ACD Advanced Cell Diagnostics

Cellular localization of RNA expression in central and peripheral nervous system using RNAscope® Technology

Detect your RNA of interest in the central and peripheral nervous system with ease

- Detection of virtually any gene in any genome in any tissue with high sensitivity and specificity
- Visualize RNA expression and distribution with morphological context
- Uncover lncRNAs and other targets for which antibodies cannot detect

MOLECULAR DETECTION + MORPHOLOGICAL CONTEXT IN A SINGLE ASSAY

Detection of RNA in nervous system using RNAscope® Technology

Neuroscience is one of the fastest growing research fields, studying the structural and functional organization and the development of the central and peripheral nervous system from the molecular and cellular level to the systems level.

One challenge in the neuroscience field is the numerous cell types in the central nervous system (CNS), many of which remain to be identified and characterized at the molecular level.

RNAscope® *in situ* hybridization (ISH) technology enables cell-specific localization of RNA transcripts precisely in the tissue to identify markers associated with neuronal and synaptic molecular pathways and to characterize specific cell types such as neuronal and glial cells.

The RNAscope® assay can be used to:

- Identify, characterize, and localize mRNAs in the central and peripheral nervous systems
- Detect mRNA expression in axons and dendrites
- Detect mRNA in the nervous system when no reliable antibodies are available
- Identify long non-coding RNA in neuronal and glial cells
- Detect mRNA in primary neuronal cell cultures

RNAscope® ISH technology provides a unique detection method for neuronal and glial cells markers that is both highly sensitive and specific for detection of any gene expressed in any neuronal and glial cell[1]. Because of its proprietary "double Z" probe, RNAscope® technology is a powerful tool to detect any gene, especially low abundance targets. And not only can RNAscope® technology be used to discern if your gene of interest is in a neuronal or glial cell, but it can also show its subcellular localization. The multiplexing capabilities of RNAscope® assay allows for detection of multiple markers at once, enabling exquisite characterization of cell populations within the nervous system as well as detection of both neuronal and glial cell markers and the signals these cells produce. RNAscope® technology also helps solve the problem when no reliable antibodies exist for your gene of interest and allows you to visualize and quantify for virtually any gene from any genome in any tissue.

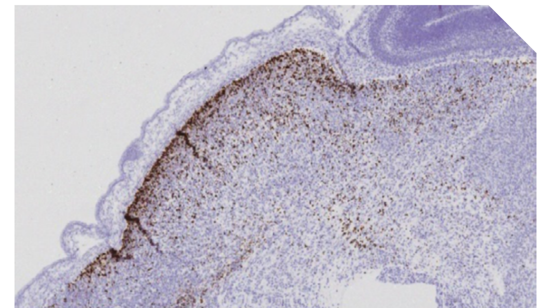

FIGURE 1. Detection of *Otx2* mRNA in mouse cerebellum FFPE tissue with the RNAscope® 2.0 HD Reagent Kit - BROWN.

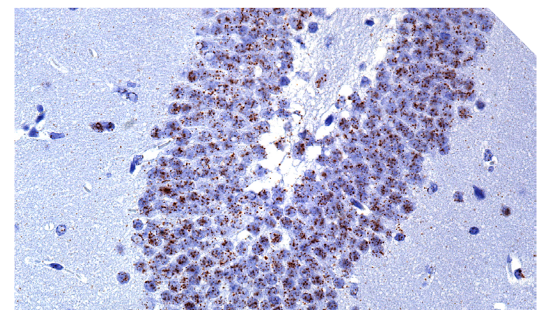

FIGURE 2. *Mash1* mRNA expression in mouse brain tissue, RNA *in situ* hybridization (ISH) using RNAscope® 2.0 HD Reagent Kit - BROWN.

Applications of RNAscope® Technology in Neuroscience

Identification, characterization, and localization of mRNA in the peripheral and central nervous systems

Numerous publications have shown that the RNAscope® technology is the method of choice to visualize mRNA in the central and peripheral nervous systems and to validate quantitative PCR and RNA sequencing data within the morphological context.

Epilepsy is a neurological disorder affecting 1% of the population worldwide. Puranam *et al.* have identified a family in which a translocation between chromosomes X and 14 was associated with cognitive impairment and a complex genetic disorder called "Genetic Epilepsy and Febrile Seizure Plus" (GEFS+)[2]. They demonstrated that the breakpoint on the X chromosome disrupted a gene that encodes an auxiliary protein of voltage-gated Na+ channels, fibroblast growth factor 13 (Fgf13), and that deletion of one *Fgf13* allele in female mice produced hyperthermia -induced seizures and epilepsy. Using the RNAscope® Fluorescent Multiplex Assay, they have shown that *Fgf13* mRNA is expressed in excitatory and inhibitory neurons (co-localization with *Gad-1*) in mouse hippocampus (Figure 3).

