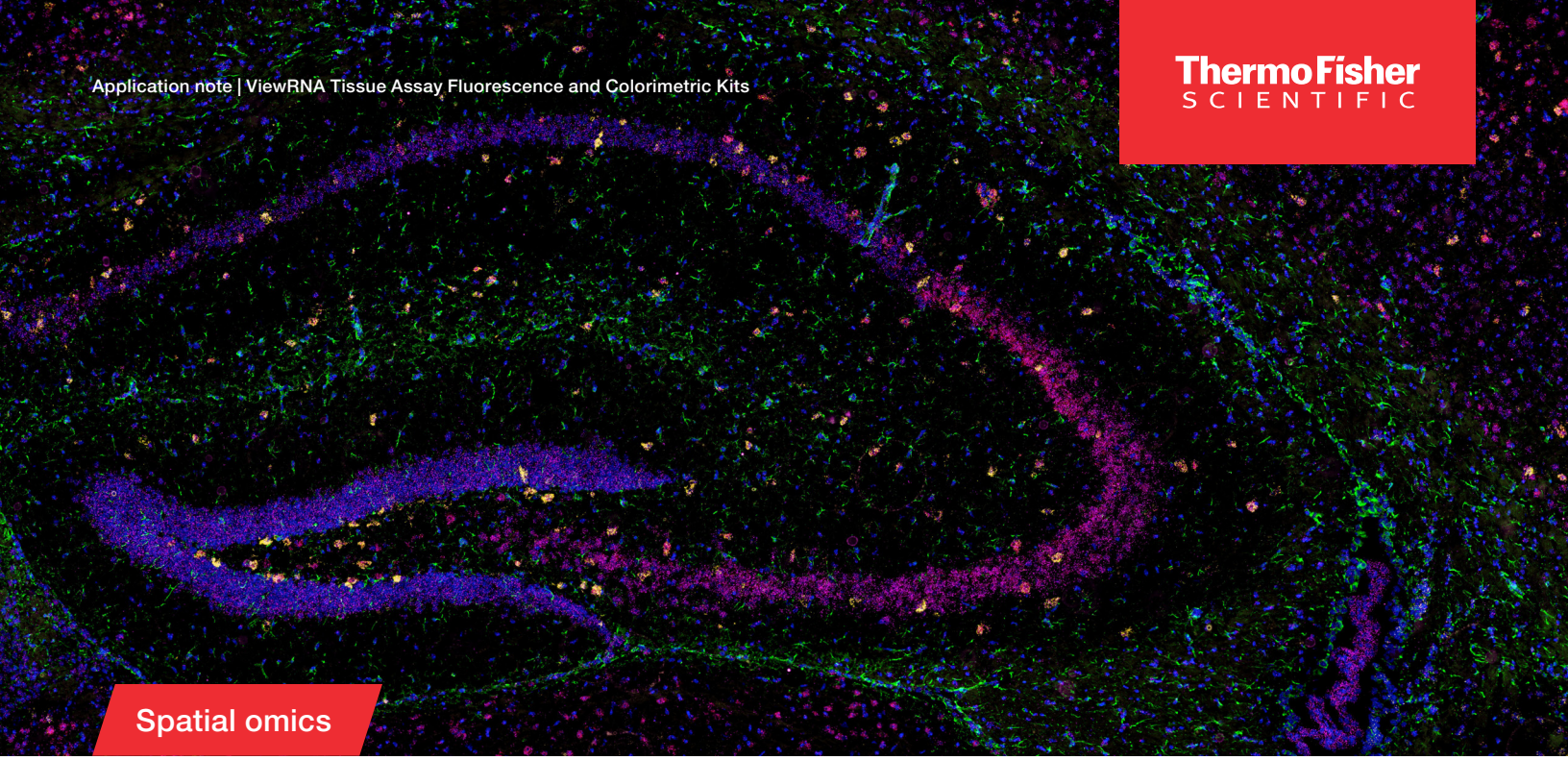

Spatial omics

Multiplexing immunohistochemistry and mRNA *in situ* hybridization with a neuroscience focus

Introduction

We present a method to address the challenges of integrating spatial proteomics and transcriptomics, specifically through the combination of RNA *in situ* hybridization (ISH) and immunohistochemistry (IHC) techniques, to achieve comprehensive insights into cellular microenvironments, cell–cell interactions, and disease mechanisms. Imaging spatial omics, which started with antibody-based detection of multiple targets, expanded to include imaging of multiple mRNA targets using new technologies with complex protocols, specialized reagents, and equipment. We've adapted existing protocols with available reagents and minimal equipment needs to demonstrate multiplex spatial omics on mouse brain sections.

