Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61709R2
Full Title:	Combining multiplex fluorescence in situ hybridisation with fluorescent immunohistochemistry on fresh frozen or fixed mouse brain sections
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
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TITLE:

Combining multiplex fluorescence *in situ* hybridization with fluorescent immunohistochemistry on fresh frozen or fixed mouse brain sections

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KEYWORDS:

RNAScope; fluorescence *in situ* hybridization; multiplex labelling, protease, fluorescent immunohistochemistry, respiratory

SUMMARY:

This protocol describes a method for combining fluorescence *in situ* hybridization (FISH) and fluorescence immunohistochemistry (IHC) in both fresh frozen and fixed mouse brain sections, with the goal of achieving multilabel FISH and fluorescence IHC signal. IHC targeted cytoplasmic and membrane attached proteins.

ABSTRACT:

Fluorescent in situ hybridization (FISH) is a molecular technique that identifies the presence and spatial distribution of specific RNA transcripts within cells. Neurochemical phenotyping of functionally identified neurons usually requires concurrent labelling with multiple antibodies (targeting protein) using immunohistochemistry (IHC) and optimization of in situ hybridization (targeting RNA), in tandem. A “neurochemical signature” to characterize particular neurons may be achieved however complicating factors include the need to verify FISH and IHC targets before combining the methods, and the limited number of RNAs and proteins that may be targeted simultaneously within the same tissue section.

Here we describe a protocol, using both fresh frozen and fixed mouse brain preparations, which detects multiple mRNAs and proteins in the same brain section using RNAscope FISH followed by fluorescence immunostaining, respectively. We use the combined method to describe the expression pattern of low abundance mRNAs (e.g., galanin receptor 1) and high abundance mRNAs (e.g., glycine transporter 2), in immunohistochemically identified brainstem nuclei.

Key considerations for protein labelling downstream of the FISH assay extend beyond tissue

preparation and optimization of FISH probe labelling. For example, we found that antibody binding and labelling specificity can be detrimentally affected by the protease step within the FISH probe assay. Proteases catalyze hydrolytic cleavage of peptide bonds, facilitating FISH probe entry into cells, however they may also digest the protein targeted by the subsequent IHC assay, producing off target binding. The subcellular location of the targeted protein is another factor contributing to IHC success following FISH probe assay. We observed IHC specificity to be retained when the targeted protein is membrane bound, whereas IHC targeting cytoplasmic protein required extensive troubleshooting. Finally, we found handling of slide-mounted fixed frozen tissue more challenging than fresh frozen tissue, however IHC quality was overall better with fixed frozen tissue, when combined with RNAscope.

INTRODUCTION:

Proteins and mRNAs that neurochemically define subpopulations of neurons are commonly identified with a combination of immunohistochemistry (IHC) and/or *in situ* hybridization (ISH), respectively. Combining ISH with IHC techniques facilitates the characterization of colocalization patterns unique to functional neurons (neurochemical coding) by maximizing multiplex labelling capacity.

Fluorescent ISH (FISH) methods, including RNAscope, have higher sensitivity and specificity compared to earlier RNA detection methods such as radioactive ISH and non-radioactive chromogenic ISH. FISH enables visualization of single mRNA transcripts as punctate stained spots[1]. Furthermore, the RNAscope assay allows an increased number of RNA targets to be labeled at a time, using different fluorophore tags. Despite these advantages, technical limitations may affect the number of fluorophores/chromogens that can be used in a single experiment. These include availability of microscope filter sets; such considerations are compounded when neurochemical identification uses combined FISH and IHC, compared to using each technique in isolation, since inherent steps optimal for one method may be detrimental to the other.

Previous application of FISH combined with IHC has demonstrated the expression of specific cellular targets in human B-cell lymphomas[2], chick embryos[3], zebrafish embryos[4], mouse retina[5] and mouse inner ear cells[6]. In these studies, tissue preparation was either formalin-fixed paraffin embedded (FFPE)[2,3,5] or fresh whole mount[4,6]. Other studies applied chromogenic RNAscope on fixed mouse and rat brain preparations[7-9]. In particular, Baleriola *et al.*[8] described two different tissue preparations for combined ISH-IHC; fixed mouse brain sections and FFPE human brain sections. In a recent publication, we combined FISH and fluorescent IHC on fresh frozen sections, to simultaneously visualize low abundance mRNA (galanin receptor 1, GalR1), high abundance mRNA (glycine transporter 2, GlyT2) and vesicular acetylcholine transporter (vAChT) protein[10] in the brainstem reticular formation.