Together with electrophysiological recordings in the hippocampal neurons of *Fgf13* mutant mice, these findings suggest that reduced expression of *Fgf13* in the hippocampus impairs excitability of inhibitory interneurons, resulting in enhanced excitability within local circuits of the hippocampus and the clinical phenotype of epilepsy.

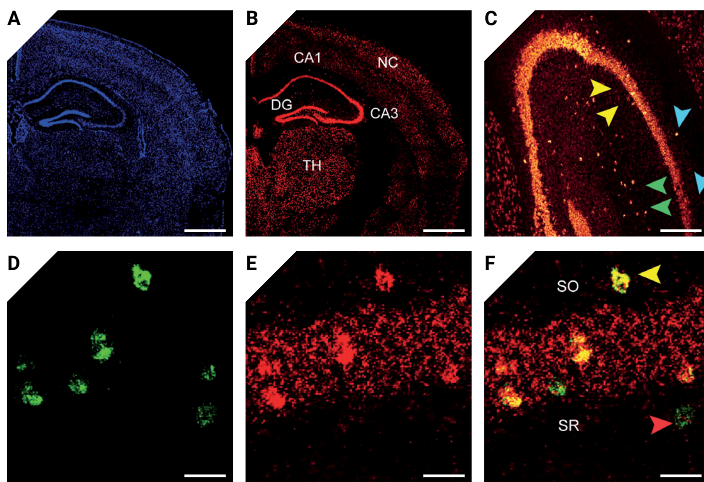

FIGURE 3. Expression of *Fgf13* mRNA in excitatory and inhibitory neurons of mouse hippocampus revealed by RNAscope® Fluorescent Multiplex technology. **A-C.** *Fgf13* mRNA expression in fresh frozen adult mouse coronal sections. DAPI staining is shown in (A). High expression of *Fgf13* in multiple regions of the brain, particularly in the hippocampus and pyriform cortex, with moderate expression in the neocortex and thalamus (B). Enlarged image reveals scattered cells in hippocampus strata oriens (blue arrowheads), radiatum (yellow arrowheads) and lacunosum moleculare (green arrowheads), consistent expression within interneurons of the CA1 region (C). Scale bars 1000 µm. **D-F.** Dual RNAscope® Fluorescent Multiplex detection using *Gad-1* (green) and *Fgf13* (red) probes, demonstrating the expression of *Fgf13* in both excitatory and inhibitory neurons. Scale bars 100 µm.

HIGHLIGHTED PUBLICATION:

Visualizing vesicular glutamate and glycine transporters expression in mouse coronal brain sections using RNAscope® Fluorescent Multiplex Assay[3].

Hackett *et al.* 2015

Vesicular transporters are packaging neurotransmitters into synaptic vesicules and therefore play important roles in the release machinery of the central nervous system. Among numerous vesicular transporters, Hackett *et al.* have studied the expression of glutamate (VGlut1, VGlut2 and VGlut3) and glycine (VGAT) transporters in the primary auditory cortex (A1) and medial geniculate body (MGB) of developing mice. To track the changes in transporter expression during development, they used RNA sequencing, immunohistochemistry and *in situ* hybridization using RNAscope® Fluorescent Multiplex Assay (Figures 4 and 5).

The study from Hackett *et al.* shows the powerful impact of RNAscope® assay on the identification of target genes from the sequencing data. RNAscope® Fluorescent Multiplex Assay provided detailed evaluation of the co-expression and co-localization of several genes in intact tissue sections, where natural anatomical features are preserved.