Research in the fields of neuroscience, cancer, and age-related disease has increasingly incorporated spatial proteomics and transcriptomics to examine the cellular microenvironment to identify cell–cell interactions, predict prognosis, and evaluate therapeutic efficacy. Spatial omics, in particular, is being utilized to investigate brain neural network heterogeneity [1], neural involvement in cancer tissue as therapeutic targets, and features in the tumor microenvironment where alterations in nerve density have been correlated with poor clinical outcome in mouse models [2,3].

Spectrally resolvable multiplexed panels of fluorescent labels have become a valuable tool in spatial omics, enabling detailed tissue analysis to identify somatic and cancer cell interactions, lymphocyte infiltration, and proliferative potential by mapping the cellular neighbor microenvironment. As a technique, this approach has provided insights into cell–cell interactions that were not previously appreciated or observed using traditional pathology approaches relying on single-antibody detection.

Expanding IHC spatial omics to include RNA ISH detection provides deeper insight into cellular biology, as mRNA and microRNA expression can reveal underlying patterns driving disease that are not apparent in protein expression alone. Protein expression can be silenced or upregulated while maintaining normal mRNA expression, and vice versa.

Multiplexing mRNA ISH and antibody-based IHC is technically challenging. Tissue protocols used for RNA ISH can adversely affect the quality of antibody-based IHC detection. Factors such as tissue source, preservation, and pretreatment practices can lead to diminished sensitivity. Methods to improve tissue accessibility for mRNA detection, such as commonly used protease digestion, results in reduction or complete loss of signal for antibody-based protein detection. Antibody-based IHC reagents can introduce RNases resulting in the degradation or loss of mRNA signal.

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Recent methods for multiplexing RNA and protein target detection aim to mitigate these effects, but most require specialized equipment, reagents, and practices that are expensive, potentially difficult to perform, and time-consuming.

We focused on using mouse brain tissue as a model for imaging patterns visible with neuron-relevant ISH and IHC labeling using examples of multiplexing two mRNA ISH targets with two antibody-based IHC labels. For IHC, we used glial fibrillary acidic protein (GFAP) to label intermediate filaments expressed in neural tissues, and HuC/HuD to label proteins found exclusively in neuronal cell bodies. For ISH, we used glutamate decarboxylase 2 (Gad2), an enzyme responsible for catalyzing the production of GABA, and peptidylprolyl isomerase B (Ppib) predicted to enable RNA polymerase binding activity.

ISH

For labeling mRNA, we use either the Invitrogen™ ViewRNA™ Tissue Assay Fluorescence Kits or Invitrogen™ ViewRNA™ Colorimetric Tissue 2-Plex Kits. These kits employ pairs of single-stranded DNA (ssDNA) oligomers complementary to the mRNA target of interest. The oligomers hybridize to the mRNA target sequence near each other allowing for a complex to form as a double-Z structure that is then selectively hybridized by successive rounds of branched ssDNA oligomers under high-stringency conditions to create a greatly amplified complex. This complex classically consists of ssDNA with label probes terminating with specific fluorophores of high signal intensity that are microscopically visible with a 10x objective. Four mRNA and/or microRNA targets can be detected simultaneously (Figure 1A).

For increased sensitivity, the Label Probe is terminated with an enzyme that can further amplify a detectable signal using either alkaline phosphatase (Fast Red Reagent, Fast Blue Reagent, or other AP substrates) or horseradish peroxidase (DAB solution or other HRP colorimetric substrates) visible by brightfield or fluorescence microscopy (Figure 1B). This signal amplification strategy results in low nonspecific background and very high signal amplification visible by fluorescence or brightfield microscopy with essentially single-molecule mRNA detection.

