

# Detection of RNA in the central and peripheral nervous system using the RNAscope® *in situ* hybridization assay

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## Highlights

- Identification, characterization, and (co-) localization of mRNA and lncRNA in the nervous system
- Identification, visualization and characterization of specific cell types in the nervous system
- Detection of mRNA in the nervous system when no (reliable) antibodies are available
- Visualization of neuronal network activity and plasticity
- Validation of target mRNA expression after high-throughput gene expression analysis
- Validation of (cell type-specific and conditional) genetic modifications

## Introduction

Neuroscience is one of the fastest growing interdisciplinary research fields that studies the central (CNS) and peripheral nervous system from the molecular and cellular levels to the systems level. Areas of research include neural development, structural and functional organization of the nervous system, cognitive and behavioral neurosciences, and clinical neurosciences including neurodegenerative diseases.

The RNAscope® *in situ* hybridization (ISH) assay provides a powerful method to detect gene expression within the spatial and morphological tissue context. The proprietary "double Z" probe design in combination with the advanced signal amplification enables a highly specific and sensitive detection of the target RNA with each dot visualizing a single RNA transcript.

Therefore, this robust signal-to-noise technology allows for the detection of gene transcripts at single molecule level with single-cell resolution analysis 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 in formalin-fixed paraffin-embedded (FFPE) and fresh/fixed frozen samples, enabling consistent characterization of cell populations within the nervous system. In summary, the RNAscope® technology allows the visualization and quantification of virtually any gene from any genome in any tissue with unprecedented specificity and sensitivity.

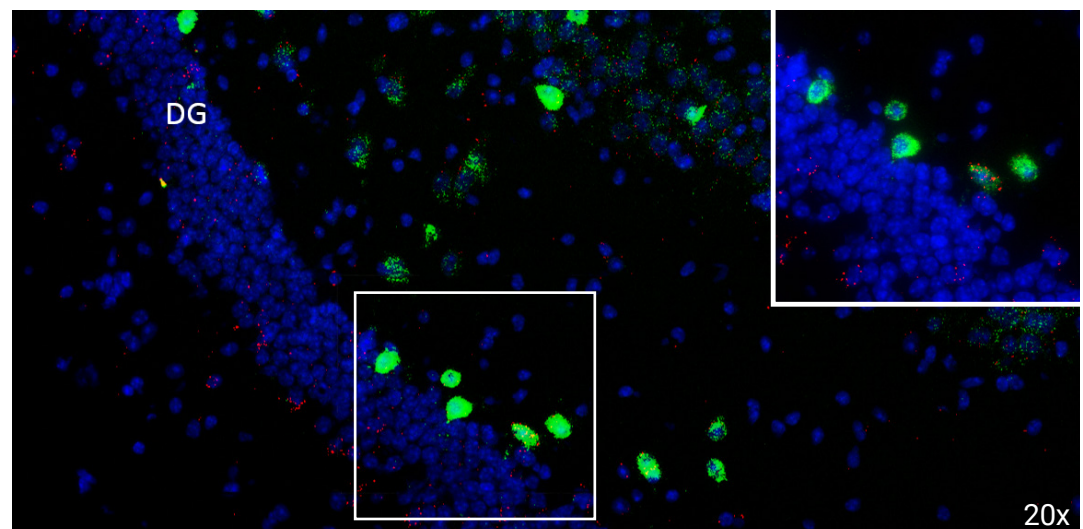


FIGURE 1. Duplex detection of G protein-coupled receptors in normal mouse brain hippocampus using the RNAscope® Multiplex Fluorescent assay on Fresh Frozen tissue samples: Cannabinoid Receptor 1 (*Cnr1*, Green) and Dopaminergic Receptor D1 (*Drd1*, Red). The delineated area is shown at higher magnification on the right. Cells are counterstained with DAPI. DG = Dentate Gyrus.



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## Identification, characterization, and (co-) localization of mRNAs in the central and peripheral nervous system

### Co-expression of target RNA of interest with specific cell type markers

The RNAscope® assay can be used for the visualization of multiple target co-expression patterns or the co-expression of the target(s) of interest with desired cell type markers that characterize particular types of neurons or glia. Commonly used cell type markers to distinguish neuronal and glial target expression patterns include *Rbfox3*, *Aif1*, and *Gfap* for the detection of neurons, microglia and astrocytes, respectively (Figure 2).

The striatum harbors two distinct neuronal populations that are involved in action selection. These medium spiny neurons either express Dopamine Receptor D1 (*Drd1*) – the striatonigral pathway – or Dopamine Receptor D2 (*Drd2*) – the striatopallidal pathway. These different neuronal cell types can be accurately detected and visualized using the RNAscope® technology (Figure 3).

Another example from the literature can be found in the research by Carstens *et al.*<sup>1</sup>. In their study for perineuronal nets in hippocampal CA2 neurons they used the RNAscope® assay to demonstrate the co-localization of *AggreCAN* mRNA – a perineuronal net component - and *Pcp4* mRNA - a marker of hippocampal CA2 neurons.