The nucleus of the solitary tract (NTS) is a major brain region involved in autonomic function. Located in the hindbrain, this heterogeneous population of neurons receives and integrates a vast number of autonomic signals, including those that regulate breathing. The NTS harbors several neuronal populations, which may be phenotypically characterized by the expression

pattern of mRNA targets including GalR1 and GlyT2 and protein markers for the enzyme tyrosine hydroxylase (TH) and the transcription factor Paired-like homeobox 2b (Phox2b).

The RNAscope proprietor recommends fresh frozen tissue preparations, but tissue prepared by whole animal transcardial perfusion fixation, along with long term cryoprotection (storage at -20 °C) of fixed frozen tissue sections, is common in many laboratories. Hence, we sought to establish protocols for FISH in combination with IHC using fresh frozen and fixed frozen tissue preparations. Here, we provide for fresh frozen and fixed frozen brain sections: (1) a protocol for combined FISH and fluorescent IHC (2) a description of the quality of mRNA and protein labelling produced, when utilizing each preparation (3) a description of the expression of GalR1 and GlyT2 in the NTS.

Our study revealed that, when combined with RNAscope methodology, IHC success varied in fresh frozen and fixed frozen preparations and, was dependent upon localization of the target proteins within the cell. In our hands, membrane bound protein labelling was always successful. In contrast, IHC for cytoplasmic protein required troubleshooting even in cases where the cytoplasmic protein was overexpressed in a transgenic animal (Phox2b-GFP)[11]. Finally, while GalR1 identify non catecholaminergic neurons, GlyT2 is not expressed within the NTS.

PROTOCOL:

A summary of tissue pre-processing steps may be found in **Figure 1**. All procedures were carried out in compliance with the Animal Care and Ethics Committee of the University of New South Wales in accordance with the guidelines for the use and care of animals for scientific purposes (Australian National Health and Medical Research Council).

1. Sample preparation of fresh frozen brain tissue

1.1. Transcardial Perfusion

1.1.1. Prepare heparinized (2500 U/L) 0.1 M phosphate buffer (PB), pH 7.5. Make dry ice ethanol slurry by mixing dry ice with ethanol. This will have a temperature of approximately −72 °C and will be used for immediate freezing of the harvested tissue.

1.1.2. Euthanize adult C57BL/6 and Phox2b-GFP[11] (Mouse Genome Informatics database ID MGI:5776545) mice by anesthetizing with sodium pentobarbital (70 mg/kg, i.p.), using a 27.5 inch needle gauge.

CAUTION**:** Pentobarbital is a barbiturate. It is acutely toxic in high doses and may cause death by respiratory arrest. Consult local healthcare, legal and material safety guidelines before use.

1.1.3. Expose the heart and cannulate the left ventricle with a drawing-up needle (23 inch gauge). Perform transcardial perfusion with heparinized 0.1 M PBS until the blood clears (2-3 minutes) at a flow rate of 11-13 mL/min. Determine blood clearing by monitoring the coloration

of the liver and the effusate from the right atrium[12].

1.1.4. Isolate the brain from the skull cavity, immediately embed it in Optimal Cutting Temperature Compound (OCT) in a cryomold or aluminum foil and place it on the dry ice ethanol bath. Store the frozen embedded tissue in an airtight container at – 80 °C for up to 3 months.

1.2. Sectioning of fresh frozen tissue

1.2.1. Set the cryostat temperature to –20 °C. Leave the OCT-embedded tissue and a cryostat chuck in the cryostat for ~30 minutes to allow for equilibration to the new temperature.

NOTE: Keep the tissue frozen at all times; transport the tissue from the –80 °C freezer to the cryostat on dry ice.

1.2.2. Secure the tissue to the pre-chilled cryostat chuck using OCT compound. In this protocol, tissue blocks were mounted onto the chuck in the coronal plane.

NOTE: Trim excess OCT from the tissue, using a razor blade, to minimize the amount of OCT being cut by the cryostat and subsequently transferred onto the glass slide.

1.2.3. Cut 14 µm thick coronal sections and mount them onto charged glass microscopy slides.

1.2.3.1. Warm the slides to room temperature before mounting the sections. Once the section has been mounted, keep the slides in a slide box in the cryostat.