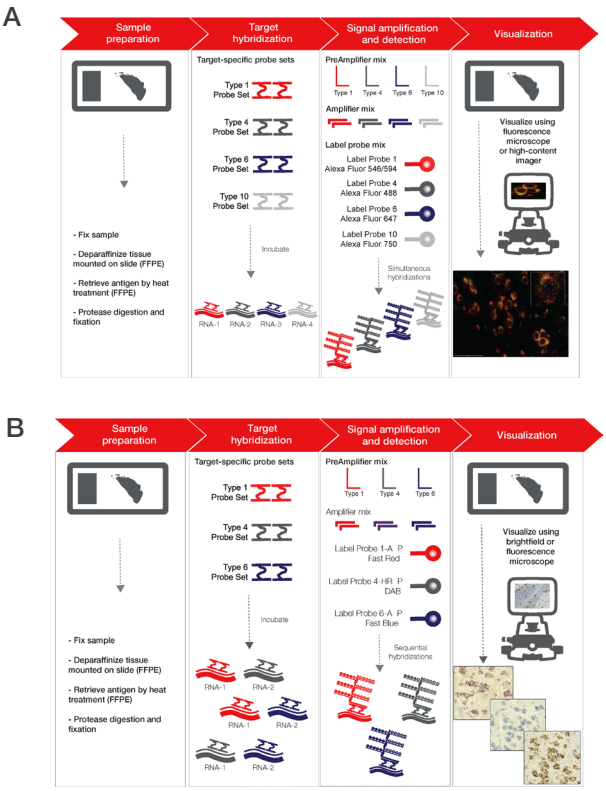


Figure 1. Overview of ViewRNA ISH assays. (A) Workflow for the ViewRNA Tissue Assay Fluorescence Kits resulting in signal amplification using four ssDNA scaffolds that can be used to detect up to four target probes simultaneously. **(B)** Workflow for the ViewRNA Colorimetric Tissue 2-Plex Kits resulting in signal amplification using three ssDNA scaffolds that can detect up to three targets sequentially using enzymatic signal amplification.

IHC

For antibody-based detection of specific protein targets, IHC-verified antibodies that have been directly labeled with spectrally separable fluorophores are used. These antibodies are available commercially from Thermo Fisher Scientific. For protein targets that do not have antibodies available as directly labeled conjugates, unconjugated antibodies were labeled with an appropriate fluorophore using the Invitrogen™ ReadyLabel™ Antibody Labeling Kits and then analytically confirmed for appropriate staining pattern and intensity (Figure 2).

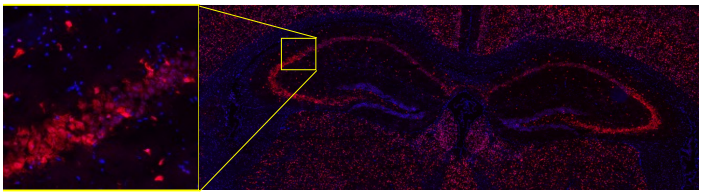


Figure 2. Functional test of the dye–antibody conjugation reaction. 20 µg of unconjugated HuC/HuD mouse antibody was labeled with Invitrogen™ Alexa Fluor™ Plus 750 dye using the ReadyLabel Antibody Labeling Kit. HuC/HuD (red) and DAPI (blue) staining are visualized on cryopreserved mouse brain. Left inset shows higher magnification of a region of dentate gyrus showing individual cell bodies labeled with HuC/HuD.

Multiplexing ISH and IHC

To combine ViewRNA mRNA detection with IHC using direct-labeled antibodies, the IHC tissue and reagents are first pretreated with RNase inhibitors. After labeling, the antibody is crosslinked to the tissue to protect against signal loss from the subsequent protease treatment step used in the ViewRNA mRNA ISH protocol [4-6]. These method modifications result in a sufficiently retained IHC signal and a robust ISH-based mRNA signal by protection from RNase degradation (Figures 3A and 3B).

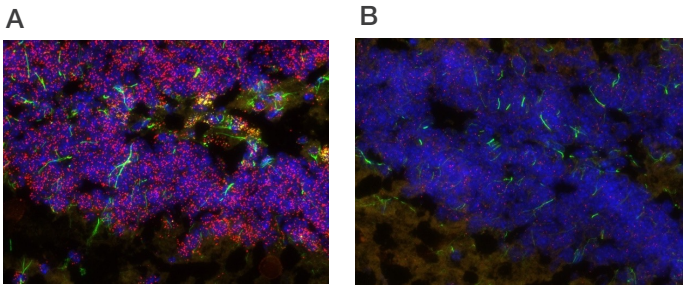


Figure 3. Comparison of the effect of antibody incubation with and without pretreatment with Invitrogen™ RNaseOUT™ Recombinant Ribonuclease Inhibitor. (A) Strong signal of GFAP (green) and mRNA signal of Gad2 (yellow) and Ppib (red) with RNaseOUT pretreatment. **(B)** Without RNaseOUT pretreatment, GFAP (green) antibody signal intensity is unaffected, but mRNA signal for both Gad2 (yellow) and Ppib (red) are greatly diminished. 40x images of the hippocampal region of a cryopreserved mouse brain coronal section are gain and exposure matched. Images were acquired on the Invitrogen™ EVOS™ M7000 Imaging System.