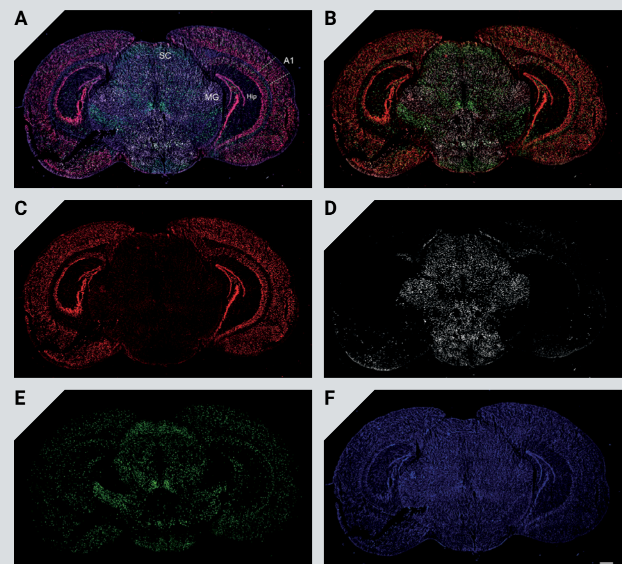

FIGURE 4. Multiplex fluorescence *in situ* hybridization using RNAscope® technology. **A.** Triple stainings of *VGAT, VGluT1* and *VGluT2* mRNA, counterstained by DAPI in fresh frozen coronal sections of adult mouse at the level of A1 and MGB. **B-F.** Single channel expression for *VGluT1* (C), *VGluT2* (D), *VGAT* (E), *DAPI* (F), and merged *VGluT1/VGluT2/VGAT* (B). SC superior colliculus, Hip hippocampus, MG medial geniculate body. Scale bars 500 µm in all panels.

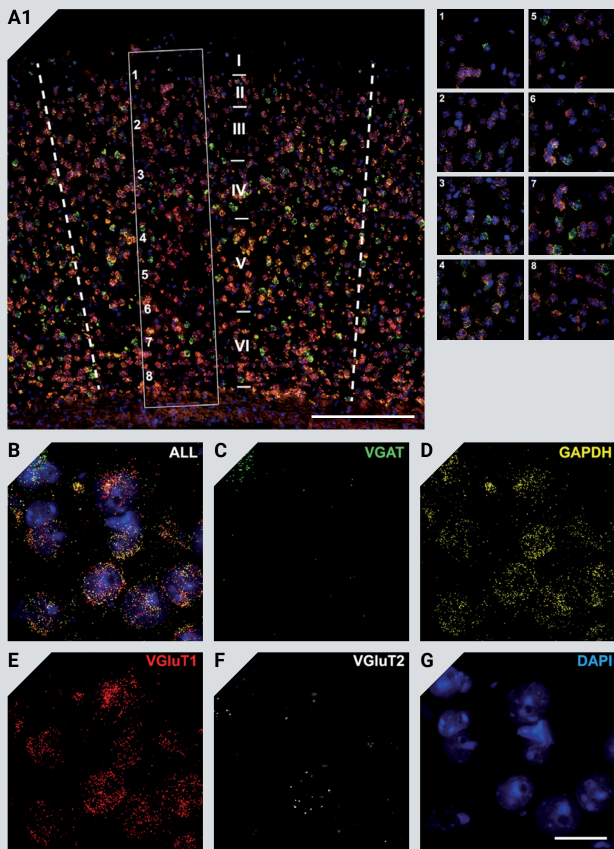

FIGURE 5. Fluorescent Multiplex *in situ* hybridization using RNAscope® technology. Quadruple staining of *VGAT, GAPDH, VGlut1* and *VGlut2* mRNA, counterstained by DAPI in fresh frozen coronal sections of adult mouse centered on **A1.** Image montage obtained at 40x magnification showing combined expression for *VGAT, GAPDH, VGluT1* and *VGluT2* mRNA, counterstained with DAPI. **Subpanels 1-8** show 100x magnification image stacks taken at sites in different layers of (A1). **B-G.** Higher resolution examples of transcript labeling from subpanel 4 shown separately for each gene. Scale bars (A) 250 µm, all other panels 20 µm.

Interneurons play a critical role in the modulation of neuronal network activity. It is therefore very important to understand the mechanisms involved in the regulation of this extremely diverse class of neurons. Local GABA (gamma aminobutyric acid)-ergic inhibitory interneurons are essential coordinators of cortical microcircuits and are implicated in neurological disorders such as epilepsy and schizophrenia. In these pathologies, the balance of excitatory and inhibitory transmission is perturbed. Local GABAergic interneurons are regulated by interactions between fast-acting synaptic transmissions and slow-acting neuro-modulators. Many research projects have been focused on two of these regulators, the N-methyl-D-aspartat receptors (NMDARs) and the neuregulin (NRG) receptor tyrosine kinase ErbB4. ErbB4 is widely expressed in GABAergic interneurons where its acute stimulation by NRGs promotes the internalization of ion channels from the cell surface. Vullhorst *et al.*, studied the NRG/ErbB4 pathway in interneurons and demonstrated that NMDARs are required to release biologically active NRG2 from cluster aggregates on the surface of interneuron cell bodies. In their study the authors have used the RNAscope® Fluorescent Multiplex Assay to study the expression of *ErbB4* and *NRG2* in mouse hippocampus interneurons (Figure 6)[4]. While most cells had weak expression of *NRG2*, cells expressing *ErbB4* had much higher levels of *NRG2*, suggesting that *ErbB4+* interneurons co-express *NRG2*.