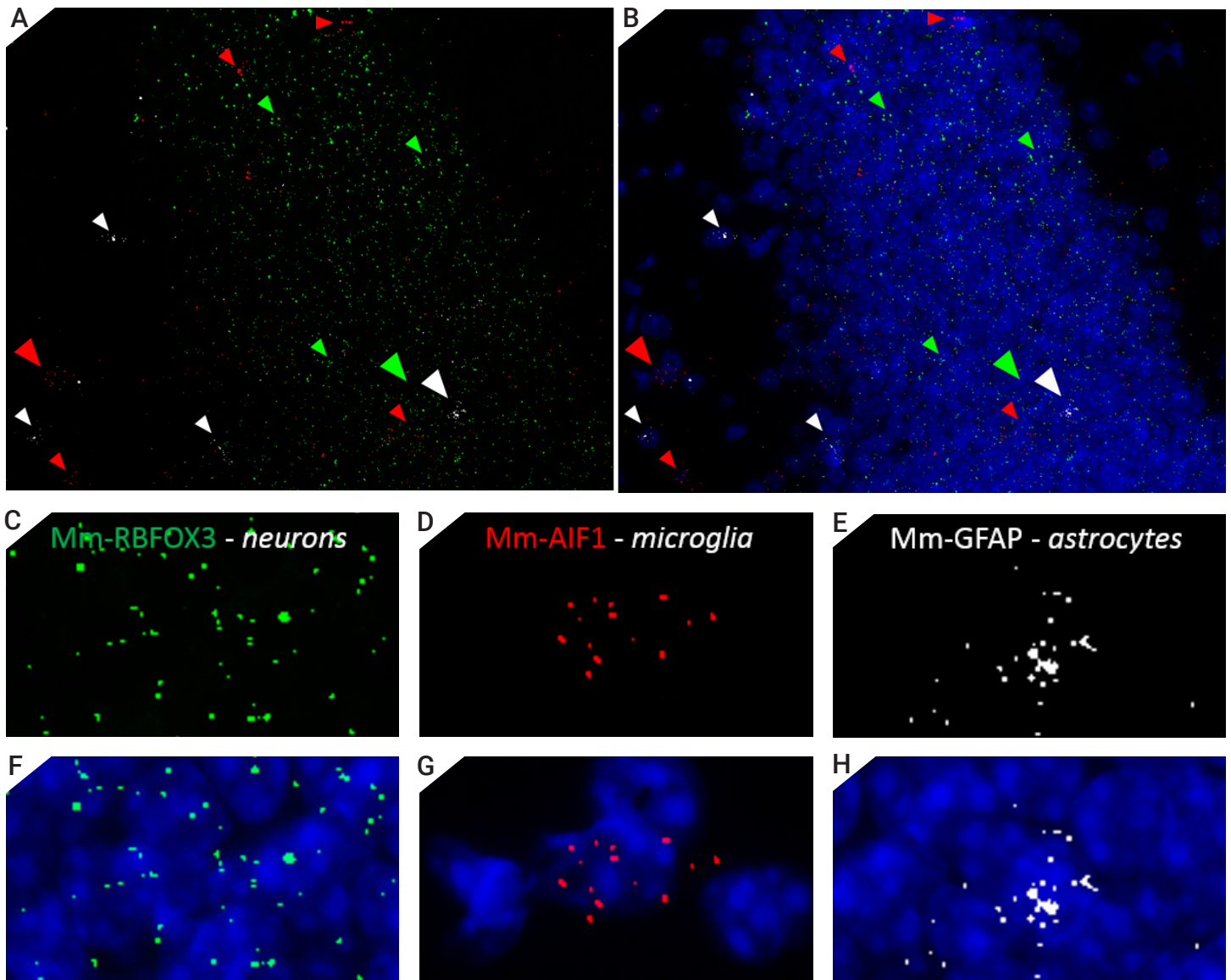


FIGURE 2. Detection of different cell type populations in normal mouse brain hippocampus using the RNAscope® Multiplex Fluorescent assay on Fresh Frozen tissue samples: Mm-Rbfox3 (neurons, Green), Mm-Aif1 (microglia, Red) and Mm-Gfap (astrocytes, White). Areas indicated by green, red and white arrows in panel A and B are shown as higher magnification in panels C-H. Cells are counterstained with DAPI.