Materials and methods

Materials

- IHC-compatible directly labeled antibodies
- Unconjugated IHC-verified antibodies
- Invitrogen™ ReadyLabel™ Antibody Labeling Kits
- Invitrogen™ ViewRNA™ Tissue Assay Fluorescence Kit
- Invitrogen™ ViewRNA™ Colorimetric Tissue 2-Plex Kits
- Invitrogen™ ProLong™ RapidSet™ Antifade Mountant
- Invitrogen™ ProLong™ Glass Antifade Mountant
- Blocking buffer (3% BSA + 5% normal goat serum in PBS)
- Gibco™ PBS, pH 7.4
- Invitrogen™ RNaseOUT™ Recombinant Ribonuclease Inhibitor
- Thermo Scientific™ DAPI, 1 mg/mL
- Invitrogen™ Aluora™ Spatial Amplification Reagent Components

Methods

Multiplexing ViewRNA ISH assays and antibody-based IHC requires careful panel design to help ensure that the fluorophores are spectrally compatible without overlapping signals in adjacent channels. Because variability in tissue quality, tissue fixation method and duration, alcohol dehydration, and paraffin embedding used on FFPE tissue can result in variable autofluorescence in the short emission wavelength, there is a need to use bright signals in the GFP channels to overcome tissue background. Additionally, bright signals adjacent to dim signals in longer wavelength channels might not be resolvable, especially if the signals co-localize. Preparation of tissue for labeling can also affect the quality of the images and panel design. FFPE tissue tends to have a low amount of RNase activity, but RNA quality may be compromised depending on tissue storage conditions and time elapsed prior to fixation. Alternatively, cryopreserved tissue often has high-quality RNA, but can be prone to degradation if not treated due to high endogenous RNase activity. The cryopreserved tissue workflow has the advantages that neither deparaffinization nor epitope retrieval are required.

For the simplest and fastest workflow, we use cryopreserved mouse brain and fix it in prechilled buffered 4% formaldehyde for 60 minutes. After washing, the tissue is permeabilized with 0.1% Triton™ X-100 detergent in PBS for 30 minutes. Then the tissue and antibody diluent are treated with RNases before and during IHC labeling. A protein blocking step is not required for IHC labeling when using directly conjugated antibodies, and multiple antibodies can be combined for a single incubation step. After labeling and washing in PBS/Tween™ 20 detergent, the tissue is crosslinked to protect the antibodies from being degraded or lost during subsequent steps. Formaldehyde fixation alone is not sufficient to protect from protease treatment used in the ISH protocol; however, fixation with a mixture of dialdehydes or bifunctional crosslinking reagents improves the retention of the antibody panel. Next, ISH labeling is performed starting with an optimized protease pretreatment described in the ViewRNA Tissue Assay Fluorescence Kits instruction manual. In this kit, labeling reagents are available as 1-plex to 4-plex fluorophore combinations consisting of Invitrogen™ Alexa Fluor™ 488, either Alexa Fluor™ 546 or Alexa Fluor™ 594, Alexa Fluor™ 647, and Alexa Fluor™ 750 dyes. Alternatively, the ViewRNA Colorimetric Tissue Kits using Fast Red Reagent, Fast Blue Reagent, and/or DAB Solution substrates for enzymatic-based deposition can be used.

Results

We present examples of images of mouse brain tissue labeled using either IHC or ISH protocols, as well as a combined IHC plus ISH protocol modified to preserve signal intensity. For IHC labeling using cryopreserved mouse brain, we present an example of an 8-plex antibody-labeled tissue plus DAPI staining that was created using iterative rounds of antibody labeling and signal deposition using an HRP enzymatic reaction with Aluora Spatial Amplification Reagent Components followed by antibody stripping and reprobing. Image acquisition and spectral deconvolution was performed on the Invitrogen™ EVOS™ S1000 Spatial Imaging System (Figure 4).