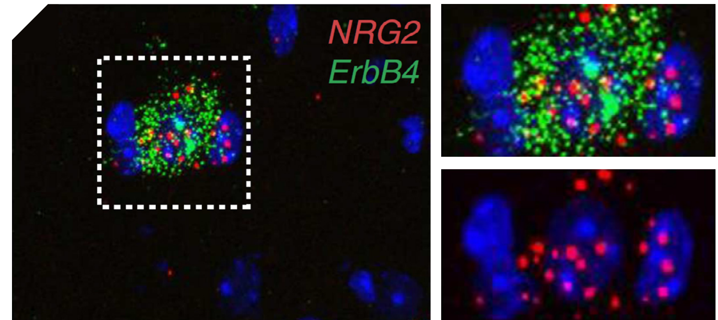

FIGURE 6. RNAscope® Fluorescent Multiplex ISH of *NRG2* and *ErbB4* in the mouse hippocampus, showing overlapping signals in a neuron located in the stratum oriens of area CA1. The pyramidal cell layer is visible in the lower right corner. DAPI was added to label nuclei (blue). Note that *ErbB4*-negative cells have much lower or no *NRG2* signal. Magnified areas on the right outlined by boundary box.

The gastrin-releasing peptide (GRP) also known as the "itch" neurotransmitter has been the subject of a continuing controversy. Before Solorzano *et al*. published their study, previous publications had demonstrated abundant GRP immunoreactivity in DRG neurons. Using *in situ* hybridization after qPCR, and immunohistochemistry to reveal the expression of the "itch" neurotransmitter, the authors showed that there is an abundant expression of *GRP* mRNA and protein in the superficial dorsal horn of the mouse spinal cord but not in DRG neurons[5]. Furthermore, using the RNAscope® Fluorescent Multiplex Assay, they have shown that GRP-expressing neurons of the superficial dorsal horn do not co-express the GRP receptor (GRPR), supporting the view that pruritogens engage spinal cord "itch" circuits via excitatory superficial dorsal horn interneuron that express GRP and that likely target GRPR-expressing interneurons (Figure 7).

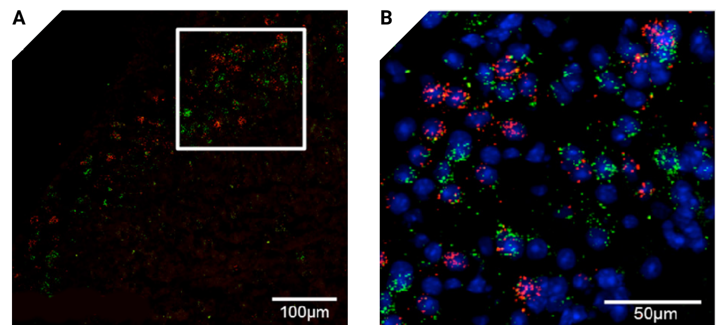

FIGURE 7. RNAscope® Fluorescent Multiplex ISH of *GRP* (green) and *GRPR* (red) in mouse spinal cord fresh frozen sections. Low power (A) and high-power (B) images of double ISH for *GRP* and *GRPR* demonstrate a close association but no overlap of these interneuron populations.

Detection of mRNA in axons and dendrites

The unique morphology of neurons raises the question of how the molecules required for the structure and function of the axon and its terminal are supplied. The discovery of protein synthesis machinery in the growth cones of mammalian neurons and the subsequent identification of various mRNAs specifically localized in axon terminals suggested that mRNA can be transported along the axon and locally translated at the nerve terminal. In addition, local translation may give autonomy to the nerve terminals to respond quickly to signals without waiting for input from the cell body. It has been suggested that axonal protein synthesis is required for axon growth as well as retrograde signaling from the tip of the axon to the cell body. Baleriola *et al.* examine whether intra-axonal protein synthesis is necessary for retrograde transmission of neurodegenerative signals in the human brain (see highlighted publication).