1.2.3.2. If more than one section needs to be mounted on one slide, warm the area for the second section by placing a finger on the opposite side of the slide for 5-10 seconds to aid adherence of the section to the slide. A cold tissue section will not attach to a cold slide. The sections should adhere to the slides flat; folding will cause them to fall off the slides during wash steps.

1.2.3.3. If cracks are noticed in the sections, increase the cryostat temperature by 1-5 °C to avoid this. It is particularly important to place tissue sections in close proximity to one another on the same slide. This will prevent wastage of FISH probes and reagents during the assay.

1.2.4. Store tissue sections mounted onto glass slides in an air-tight container at –80 °C for up to 6 months.

NOTE: Keep the sections frozen at all times and avoid freeze thaw cycles, to prevent RNA degradation. Transport the slide box from inside the cryostat to the –80 °C freezer on dry ice.

1.3. Fixation of fresh frozen tissue

1.3.1. On the day the FISH probe assay is to be performed, prepare 4% paraformaldehyde (PFA)

in 0.1 M phosphate buffer (PB), pH 7.5 (4% PFA solution). Filter by passing through filter paper (Grade 1: 11 µm, **Table of Materials**) in a Buchner funnel or crucible filter.

CAUTION**:** PFA is harmful and toxic by skin contact or inhalation. All procedures with PFA solution should be performed in a fume hood cabinet. PFA solution waste should be disposed of carefully following institutional safety protocols.

1.3.2. Cool the 4% PFA solution to 4 °C. Transport the slide-mounted tissue from the –80 °C freezer in dry ice and immediately immerse it in the pre-chilled fixative for 15 minutes.

NOTE: It is important that this fixation step does not exceed 15 minutes as over-fixation will result in non-specific background labelling.

1.4. Dehydration of fresh frozen tissue

1.4.1. Dehydrate tissue sections by submerging the slides in graded concentrations of ethanol. In a Coplin jar, first submerge in 50%, then 70% and finally absolute ethanol, for 5 minutes each at room temperature. Repeat the final absolute ethanol incubation a second time.

1.4.2. Air dry slides and, outline each section using a hydrophobic barrier pen, ensuring that the internal area is kept to a minimum.

NOTE: Make sure that the glass slide is completely dry before drawing the hydrophobic barrier. The hydrophobic barrier should surround the tissue sections completely without gaps and must be dry before further processing.

2. **Sample preparation of fixed frozen brain tissue**

2.1. Transcardial perfusion fixation

2.1.1. Euthanize mice by anesthetizing with sodium pentobarbital (70 mg/kg, i.p) followed by transcardial perfusion, first with 0.1 M PB then 4% PFA solution. Fix with 10 minutes of perfusion at 11-13 mL/min.

2.1.2. Isolate the brain from the skull cavity following perfusion-fixation and submerge overnight in 4% PFA solution, at 4 °C.

2.2. Tissue sectioning of fixed tissue

2.2.1. Rinse the brain in sterile 0.1 M PBS before removing the meningeal layers, with the aid of a dissecting microscope, using fine forceps.

2.2.2. Cut the brain precisely into blocks (separate the brainstem from the forebrain prior to vibratome sectioning) using a brain matrix (**Table of Materials**). Specifically, cut the brainstem

caudally at the pyramidal decussation and dissect away the cerebellum. Similarly, cut the forebrain immediately rostral to the optic chiasm.

2.2.3. Secure the tissue onto a vibrating microtome chuck using cyanoacrylate and embed in 2% agar solution.

2.2.4. Cut 30 µm thick tissue sections using a vibrating microtome and store cut sections in cryoprotectant solution (30% RNase free sucrose, 30% ethylene glycol, 1% polyvinylpyrrolidone -PVP-40, - in 0.1 M sodium phosphate buffer, pH 7.4). Tissue sections may be stored in cryoprotectant at –20 °C for up to 6 months.

2.3. Preparation of fixed sections prior to FISH

2.3.1. On the day of FISH, wash free floating sections three times, for 10 minutes per wash, to remove the cryoprotectant solution. To wash, place sections in 0.1 M PBS in a 12 well cell culture plate and agitate on a rotating platform shaker (90 – 100 rpm).

2.3.2. After washes, use a paintbrush to mount sections on glass microscopy slides and air dry for at least 2 hours.

NOTE: The sections should adhere flat onto the slides as any pronounced folds will cause them to detach during washes.