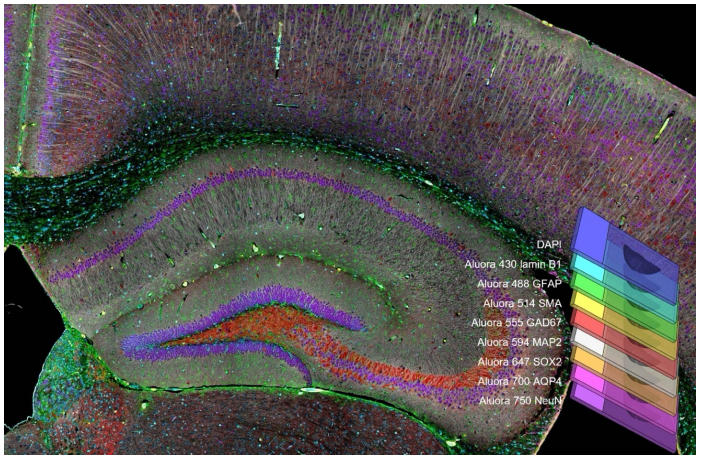


Figure 4. Multiplex IHC using Aluora reagents. Iterative rounds of labeling were performed on mouse brain tissue using primary antibodies and Aluora Spatial Amplification Reagent Components. Nuclei were stained with DAPI. Dye and protein combinations in the 8-plex assay are Aluora 430 lamin B1, Aluora 488 GFAP, Aluora 514 SMA, Aluora 555 GAD67, Aluora 594 MAP2, Aluora 647 SOX2, Aluora 700 AQP4, and Aluora 750 NeuN. Image was acquired on the EVOS S1000 Spatial Imaging System.

The ISH protocol was performed on cryopreserved mouse brain tissue using the ViewRNA Tissue Assay Fluorescence 4-Plex Kit using mRNA target probes of Ppib, Gad2, Polr2a, and Gapdh (Figure 5).

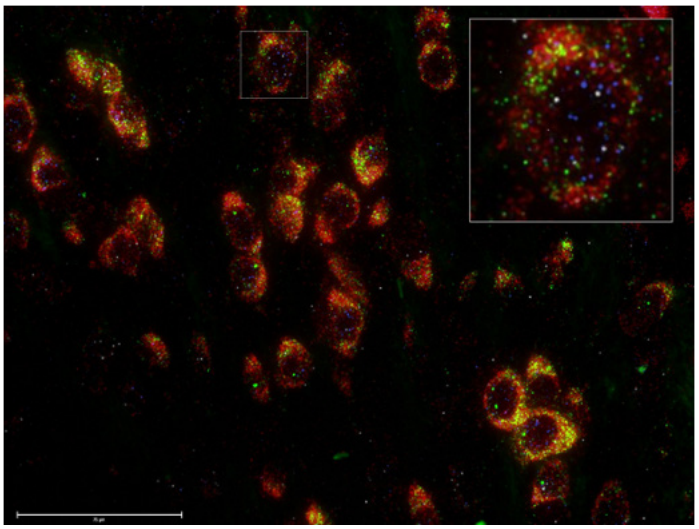


Figure 5. RNA ISH in mouse brain tissue using the ViewRNA kit. Mouse brain (FFPE) tissue was processed and peptidyl-prolyl cis-trans isomerase B (Ppib), glutamate decarboxylase 2 (Gad2), RNA polymerase II subunit A (Polr2a), and Gapdh mRNA were labeled using ViewRNA probe sets. These probe sets were detected using the ViewRNA ISH tissue assay while using Type 4 Alexa Fluor 488 labeling for Gad2 (shown in green), Type 1 Alexa Fluor 594 labeling for Gapdh (shown in red), Type 6 Alexa Fluor 647 labeling for Ppib (shown in blue), and Type 10 Alexa Fluor 750 labeling for Polr2a (shown in white). The fluorescent image was taken using the EVOS M7000 Imaging System and post-processed for visualization.

The ISH protocol was also performed on cryopreserved mouse brain tissue using ViewRNA Colorimetric Tissue 1-Plex or 2-Plex Kits. A 20x stitched image of a 2-plex assay shows one-half of a C8 coronal section of mouse brain with Gad2 mRNA labeled using Fast Red Reagent, and Ppib mRNA labeled using Fast Blue Reagent. For this image, sequential labeling is required only when using 2-plex AP labeling (Figure 6).