Detection of neuronal and glial markers when no reliable antibodies are available

Most proteins have no antibody and many have no reliable antibody for detection, limiting studies of their expression[7]. Among them are numerous proteins acting in the nervous system such as G-protein coupled receptors (GPCRs), ion channels, and neurotransmitter transporters and receptors. While conventional *in situ* hybridization stays challenging to establish for new users, RNAscope® technology is a method of choice to visualize these mRNAs in morphological context in the central and peripheral nervous systems.

Kappa-opioid receptor (KOR) agonists have disphoric properties in aversive properties in rodents. This has been attributed to the activation of KORs within the mesolimbic dopamine (DA) system. However, the role of DA in KOR-mediated aversion and stress remains controversial as recent studies have suggested that activation of KORs on serotoninergic neurons may be sufficient to mediate aversive behaviors. Chefer *et al.* have addressed this question using conditional knock-out (KO) mice with KORs deleted on DA neurons[8]. Control mice displayed conditioned place aversion (CPA) to the systematically administered KOR agonist U69,593 but, in contrast, DATCre-KOR KO mice did not exhibit CPA with the same agonist. Their results have provided evidence that KORs on ventral tegmental area (VTA) DA neurons are necessary to mediate KOR-mediated aversive behavior. They characterized the expression of *KOR* in DAT neurons using RNAscope® Fluorescent Multiplex Assay and demonstrated that Cre-mediated recombination of the *KOR* gene is specific to dopamine transporter mRNA expressing neurons in the VTA region (Figure 9).

HIGHLIGHTED PUBLICATION:

Axonally Synthesized ATF4 Transmits a Neurodegenerative Signal across Brain Regions. Visualization of axonal expression of Atf4 mRNA in cultured neurons and in post mortem human brain[6].

Baleriola *et al.* 2014

In Alzheimer's disease (AD) brain, exposure of axons to Aβ causes pathogenic changes that spread retrogradely by unknown mechanisms, affecting the entire neuron. Baleriola *et al.* found that locally applied $A\beta_{1-42}$ initiated axonal synthesis of a defined set of proteins including the transcription factor Atf4. In their study, they used fluorescent *in situ* hybridization to visualize the axonal *Atf4* mRNA in mouse hippocampal neurons grown in microfluidic chambers.

To confirm these findings, they used RNAscope® BROWN chromogenic assay to visualize the expression of *Atf4* mRNA in post mortem brains of 8 AD patients and 8 age-matched controls. Axons and cell bodies containing *Atf4* mRNA granules were found in the hippocampal formation in all cases. However, AD brains exhibited a higher frequency of *Atf4*-containing axons in the hippocampus, subiculum, and entorhinal cortex (Figure 8).

These results reveal an active role for intra-axonal translation in neurodegeneration and identify Atf4 as a mediator for the spread of AD pathology.

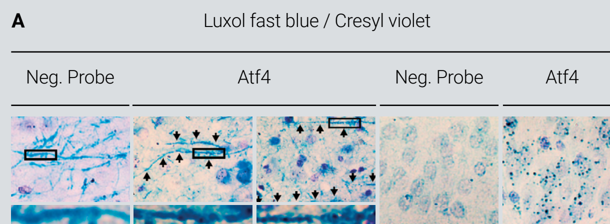

FIGURE 8. Visualization of axonal *Atf4* mRNA in AD post mortem human brain FFPE tissue samples by RNAscope® 2.0 HD Reagent Kit-BROWN Chromogenic Assay. Representative micrographs of *Atf4* mRNA granules in axons and cell bodies in human brain samples. **Panels 1-3:** axons stained with Luxol fast blue with a negative probe or an *Atf4*-targeting probe. *Atf4*-containing axons are indicated with arrows. **Panels 4-5:** examples of granule cells stained with cresyl violet and a negative or *Atf4*-targeting probe. The scale bars represent 20 µm and 5 µm (insets).

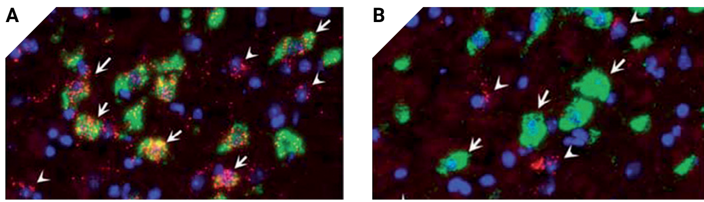

FIGURE 9. Detection of *KOR* in DAT positive neurons using RNAscope® Fluorescent Multiplex Assay on fresh frozen sections of mouse midbrain. Representatives images of dual fluorescence ISH of *DAT* mRNA (green) and *KOR* mRNA (red) in the VTA of a DAT Cre-KOR KO (B) and control (A) mouse. *DAT* positive neurons do not express *KOR* mRNA in DAT-KOR KO mice (B). *KOR* mRNA was present in DAT-negative neurons in both control and KO animals.