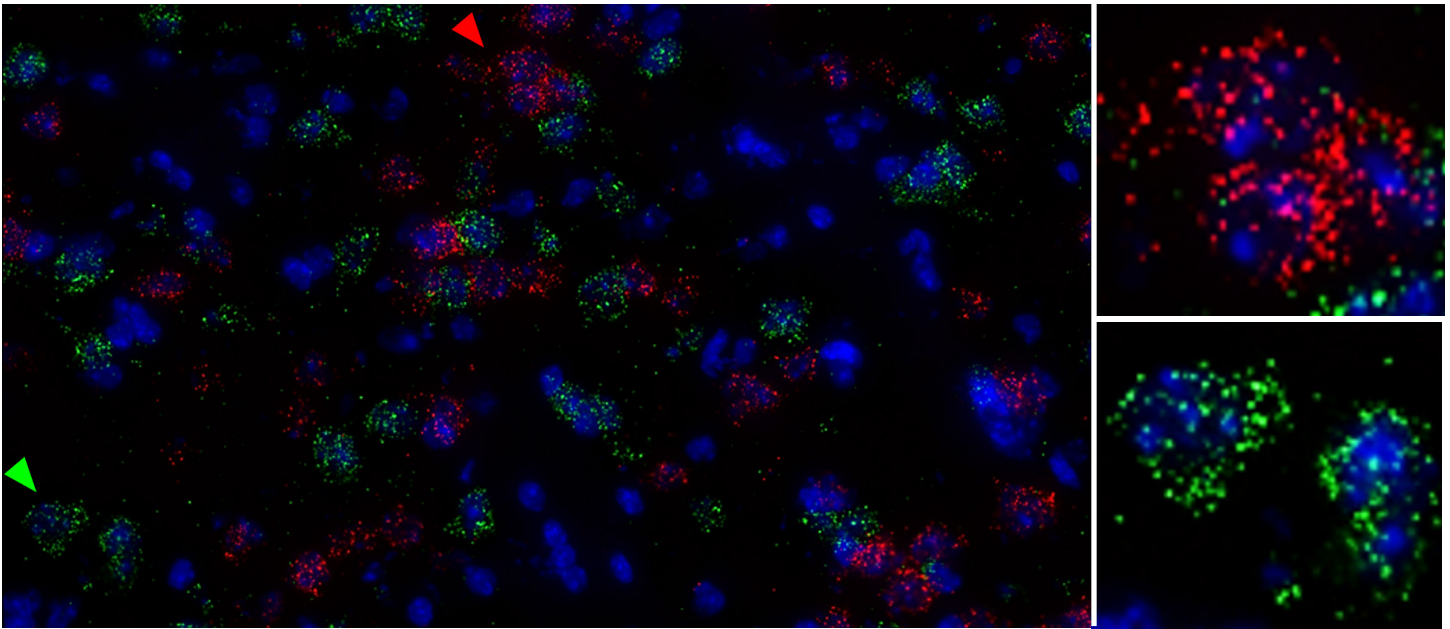


FIGURE 3. Detection of two distinct striatal neuronal populations expressing either Dopamine Receptor D1 (*Drd1*, Red) or Dopamine Receptor D2 (*Drd2*, Green) using the RNAscope® Multiplex Fluorescent assay on Fresh Frozen tissue samples. Areas indicated by green and red arrows are shown as higher magnification images in the right panels. Cells are counterstained with DAPI.

## The sub-cellular localization of mRNA expression

The detection of single-molecules and the single-cell resolution of the RNAscope® technology coupled with morphological context allows for the sub-cellular localization of mRNA expression in the cell body, axons or dendrites. Using the RNAscope® assay to confirm RT-PCR findings, Gervasi *et al.*<sup>2</sup> provided the first direct evidence that *TH* mRNA is trafficked to the axon and that the mRNA is locally translated in the distal axons of SCG neurons.

## Detection of long non-coding RNA (lncRNA)

Long non-coding RNAs (lncRNAs) are a large and diverse class of RNA transcripts with a length of more than 200 nucleotides and no protein-coding potential. Most recent statistics from GENCODE<sup>3</sup> (v25, March 2016) show that the human genome contains 23,025 non-coding genes, surpassing the number of protein-coding genes (19,950). About 70% (15,767) of these non-coding genes code for long non-coding RNAs (lncRNAs). Recent studies have suggested the involvement of lncRNAs in neurodevelopment and brain function. Despite their functional importance, the mechanisms by which lncRNAs control cellular processes are still elusive. RNAscope® technology can shed light on the brain-enriched lncRNA functionality by visualizing tissue-restricted expression patterns, localization of lncRNAs to distinct subcellular structures, and expression regulation. Figure 4 depicts the expression for two lncRNAs, i.e. *Neat1* and *Malat1*, in the mouse hippocampus.

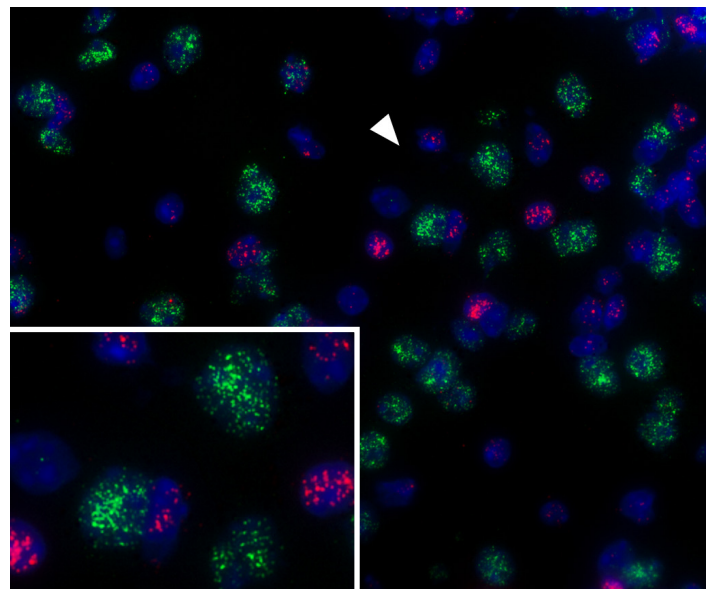


FIGURE 4. Detection of lncRNA in normal mouse brain hippocampus using the RNAscope® Multiplex Fluorescent assay on Fresh Frozen tissue samples: *Neat1* (Red) and *Malat1* (Green). Cells are counterstained with DAPI.

## Detection of mRNA in the nervous system when no (reliable) antibodies are available

### G-protein-coupled receptors (GPCRs) and ion channels

IHC is a well-established method for a broad range of applications from discovery to diagnostics and prognostics. However, raising antibodies to G-protein coupled receptors (GPCRs) has been challenging due to difficulties in obtaining suitable antigen accessibility as GPCRs are transmembrane proteins often expressed at low levels and very unstable when purified (Hutchings *et al.*<sup>4</sup>). Figures 5-9 provide both fluorescent and chromogenic images for four types of GPCRs - readily detected as mRNA targets by the RNAscope® assay - in hippocampal, striatal and cortical normal mouse brain areas: Dopaminergic Receptors D1 and D2 (*Drd1* and *Drd2*), Cannabinoid Receptor 1 (*Cnr1*), and Cholinergic Receptor Muscarinic 3 (*Chrm3*).

Ion channels, which are another class of membrane proteins, also constitute a challenging class of targets for antibody development since they must remain membrane-associated to maintain their native conformation. Examples for the detection of ion channels can be found in Figure 10. Altogether, the RNAscope® technology is an ideal method to visualize these targets within their morphological context in the central and peripheral nervous system.