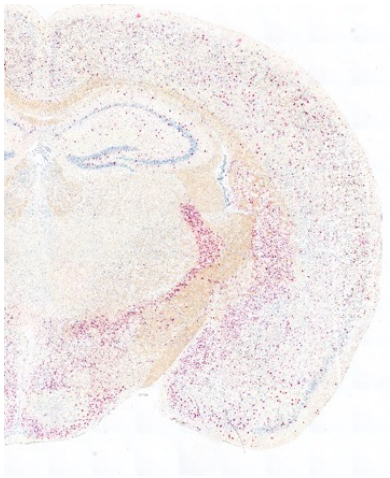
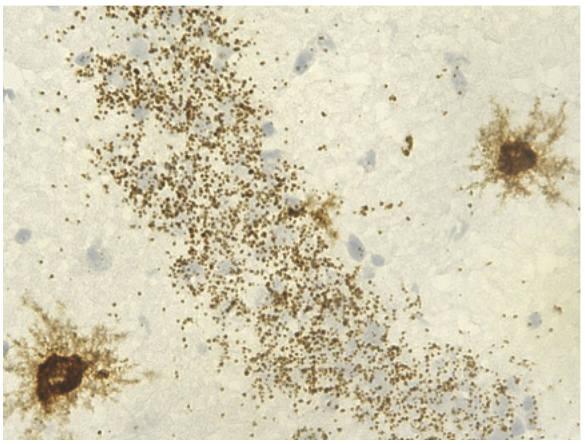


Figure 6. Coronal section of cyropreserved mouse brain labeled with the ViewRNA Colormetric Tissue 2-Plex Kit. Fast Red Reagent was used for Gad2 mRNA, and Fast Blue Reagent was used for Ppib mRNA. Stitched image was captured on the EVOS S1000 Spatial Imaging System using a 20x objective.

The recently introduced DAB substrate enzymatic labeling using HRP allows for highly sensitive detection, shorter development time, as well as an image that is stable in organic mountant. Imaging potassium voltage-gated channel interacting protein 2 (Kcnip2) using a 40x objective demonstrates how sensitive the DAB colorimetric signal is, clearly showing separate well-defined individual puncta along with dense clusters of puncta of the pyramidal cells of the dentate gyrus (Figure 7A). DAB (Gad2) used with Fast Red Reagent (Ppib) or Fast Blue Reagent in a 2-plex workflow for colorimetric labeling is also much faster and sensitive with a shorter protocol because no quenching or re-incubation with enzyme is required (Figure 7B). These same targets are also labeled with 2-plex Fast Red Reagent and Fast Blue Reagent and imaged in brightfield on the Invitrogen™ EVOS™ M5000 (Figure 8A), the EVOS™ M7000 (Figure 8B), and the EVOS™ S1000 (Figure 8C) imaging systems.

A



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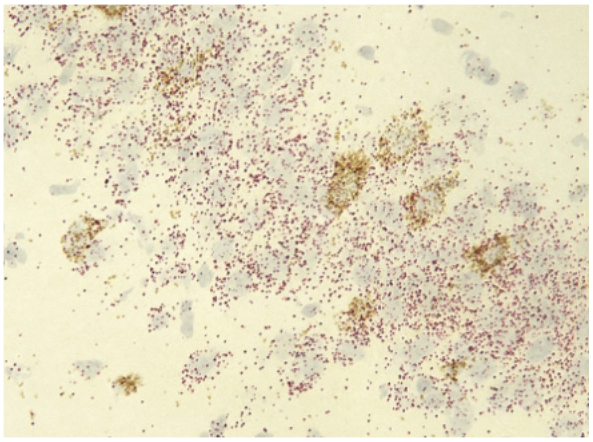

Figure 7. Imaging of Kcnip2 using the DAB colorimetric signal. (A) Kcnip2 labeling with the ViewRNA Colorimetric Tissue 1-Plex Kit using DAB substrate. Hematoxylin-labeled nuclei are visualized in light gray. Image was captured with the EVOS M7000 Imaging System using a 40x objective. **(B)** Gad2 (brown) plus Ppib (red) labeling with the ViewRNA Colorimetric Tissue 2-Plex Kit using DAB and Fast Red substrates. Hematoxylin-labeled nuclei are visualized in light gray. Image was captured with the EVOS M7000 Imaging System using a 40x objective.