2.3.3. Using a hydrophobic barrier pen, draw a hydrophobic barrier around the sections to contain the FISH reagents around the sections. Once more, it is important to minimize the internal area of the outline drawn with the barrier pen.

POSSIBLE BREAK POINT: The sections could be stored at room temperature, overnight, to continue the assay the next day.

3. FISH assay

NOTE: The rest of the protocol applies to both fresh frozen and fixed frozen tissue.

3.1. Prepare the reagents and instruments for hybridization and amplification steps.

3.1.1. Set a benchtop incubator and water bath to 40 °C.

3.1.2. Prepare a humidified, light-protected chamber for incubating slides. Humidification prevents drying of tissues - slides are securely located above a moist reservoir. Ideally, the chamber is made of heavy-duty polystyrene, it is light-proof and airtight to maintain a saturated water vapor atmosphere. Closure of the chamber relies on minimal friction to avoid movement. We used a slide box lined with wet laboratory wipes (**Table of Materials**) at the bottom. Place the slide box inside the incubator to prewarm it to 40 °C.

3.1.3. Warm the 50x Wash Buffer (**Table of Materials**) and probes to 40 °C for 10 minutes, using the water bath, then cool to room temperature.

3.1.4. Prepare 1 L of 1x Wash Buffer from the 50x stock concentration.

3.1.5. Prepare probe mixture (**Table of Materials**): the C1 probe is ready to use at stock concentration whereas C2 and C3 probes are shipped as 50x concentration and require dilution with the diluent supplied in the kit.

NOTE: Probe mixtures can be stored at 4 °C for up to 6 months.

3.2. Protease treatment

3.2.1. Incubate sections with Protease III (**Table of Materials**) at room temperature for 30 minutes.

NOTE: Ensure that Protease III and incubation reagents in downstream processes (probe mixture, amplification solutions, blocking buffer and antibody sera) cover the sections entirely. A pipet tip may be used to spread the reagent onto the section to cover the entire area inside the hydrophobic barrier.

3.2.2. Wash slides twice, for 2 minutes each time, with 0.1 M PBS with in a large plastic square petri dish. Here a 245 mm x 245 mm square bioassay dish was used (**Table of Materials**). Hold from one side of the dish and tilt gently 3-5 times. After washes, flick excess 0.1 M PBS from slide and immediately add the next reagent. Do not let tissue sections dry.

NOTE: During each wash, the slides are immersed in solution at room temperature. This is the workflow for all subsequent wash steps. The fixed 30 µm thick sections dislodge from slides more easily that 14 µm thick sections, be gentle during the washes.

3.3. Hybridization and amplification

3.3.1. After washing off the protease solution, place the slides in the humidified, prewarmed chamber. Incubate sections with probe mixture (**Table of Materials**) for 2 hours at 40 °C inside a benchtop incubator.

NOTE: Ensure there are at least 2 sections set aside for positive and negative control probes to assess sample RNA quality and optimal permeabilization. Positive control probes target house-keeping genes; here, positive control probes were a cocktail of RNAs targeting ubiquitin C (UBC; high-abundance), peptidylpropyl isomerase B (PPIB; moderate-abundance) and RNA polymerase 2a (POLR2A; low-abundance). Negative control probes target the bacterial 4-hydroxy-tetrahydrodipicolinate reductase (DapB) gene, which is normally absent in mouse brain samples. Positive DapB signal indicates non-specific signal and/or bacterial contamination of the sample.

3.3.2. Following hybridization with the probe mixture, the signal amplification steps consist of incubation with Amp 1-FL (30 minutes), then with Amp 2-FL (15 minutes), followed by Amp 3-FL (30 minutes) and finally Amp 4-FL (15 minutes) - each at 40 °C. Using the dropper bottles provided add enough Amplification solution to cover tissue sections entirely. Proceed to IHC assay following the last amplification step.

3.3.3. Rinse slides with Wash Buffer twice for 2 minutes between probe hybridization and each amplification step.

4. IHC Assay

4.1. IHC blocking step

4.1.1. To prevent non-specific binding of antibodies, incubate the sections for 1 h at room temperature with blocking solution containing 10% normal horse serum, 0.3% Tween20 in 1x TBSm (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% merthiolate) following the FISH assay. Prepare primary antibodies in a dilution buffer containing 1x TBSm, 5% normal horse serum and 0.1% Tween20. Primary antibody suppliers are listed in the **Table of Materials**.