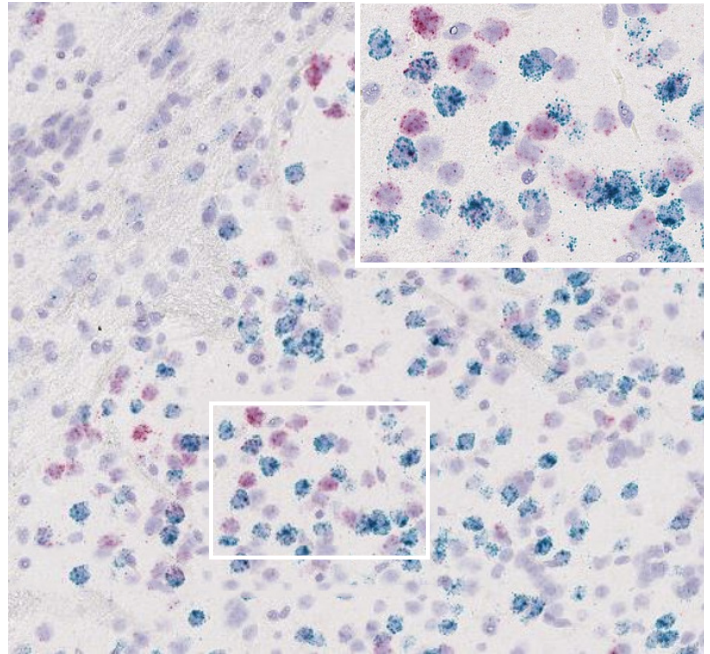


FIGURE 5. Detection of G protein-coupled receptors in normal mouse brain striatum using the RNAscope® 2.5 HD Duplex Chromogenic assay on FFPE tissue samples: Cannabinoid receptor 1 (*Cnr1*, Red) and Dopaminergic receptor D1 (*Drd1*, Green). Cells are counterstained with hematoxylin. The area indicated by the box is shown as higher magnification inset.

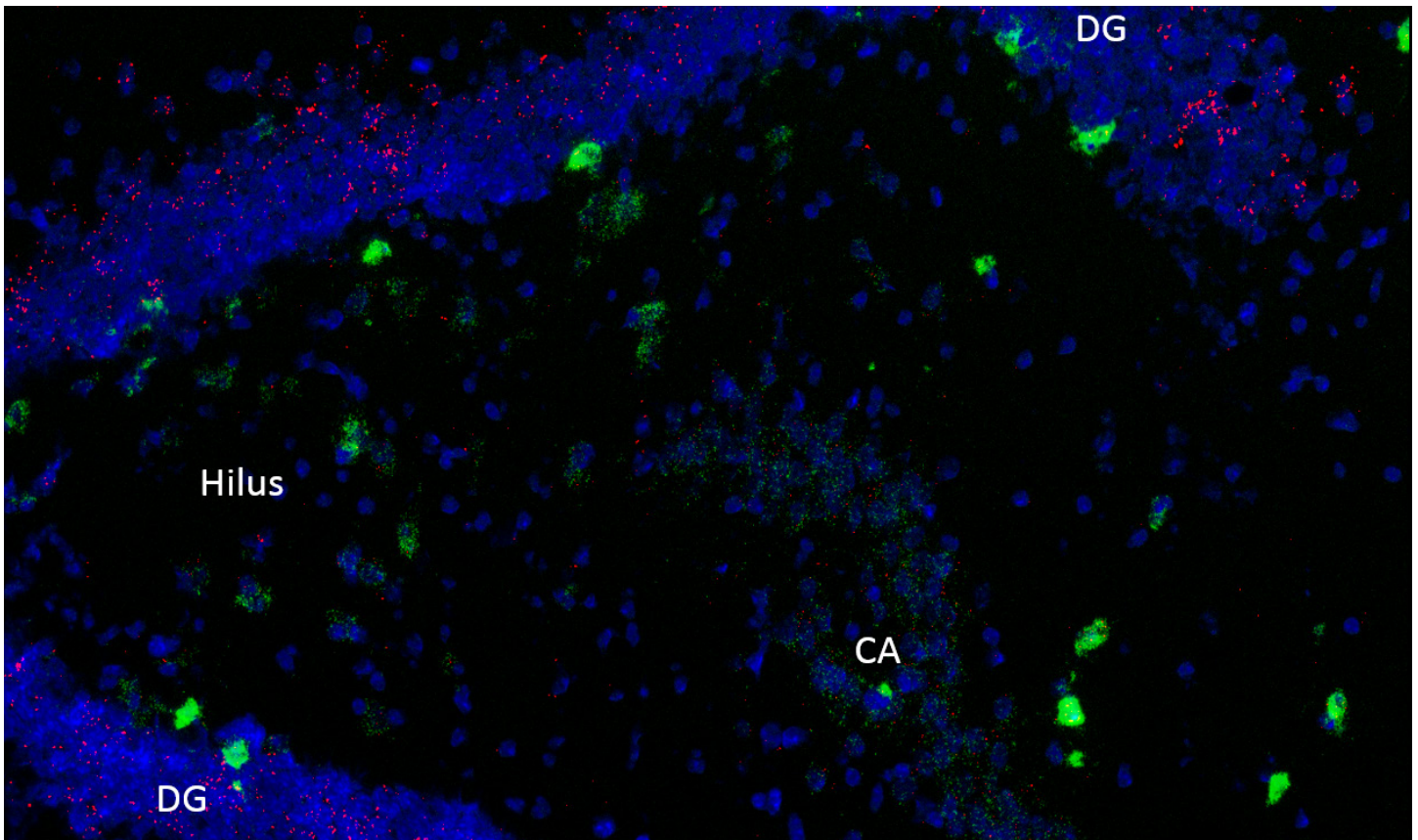


FIGURE 6. Duplex detection of G protein-coupled receptors in normal mouse brain hippocampus using the RNAscope® Multiplex Fluorescent assay on Fresh Frozen tissue samples: Cannabinoid Receptor 1 (*Cnr1*, Green) and Dopaminergic Receptor D1 (*Drd1*, Red). Cells are counterstained with DAPI. CA = Cornu Ammonis. DG = Dentate Gyrus.