Although early studies suggested that cannabinoid CB_2 receptors (CB_2Rs) are absent in the brain, these data have been challenged by recent findings of significant CB_2R involvement in several dopamine (DA)-related CNS disorders. Zhang *et al.* have studied the cellular mechanisms underlying these actions and have found that CB_2R genes and receptors are expressed in midbrain DA neurons, and that activation of CB_2Rs receptors inhibits DA neuronal firing and intravenous cocaine self administration[11]. In their study, they used RNAscope® Fluorescent Multiplex Assay to reveal the expression of CB_2R mRNA in DA neurons. They performed dual ISH using a CB_2 probe to detect their target and a tyrosine hydroxylase (TH) probe to identify the phenotype of the CB_2-positive neurons (Figures 10 and 11). The findings of Zhang *et al.* completely change the view that brain CB_2Rs are not expressed in neurons and suggest that neuronal CB_2Rs modulate DA neuronal activity and DA-regulated behavior.

Energy homeostasis is regulated by the release of anorexigenic α-melanocyte stimulating hormone and orexigenic agouti-related peptide (AgRP) from discrete hypothalamic arcuate neurons onto common target sites in the central nervous system. These two peptides are ligands to the melanocortin-4-receptor (MC4R): α-MSH is an agonist that couples to the receptor in the Gαs signaling pathway while AgRP binds competitively to block α-MSH binding. Ghamari-Langroudi *et al.* have recently shown that, in mice, regulation of the firing activity of neurons from the paraventrical nucleus of the hypothalamus (PVN) by α-MSH and AgRP can be mediated independently from Gαs signaling by ligand induced coupling of MC4R to the closure of inwardly rectifying potassium channel Kir 7.1[9]. Using RNAscope® Fluorescent Multiplex Assay they performed dual fluorescent ISH in sections of mouse PVN and determined that approximately 90% of PVN expressing *MC4R* messenger co-express *Kir7.1* mRNA (Figure 12). They concluded that the coupling of MC4R and Kir7.1 might explain unusual aspects of the control of energy homeostasis by melanocortin signaling.

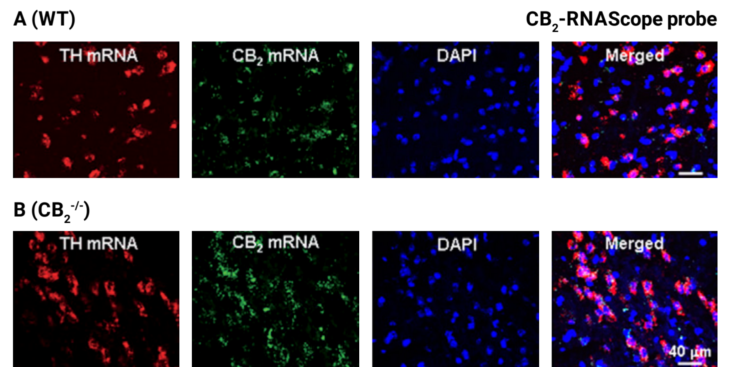

FIGURE 10. *CB_2* mRNA expression in VTA neurons visualized by RNAscope® Fluorescent Multiplex Assay. **A.** *CB_2* mRNA and the location detected by a *CB_2* RNAscope® probe. **B.** CB_2 RNAscope® probe detected *CB_2* mRNA in VTA DA neurons in WT and $CB_2^{-/-}$ mice. This probe detected *CB_2* mRNA in $CB_2^{-/-}$ mice because it targets the downstream UTR region rather than the upstream gene-deleted region.