4.2. Immunohistochemistry

4.2.1. Remove excess blocking buffer by flicking the slide and incubate sections with primary antibodies overnight at 4 °C.

4.2.2. Wash slides 3 times (5 minutes each) with 1x TBSm and incubate with secondary antibody in diluent containing 1x TBSm, 1% normal horse serum and 0.1% Tween20 for 2 hours at room temperature. Secondary antibodies used in this protocol are listed in the **Table of Materials.**

4.2.3. Wash slides 3 times with 1x TBSm (5 minutes each) before coverslipping with mounting medium with or without DAPI (**Table of Materials**).

5. Imaging

5.1. Examine immunostaining under an epifluorescence microscope equipped with a camera (see **Table of Material** for details). Acquire representative images at 20x magnification and save as TIFF files.

5.2. Export representative images into an image processing software (**Table of Materials**) for brightness/contrast adjustment to increase the clarity and to reflect true rendering.

6. OPTIONAL: Quantitative analysis of target transcripts

NOTE: This is a methods article and quantitative results are not provided. The method of

quantification presented here is sourced from Dereli *et al.*[10].

6.1. Acquire images from the regions of interest as explained in 5.1 and apply the same microscope and camera settings (such as exposure time and light intensity) to all images of the same fluorophore.

6.2. Plot the neuronal profiles using an image analysis software (**Table of Materials**).

6.3. Align the sections with reference to Bregma level according to a stereotaxic brain atlas[13].

6.4. Apply the same brightness and contrast to all the images of the same fluorophore. Only consider the neurons with DAPI-stained nuclei.

6.5. Manually count the number of mRNA, protein expressing, mRNA/mRNA, protein/protein and mRNA/protein coexpressing cells within the region of interest.

6.6. To decrease bias in the experimental results, have the person quantifying experimental outcomes blinded to the experimental groups.

6.7. Apply Abercrombie correction[14] to total cell counts by using the following Abercombie equation:
Corrected cell count = manual cell count x section thickness / (section thickness + nuclear size)
For example, for 14 µm thick sections, the average nuclear width is calculated to be 7.7 ± 0.3 µm and average section thickness is 14 ± 1 µm based on 30 cells and 10 sections respectively in 5 animals[10]. According to the Abercrombie equation, corrected cell count would be manual cell count x 14/(14+7.7).

REPRESENTATIVE RESULTS:

Here, we outline a method for combining multiplex FISH with fluorescent IHC to localize mRNA expression for GalR1 and GlyT2 using fresh-frozen and paraformaldehyde fixed tissues respectively in the mouse NTS. A pipeline of the tissue processing, FISH and IHC procedures described in the methods is displayed in **Figure 1** and **Figure 2**. **Table 1** provides a summary of the FISH probe and antibody combinations used in each figure.

Control probes are routinely assayed concurrently with target probe, to ensure integrity of the workflow and confirm sample quality. The absence of DapB labelling confirms sound tissue quality and integrity, and the absence of bacterial contamination (**Figure 3A**). Labelling from positive control probes targeting ubiquitin C (UBC, high abundance), peptidylpropyl isomerase B (PPIB, moderate abundance) and RNA polymerase 2a (POLR2A, low abundance) mRNA confirms RNA integrity and the signal observed between assays may be used to calibrate inter assay variability (**Figure 3B**). To validate FISH probe expression, we used control tissues that have been previously described to express the mRNA transcript. For example, GalR1 mRNA expression, was confirmed to be positive in the thalamus as previously described[10,15]. Phox2b mRNA distribution was additionally verified by colabelling with Phox2b antibody; we confirmed that FISH labelling

was present only in neurons that were also positively stained using the Phox2b antibody (**Figure 5**).

To distinguish GalR1+ neurons in the NTS from neighbouring nuclei, we used additional neurochemical markers. TH, Phox2b or Phox2b-GFP immunoreactivity (**Figure 4-6**), and Phox2b FISH (**Figure 5** and **Figure 6**) differentiated the NTS from other nuclei in the dorsal brainstem as NTS neurons have previously been reported to express Phox2b and TH[16,17]. Since the NTS is nestled by cholinergic nuclei - it lies dorsal to the hypoglossal nucleus and dorsal motor nucleus of the vagus (DMNX), and ventral to the vestibular nucleus - we co-labelled with the cholinergic marker vAChT[18] (**Figure 4**). Therefore, the expression of GalR1 within the NTS was assessed in relation to TH and Phox2b, whilst vAChT labelling aided spatial orientation with respect to rostrocaudal, dorsoventral and mediolateral coordinates. We found all TH immunoreactive and GalR1 mRNA positive neurons in the NTS were Phox2b-GFP immunoreactive, but not all Phox2b-GFP immunoreactive neurons in the NTS were TH immunoreactive or GalR1 mRNA positive (Figure 4). Also, we demonstrated that mRNA for the low abundance receptor GalR1 was absent in TH and vAChT immunoreactive neurons.