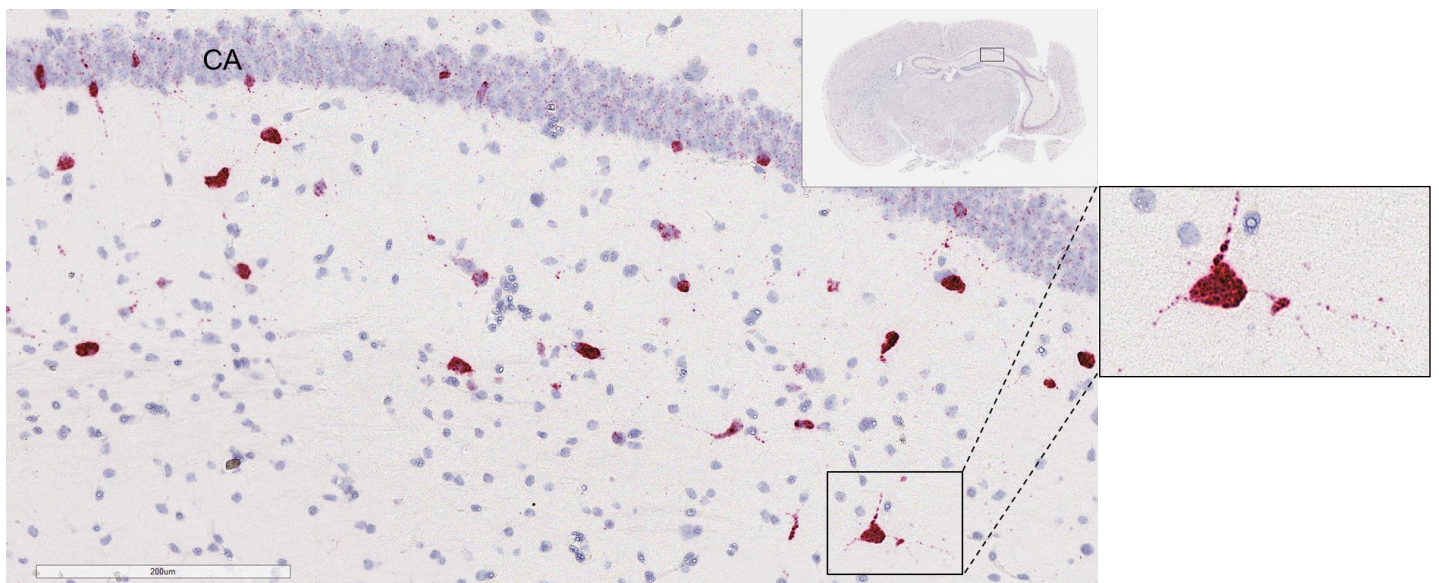
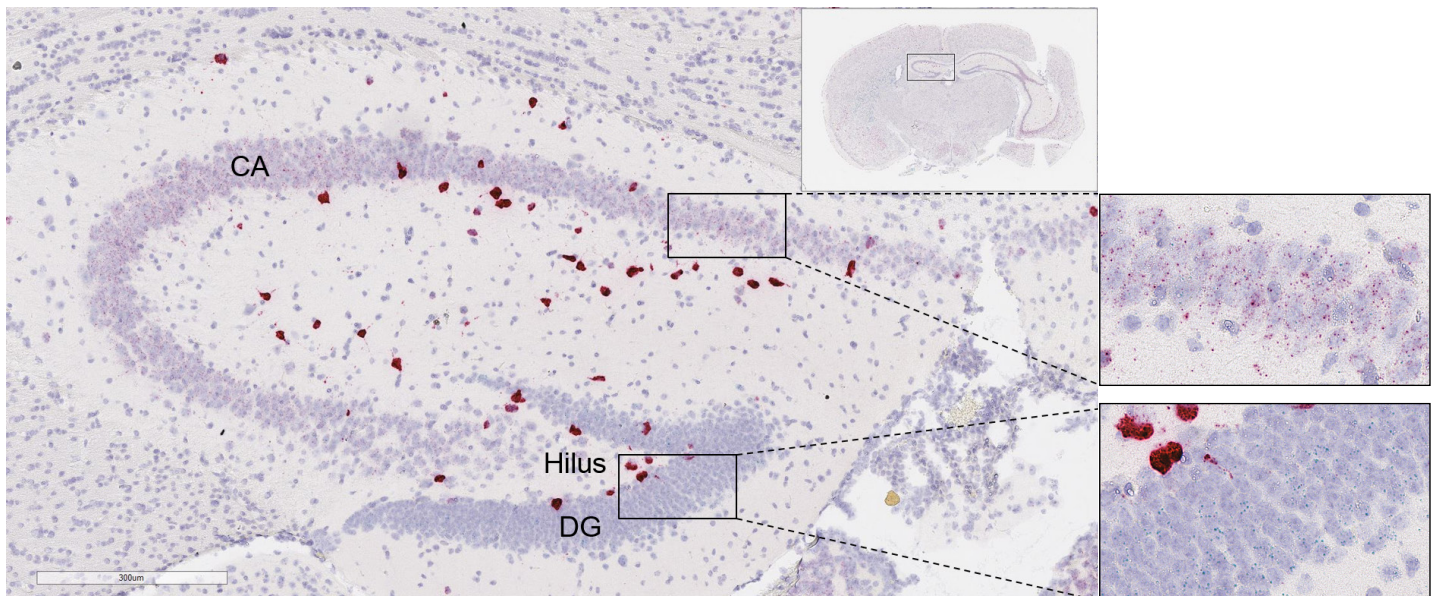


FIGURE 7. Detection of G protein-coupled receptors in normal mouse brain hippocampus using RNAscope® 2.5 HD Duplex Chromogenic assay on FFPE tissue samples: Cannabinoid receptor 1 (*Cnr1*, Red) and Dopaminergic receptor D1 (*Drd1*, Green). Cells are counterstained with hematoxylin. CA = Cornu Ammonis. DG = Dentate Gyrus.