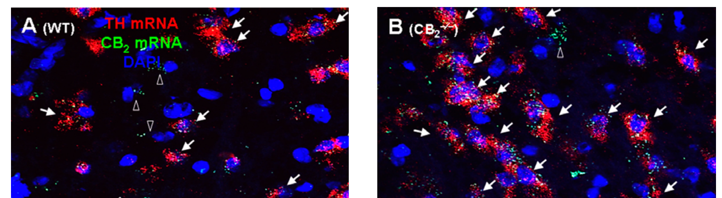

FIGURE 11. Detection of *CB_2* and *TH* mRNA in fresh frozen brain sections using RNAscope® Fluorescent Multiplex Assay. Representative confocal images under high magnification illustrating colocalization of *CB_2* mRNA (green) and *TH* mRNA (red) in VTA DA neurons (white arrows) in WT (A) and Zimmer $CB_2^{-/-}$ (B) mice. *CB_2* mRNA is also expressed in TH-negative VTA non-DA neurons (open triangles).

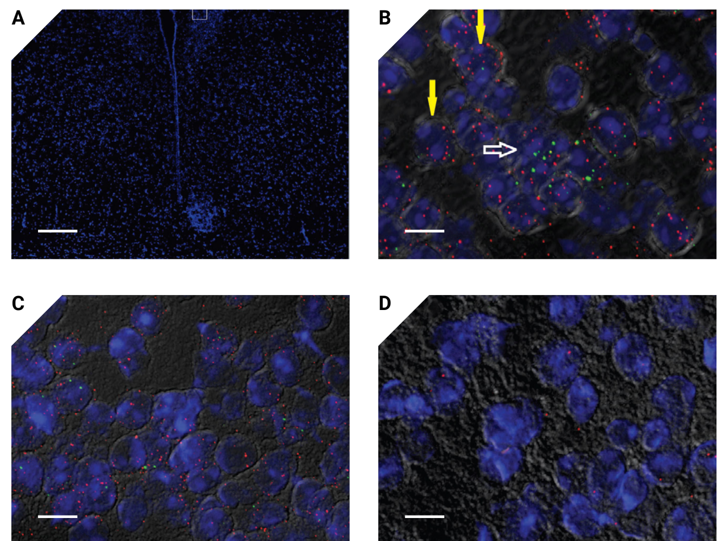

FIGURE 12. Co-expression of *MC4R* and *Kir7.1* mRNA in the paraventrical nucleus of the hypothalamus (PVN) determined by fluorescent ISH using RNAscope® Fluorescent Multiplex Assay on fresh frozen PVN sections. Images demonstrate the region of the hypothalamus under study (**A.** scale bar, 200 µm), co-localization of *MC4R* (green) and *Kir7.1* (red) mRNAs (**B.** white open arrows, double labeled cells; yellow arrows, *Kir7.1* expression only, scale bars 10 µm), and negative controls (**C.** *MC4R* probe with tissue from MC4R knockout mice, **D.** bacterial probe with tissue from wild-type mice, scale bars 10 µm).

Identifying the role of long non-coding RNA in the nervous system

Recent genome-wide studies have shown that only 2% of transcribed RNAs could be translated into proteins, making the vast majority of transcripts “non-coding”[7]. Except for the RNAs involved in translation, such as rRNA and tRNA, other non-coding RNAs (known as regulatory RNAs), can play a variety of roles in transcriptional and postranscriptional regulation. Non-coding RNAs vary in length and function, with long non-coding (lnc) RNAs greater than 200 nucleotides in length. Emerging data indicate that lncRNAs can have critical biological functions.

Ramos *et al.* have identified a novel neural-specific lncRNA, Pinky (*Pnky*), which regulates neurogenesis from neural stem cells (NSCs) in the embryonic and postnatal brain[10]. Using RNAscope® technology, they demonstrated the nuclear expression of *Pnky* in ventricular-subventricular (V-SVZ) NSC cultured cells and in the V-SVZ of the adult mouse brain (Figure 13).

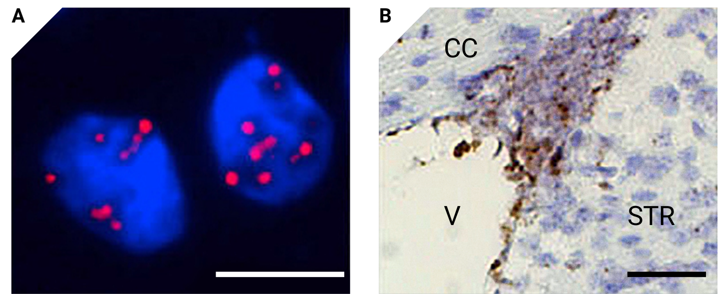

FIGURE 13. **A.** Detection of *Pnky* lncRNA in V-SVZ NSC cultured cells using RNAscope® Fluorescent Multiplex Assay. Nucleus are counterstained with DAPI, scale bar 10 µm. **B.** Detection of *Pkny* lncRNA in adult mouse coronal brain sections using RNAscope® 2.0 HD Reagent Kit-BROWN. Nuclei are counterstained with hematoxylin. V: ventricle, CC: corpus collosum, STR: striatum. Scale bar 50 µm.