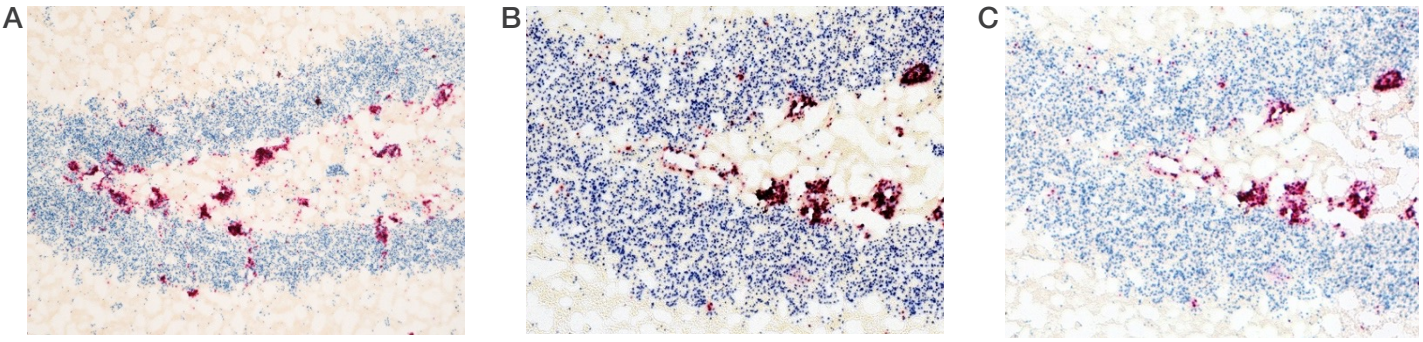

Figure 8. mRNA ISH 2-plex image of Gad2 (red) and Ppib (blue) labeling region of hippocampus of a cryopreserved mouse brain using the ViewRNA Colorimetric Tissue 2-Plex Kit with Fast Red and Fast Blue substrates. (A) Tissue mounted in ProLong RapidSet Antifade Mountant. 20x brightfield image acquired using the EVOS M5000 Imaging System. **(B,C)** Tissue mounted in ProLong Glass Antifade Mountant with the same region of interest acquired using the EVOS M7000 Imaging System and the EVOS S1000 Spatial Imaging System, respectively.

Multiplexing of IHC and ISH is presented using Ppib and Gad2 for mRNA ISH targets combined with GFAP and HuC/HuD for IHC antibody labeling of protein targets. Modified protocols were used to allow for retention of IHC signal after protease digestion and protection of mRNA quality during the IHC incubation steps with the use of RNase inhibitors. Images of the multiplexed tissue were acquired on the EVOS S1000 Spatial Imaging System using a 20x objective (Figures 9A and 9B) while images of the same tissue sections were acquired on the EVOS M5000 Imaging System using a 40x objective (Figures 10A and 10B).

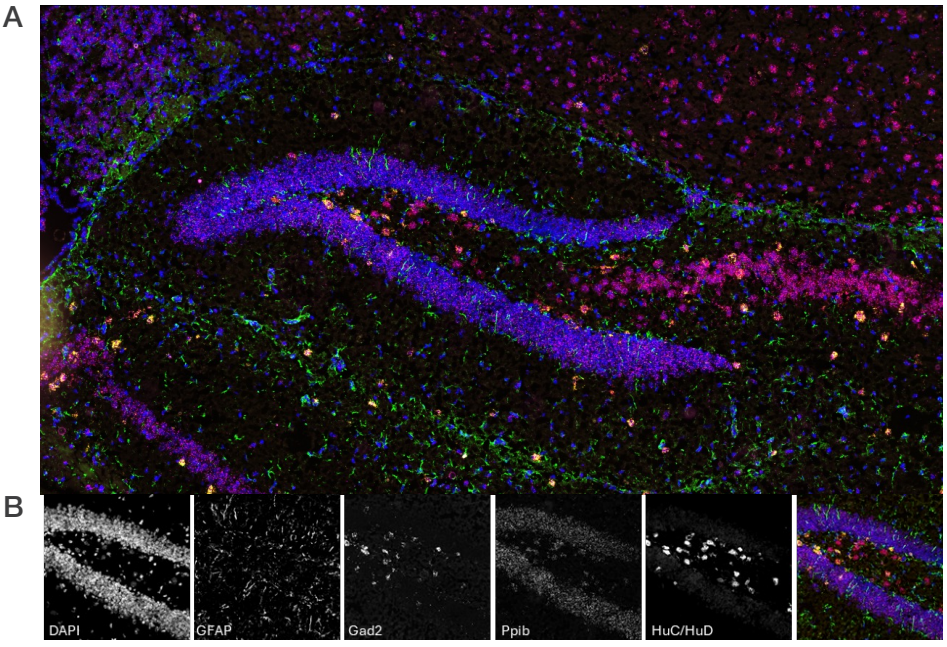

Figure 9. Multiplexing of IHC and ISH visualized on the EVOS S1000 Spatial Imaging System. (A) Stitched image of hippocampal region of cryopreserved mouse brain showing IHC and ISH multiplexing of DAPI (blue), GFAP (green), Gad2 (yellow), Ppib (magenta), and HuC/HuD (red) was acquired using a 20x objective. **(B)** Panel showing the individual and combined channels of a small region of interest.