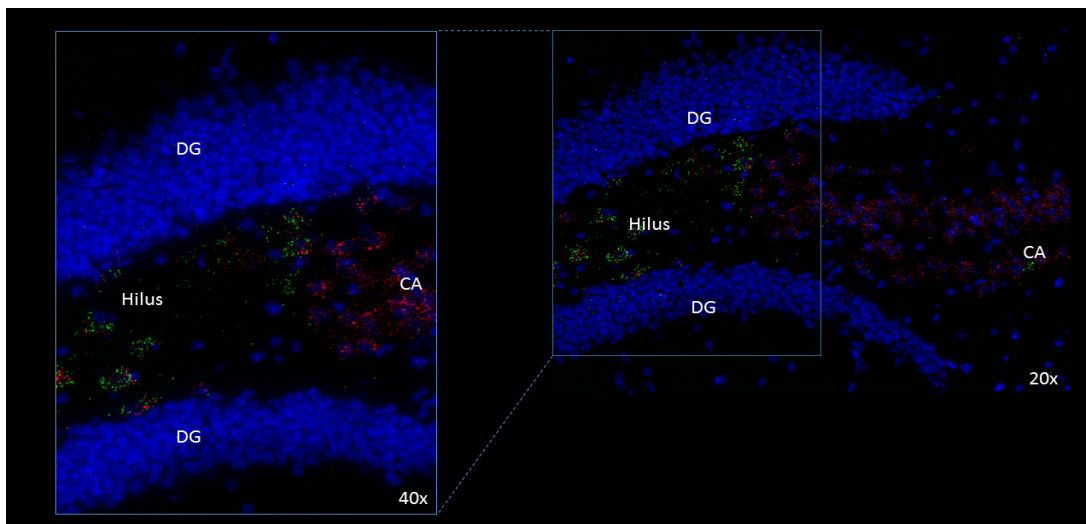


FIGURE 8. Detection of G protein-coupled receptors in normal mouse brain hippocampus using the RNAscope® Multiplex Fluorescent assay on Fresh Frozen tissue samples: Cholinergic Receptor Muscarinic 3 (*Chrm3*, Red) and Dopamine Receptor D2 (*Drd2*, Green). Cells are counterstained with DAPI. CA = Cornu Ammonis. DG = Dentate Gyrus.

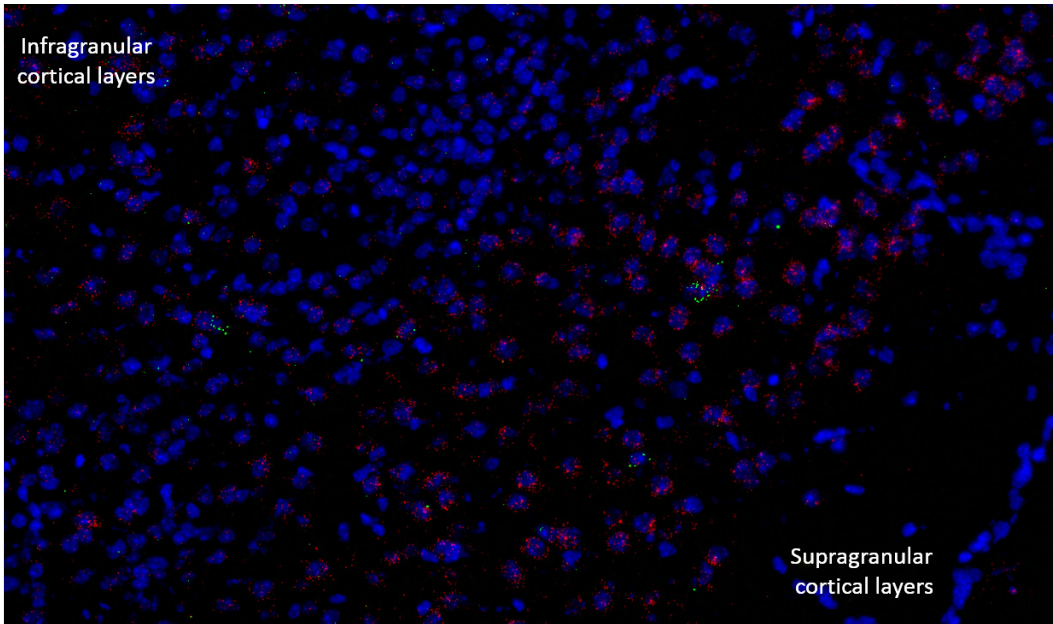


FIGURE 9. Detection of G protein-coupled receptors in normal mouse brain cortex using the RNAscope® Multiplex Fluorescent assay on Fresh Frozen tissue samples: Cholinergic Receptor Muscarinic 3 (*Chrm3*, Red) and Dopamine Receptor D2 (*Drd2*, Green). Cells are counterstained with DAPI.