In fresh frozen preparations, when combined with the FISH probe assay, IHC success was dependent on subcellular location of the target protein. For example, vAChT (a synaptic vesicle membrane-bound protein) was clearly immunolabelled, whereas TH and GFP (cytoplasmic proteins) were indefinitely immunolabelled and only faintly observed (**Figure 4**). We describe this indefinite labelling as 'flocculent' because cells lacked a clear outline and proved difficult to distinguish from the background. On the same fresh frozen tissue section, GalR1 FISH probe labelling of cytoplasmic GalR1 mRNA was punctate and clearly observed (**Figure 4**).

Furthermore, since the TH and vAChT antibodies are raised in the same host, both proteins were labelled using the same secondary antibody and therefore the same colour fluorophore (594). They are easily distinguished for two reasons: they never co-label in the same neurons, and the subcellular localisation is different for these proteins; vAChT in vesicles exhibiting a punctate appearance, and TH in the cytoplasm and neuronal processes.

To support our hypothesis that IHC quality (in fresh frozen preparations) is dependent on protein subcellular localisation, we compared labelling for Phox2b mRNA (located in the cytoplasm), GFP (over-expressed in cytoplasm) and Phox2b protein (primarily found in the nucleus) in neurons. As expected, our results show overlap of Phox2b mRNA, GFP and Phox2b antibody labelling in individual neurons of the NTS (**Figure 5**). Cells with cytoplasmic mRNA labelling corresponded with cells exhibiting nuclear labelling of the Phox2b protein providing validation of the combined FISH-IHC method. Although cytoplasmic Phox2b-GFP had a flocculent appearance, nuclear Phox2b protein signal was clear and specific. In conclusion, when combined with FISH on fresh frozen preparations, membrane-bound proteins including vAChT and Phox2B exhibit higher quality immunolabelling than cytoplasmic proteins.

In contrast, IHC was reliable irrespective of subcellular localization, when performed on fixed frozen sections in combination with FISH. Multiplex FISH for GlyT2 mRNA and Phox2b mRNA was

successful, as shown in **Figure 6**. GlyT2 mRNA positive neurons were located ventral to the NTS and not within the NTS. GlyT2+ and Phox2b+ neurons did not colocalize. A subpopulation of Phox2b+ NTS neurons was TH immunoreactive and none contained GlyT2 mRNA. TH immunoreactive neurons are apparent on the same tissue section, exhibiting positively labelled soma and neuronal processes (**Figure 6**). This contrasts with the 'flocculent' appearance of TH immunoreactive neurons in fresh frozen tissue sections. Thus, the fixed frozen preparation described here is an alternative method of tissue preparation which enables reliable targeting of cytoplasmic proteins immunohistochemically, in combination with RNAscope.

FIGURE AND TABLE LEGENDS:

Figure 1: Parallel workflow of tissue pre-processing steps for both fresh-frozen and paraformaldehyde fixed tissue. Processing steps for fresh-frozen tissue are displayed in the red outlined boxes, whereas those for paraformaldehyde (PFA) fixed tissue are displayed in the blue outlined boxes.

Figure 2: Summary of combined FISH probe and immunohistochemistry procedure. Following tissue pre-processing, the slide mounted tissue is encircled using a hydrophobic barrier pen, as seen in the first frame, and incubated in a protease solution at room temperature. Following washes, tissue is transferred to a benchtop incubator for hybridization for 2 hours before sequential amplification steps. The *in situ* hybridization system utilizes a proprietary 'Z probe' design, preamplifiers and amplifiers as seen in frames 3-6[6]. Once tissue has undergone FISH probe processing, it is washed before blocking with normal horse serum. The primary antibody incubation is carried out overnight at 4^{o}C to maximize antibody-antigen binding. The secondary antibody incubation (2 hours) was and carried out at room temperature.