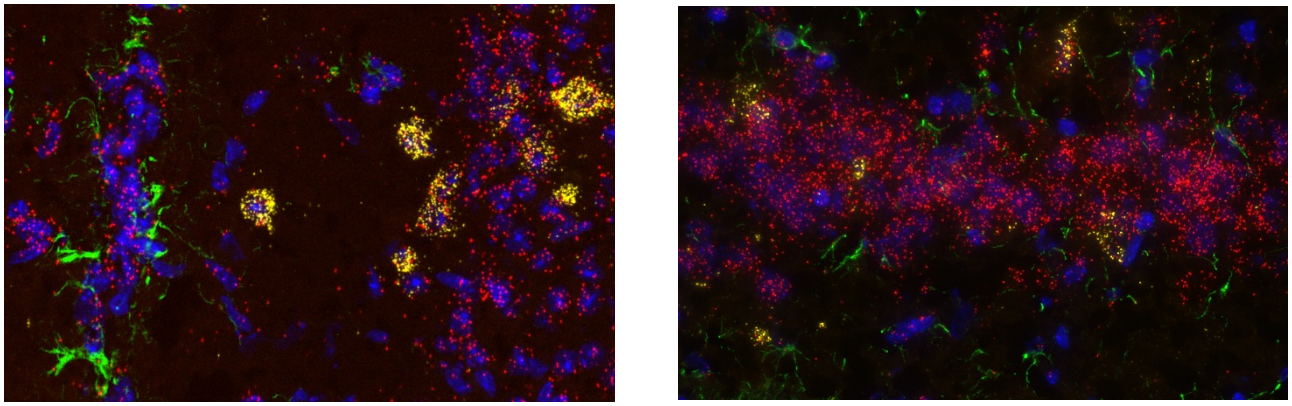

Figure 10. Multiplexing of IHC and ISH visualized on the EVOS M5000 Imaging System. Hippocampal regions of cryopreserved mouse brain tissue labeling with GFAP (green) for IHC, combined with ISH mRNA detection of Gad2 (yellow) and Ppib (red) using the ViewRNA Tissue Assay Fluorescence Kit and mounted in ProLong RapidSet Antifade Mountant. Nuclei were stained with DAPI (blue). Images were acquired on the EVOS M5000 Imaging System using a 40x objective.

Conclusions

Customizing the multiplexing of IHC with ISH requires consideration of the impact of the individual protocols and modifications to existing protocols to minimize loss of signal from either protein and mRNA detection. In addition to the protocol modifications that are described, other considerations are necessary for panel design. The signal intensities of fluorophores need to be optimized in relation to adjacent channels. Tissue type, fixation, and protocol-generated autofluorescence can result in low signal-to-background ratios around the green emission wavelengths. There are several methods used in reducing autofluorescence in tissue; however, we have found that the most effective is pretreatment with light exposure in an alkaline hydrogen peroxide solution prior to IHC labeling [6,7].

We present multiplexed IHC and ISH techniques that are useful for quantitative and qualitative evaluation of mRNA expression in neurological tissue using microscopic instruments ranging from the versatile, broad-application EVOS M5000 Imaging System, the fully automated EVOS M7000 Imaging System, and the EVOS S1000 Spatial Imaging System, a high-performance multimodal imaging instrument capable of simultaneously resolving nine fluorophores during a single acquisition with integrated spectral unmixing.

References

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Ordering information

Description	Quantity	Cat. No.
ViewRNA Tissue Assay Fluorescence 4-Plex Kits	1 kit	QVT0700
ViewRNA Colorimetric Tissue 2-Plex Kits	1 kit	QVT0412C
ReadyLabel Antibody Labeling Kit, Alexa Fluor Plus 750	1 kit	R10724
GFAP Monoclonal Antibody (GA5), Alexa Fluor 488, eBioscience	1 unit	53-9892-82
HuC/HuD Monoclonal Antibody (16A11)	1 unit	A-21271
EVOS M5000 Imaging System	1 system	AMF5000
EVOS M7000 Imaging System	1 system	AMF7000
EVOS S1000 Spatial Imaging System	1 system	AMFS1000
ProLong Glass Antifade Mountant	1 each	P36982
ProLong RapidSet Antifade Mountant	50 tests	P38930
Normal Goat Serum (10%)	1 each	50062Z
PBS, pH 7.4	1 each	10010023
RNaseOUT Recombinant Ribonuclease Inhibitor	5,000 units	10777019
Aluora Spatial Amplification Reagent Components	8 x 1,000 reactions	A40002450
DAPI, 1 mg/mL	1 mL	62248

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