Detection of RNA in primary neuronal cell cultures

Central nervous system-related diseases are extremely difficult to study due to the unavailability of human neurons. To study neurological disorders, human iPSC (induced pluripotent stem cells)-derived neurons have been characterized as a highly pure population of GABA-ergic and glutamatergic subtypes. Menghello *et al.* from Janssen Pharmaceuticals have used RNAscope® Assay to characterize iCell, commercially available iPSC-derived neurons. Using RNAscope® Fluorescent Multiplex Assay, they have demonstrated that these cells are expressing the neuronal marker *MAP2* as well as the GABAergic marker *VIAAT* (vesicular inhibitor amino acid transporter *SLC32A1*)[12] (Figure 14).

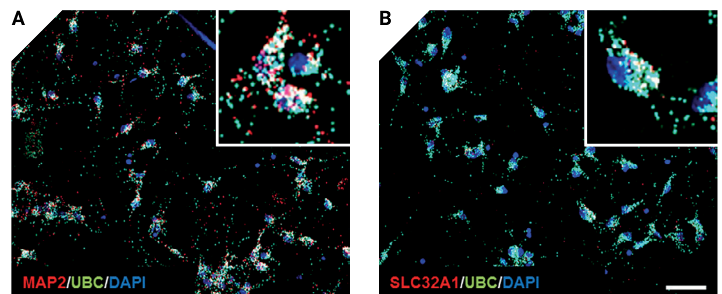

FIGURE 14. Characterization of iCell® iPSC-derived neurons using RNAscope® Fluorescent Multiplex Assay. **A.** *MAP2* mRNA (red) in DIV14 iPSC-derived neurons, positive control ubiquitin (*UBC*) is represented in green together with DPAI as nuclear staining. **B.** *SLC32A1* mRNA positive signal (red).

Primary neuronal cell cultures of rodent neurons also represent an indispensable tool to better understand neurological disorders and to study neuronal molecular pathways. Grabinski *et al.* have developed a method for combining RNAscope® ISH with immunohistochemistry (IHC) in thick free-floating brain sections and in primary neuronal cell cultures[13] (Figure 15). IHC is widely used in neuroscience to detect critical markers of neuronal or glial cells while RNAscope® *in situ* hybridization allows semi-quantitative detection of one or several targets of interest. Combination of both methods is a powerful tool to study molecular pathways within the morphological context of the central and peripheral nervous system.

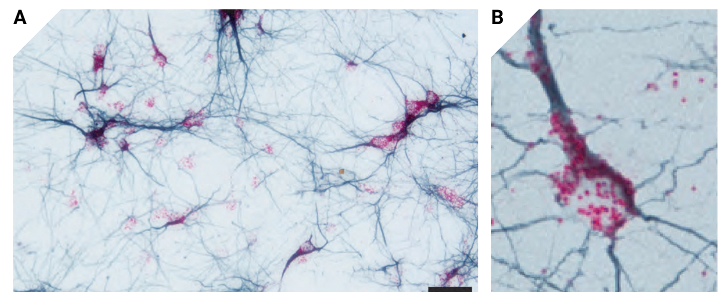

FIGURE 15. RNAscope® 2.0 HD Reagent Kit-RED combined with IHC in primary neuron cultures. Primary rat neurons were processed for *tau* ISH and βIII-tubulin IHC. Clear puncta are present for the *tau* ISH signal (red), while neuronal cell bodies and processes are labeled with tubulin IHC (blue). Scale bar 50 µm.

Summary

Identification and characterization of the numerous cell types of the nervous system is critical to neuroscience research field. RNAscope® technology not only detects neuronal markers and molecular pathways, but also provides information regarding morphological context. Due to the high resolution capabilities of RNAscope® ISH, the technology can offer additional information to gene profiling studies by identifying the cellular distribution and anatomical regions of gene expression. RNAscope® technology is also an excellent resource to detect markers for which no reliable antibodies are available, such as GPCRs, or antibodies are unable to detect, such as lncRNAs.

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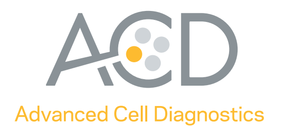

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