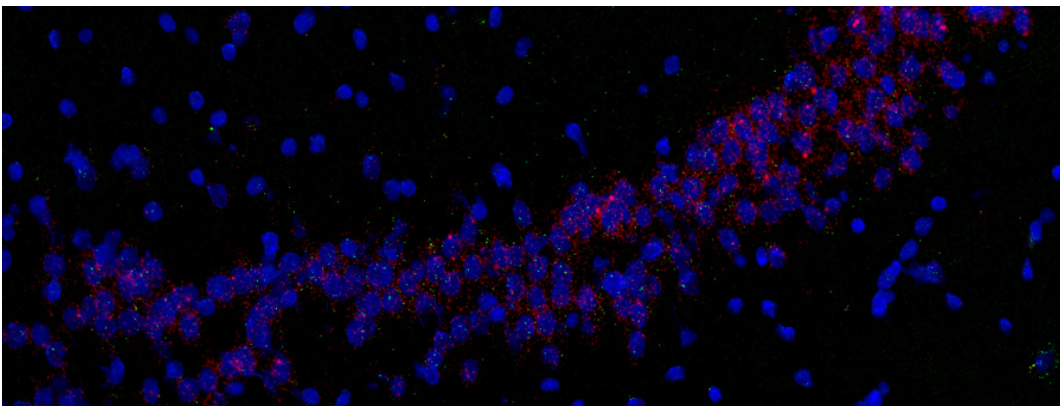


FIGURE 10. Fluorescent detection of ion channels. Detection of sodium Acid-Sensing Ion Channel 1 (*Asic1*, Green) and Inwardly-Rectifying Potassium Channel 3 (*Kcnj3*, Red) in normal mouse brain hippocampus using the RNAscope® Multiplex Fluorescent assay on Fresh Frozen tissue samples.

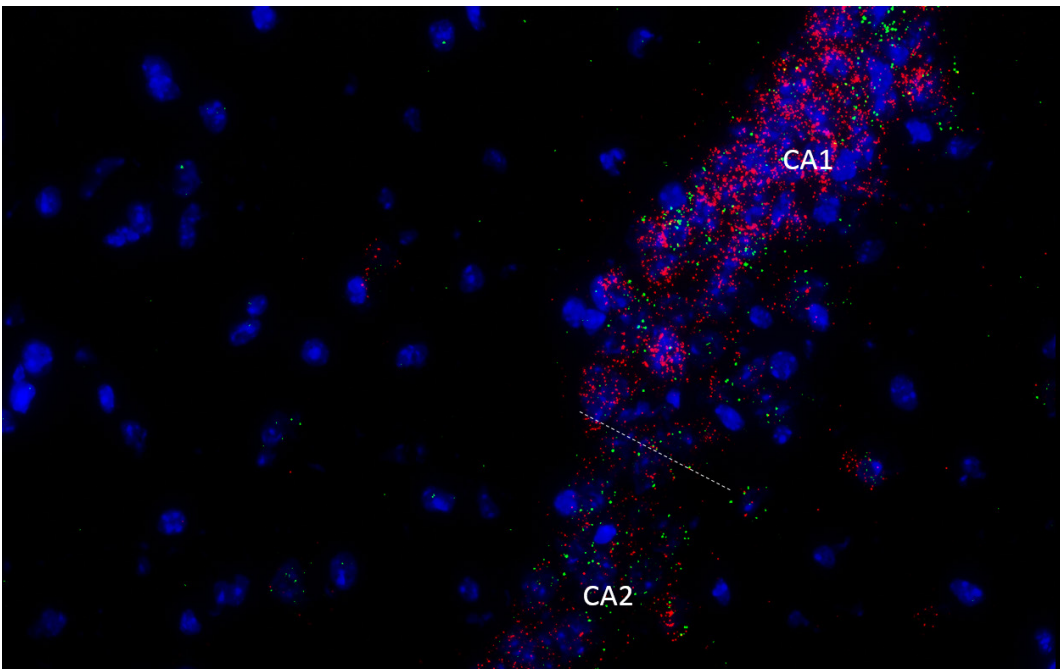


FIGURE 11. Detection of the transcription factor immediate early gene and activity marker *Cfos* (Green) in combination with the G protein-coupled receptor Cholinergic Receptor Muscarinic 3 (*Chrm3*, Red) in normal mouse brain hippocampus using the RNAscope® Multiplex Fluorescent assay on Fresh Frozen tissue samples. Cells are counterstained with DAPI. CA = Cornu Ammonis.

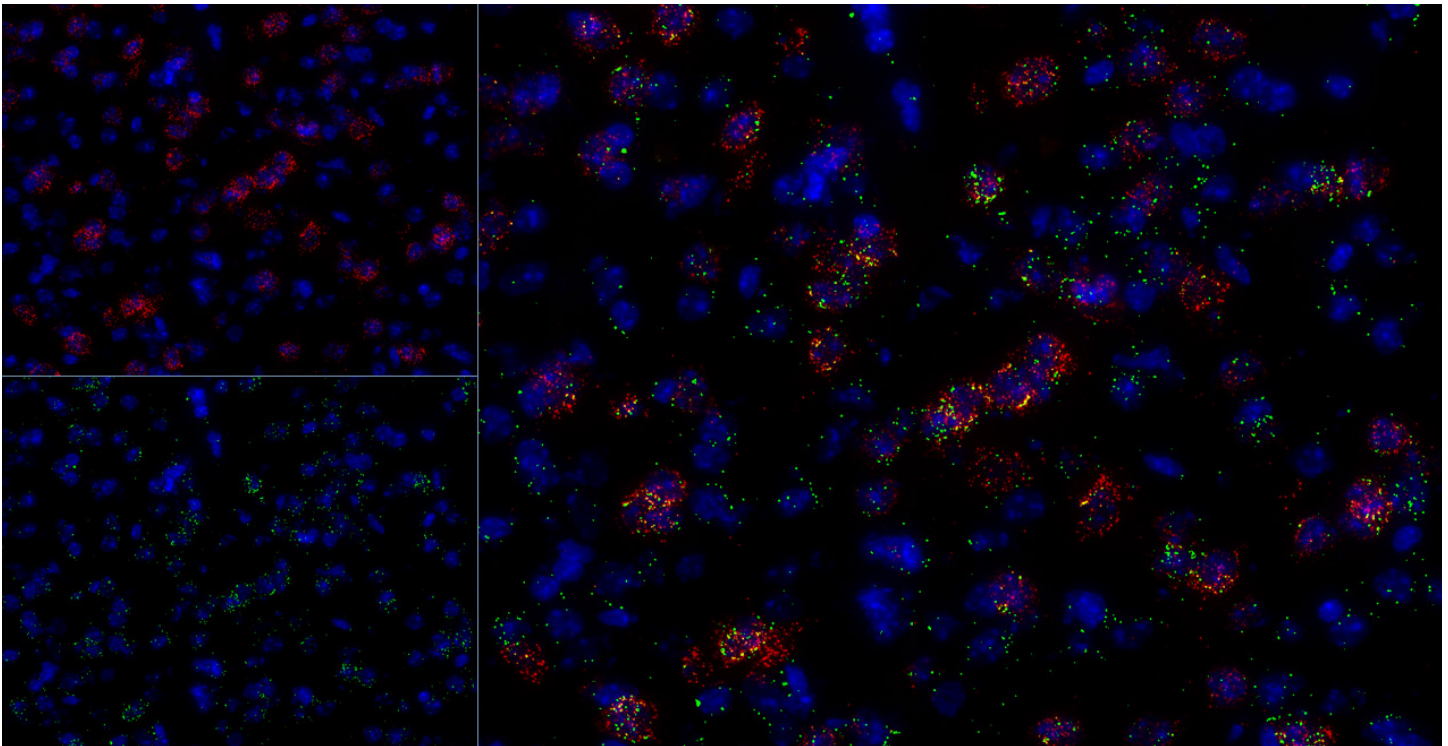


FIGURE 12. (Right panel) Detection of the effector immediate early gene and plasticity marker *Arc* (Green) in combination with the G protein-coupled Dopamine Receptor D1 (*Drd1*, Red) in normal mouse brain striatum using the RNAscope® Multiplex Fluorescent assay on Fresh Frozen tissue samples. (Top left panel) Single-channel image for *Drd1* (Red). (Bottom left panel) Single-channel image for *Arc* (Green).

## Visualization of neuronal network activity and plasticity

RNAscope® ISH can be applied for the visualization of activity and plasticity markers in the brain by means of detecting immediate early genes. Immediate early genes (IEGs) are genes which are transiently and rapidly activated at the transcriptional level in response to a wide variety of stimuli without the need for *de novo* protein synthesis (Guzowski *et al.*<sup>5</sup>). The detection of neuronal activity in the mouse brain hippocampus is shown by using a probe for *Cfos* which is a proto-oncogene and transcription factor that is induced within 15 minutes of stimulation (Figure 11). To detect neuronal plasticity, the expression of the effector immediate early gene Activity-Regulated Cytoskeleton-associated protein *Arc* (also known as *Arg3.1*) is visualized in Figure 12.

## Validation of target mRNA expression

The RNAscope® technology can be used to validate gene expression profiling studies or quantitative RT-PCR results. Examples from literature can be found in the research by Lake *et al.*<sup>6</sup> and Greer *et al.*<sup>7</sup>. Lake *et al.*<sup>6</sup> used both RNAscope® chromogenic and multiplex fluorescent ISH for a set of selected markers and confirmed subtype- and layer-specific expression patterns in the cortex revealed by single-cell transcriptomics for the human brain. Quantitative analysis showed that RNAscope® gene expression levels were consistent with

RNA-seq data. Greer *et al.*<sup>7</sup> employed the RNAscope® technology to validate RNA profiling expression data and to interrogate the expression pattern of 12 members of the Ms4a family in necklace sensory neurons.

## Validation of (cell type-specific and conditional) genetic modifications

RNAscope® ISH can be used to validate knock-out models or transgene expression patterns. In their study investigating the influence of the orphan GPCR GPR88 in A2AR-expressing *Drd2* neurons on anxiety-like behaviors, Meirman *et al.*<sup>8</sup> used multiplex fluorescent RNAscope® ISH for *Gpr88*, *Drd1* and *Drd2* to verify the specific excision of *Gpr88* in striatal *Drd2*-medium spiny neurons of the conditional A2AR-driven *Gpr88* knock-out. Seidemann *et al.*<sup>9</sup> reports results of a new method using the genetically encoded calcium indicator GCaMP6f for chronic imaging of neural population responses in the primary visual cortex V1 of behaving monkeys. The RNAscope® Multiplex Fluorescent assay was used for the spatial analysis and validation of virus-mediated GCaMP expression in tissue samples from a GCaMP6f expressing site in a macaque 10 weeks after viral injection. They showed that 80% of cells were CaMKIIa-positive and all neurons expressing GCaMP were CaMKIIa-positive (attesting to promotor fidelity).

## Conclusions

In this study we demonstrate the outstanding specificity and sensitivity of the RNAscope® assay as a method to chromogenically and fluorescently examine gene expression within the morphological tissue environment. Therefore, the RNAscope® technology is broadly applicable across various neuroscience research fields and especially in combination with cell type markers or when antibody resources are non-existent or not reliable including lncRNA and GPCR detection.

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