Figure 3: Representative microscopic images from coronal mouse forebrain sections at the level of the lateral septum (Bregma 1.1 to -0.1) showing labelling of positive and negative control probes. (**A**) a lack of signal following ISH with bacterial 4-hydroxy-tetrahydrodipicolinate reductase (DapB) confirms the absence of background signals. (**B**) Labelling with positive control probes targeting ubiquitin C (UBC), peptidylpropyl isomerase B (PPIB) and RNA polymerase 2a (POLR2A) illustrates the signal to be expected from high, moderate and low abundance targets respectively. Scale bars are 50 µm. All images were acquired with 20x objective.

Figure 4: Representative microscopic images of a fresh frozen coronal brainstem section from a Phox2b-GFP mouse showing combined labelling of GalR1 mRNA (FISH) and 3 proteins (IHC) in the nucleus of the solitary tract (NTS) region. Insets in A are enlarged in B. GalR1 mRNA is indicated by punctate FISH probe labelling (arrowheads). Antibodies targeting the cytoplasmic proteins GFP and tyrosine hydroxylase (TH) exhibited "flocculent" labelling (arrows). Vesicular acetylcholine transporter (vAChT) immunoreactivity is demonstrated (red punctate labelling) in the hypoglossal nucleus (XII). Scale bars are 100 µm in A and 25 µm in B. All images were acquired with 20x objective. Other abbreviations: area postrema (AP), medial vestibular nucleus (MVe).

Figure 5: Representative microscopic images of a fresh frozen coronal brainstem section from a Phox2b-GFP mouse, illustrating targeting of Phox2b in the nucleus of the solitary tract (NTS)

with three different approaches: Phox2b mRNA (FISH), GFP (IHC) and Phox2b protein (IHC). Phox2b protein is localized to the nucleus. Insets in A are enlarged in B. Arrows indicate neurons that are triple labelled with Phox2b probe (orange-550), GFP antibody (green-488) and Phox2b antibody (red-647). Scale bars are 100 µm in A and 25 µm in B. All images are acquired with a 20x objective. Other abbreviations: area postrema (AP).

Figure 6: Representative images from fixed frozen coronal brainstem sections demonstrating successful FISH combined with reliable immunolabelling of cytoplasmic proteins (tyrosine hydroxylase [TH]). Double FISH showing glycine transporter 2 (GlyT2-red-647, filled arrowheads) and Phox2b (yellow-550, arrows) mRNA labelling in nucleus of the solitary tract (NTS) region. FISH was combined with IHC for TH protein (blue-346, empty arrowheads). Scale bars are 25 µm. All images were acquired with a 20x objective.

Table 1: FISH probe, antibody and corresponding flurophore combination combinations used in Figures 3-6.

DISCUSSION:

In neuroscience, FISH and IHC are routinely used to investigate the spatial organization and functional significance of mRNA or proteins within neuronal subpopulations. The protocol described in this study enhances the capacity for simultaneous detection of mRNAs and proteins in brain sections. Our combined multiplex FISH-IHC assay enabled phenotypic identification of distinct neuronal subpopulations in the NTS in both fresh frozen and fixed brain preparations. FISH-IHC in fixed frozen tissue preparations produced reliable IHC outcomes. For example, multiplex FISH for low and high abundance mRNAs (GalR1 and GlyT2 respectively) and IHC (targeting tyrosine hydroxylase) revealed that GalR1 and GlyT2 are expressed in non-catecholaminergic NTS. IHC for TH was not successful in fresh frozen tissue, highlighting the limited capacity for FISH-IHC in fresh frozen preparations.

ISH may be more appropriate than IHC in a range of scenarios. First, IHC may not perform well when detecting low abundance proteins, such as receptors. Using ISH to target relatively higher abundance mRNAs for these proteins improves detectability[1]. Second, proteins such as neuropeptides are trafficked to the axonal terminals following translation in the cell soma[19]. When neuropeptides are targeted with IHC, the axonal processes and terminals label with the antibody, but not the cell somas, reducing the capacity to identify the cell of origin or perform quantitative analysis. However, since mRNAs which code for all proteins are found localized to the soma, the ISH technique is advantageous. Finally, antibodies are not readily available for some protein species, or the available antibodies are appropriate for other proteomics techniques (e.g., western blot) but not IHC. In these circumstances, mRNA labelling methods prove useful. A caveat is that mRNAs may not always be translated into protein, and so they only provide a proxy for protein identification. Since commercial FISH kits can be costly and the ISH probes are less likely to be commercially available (compared to antibodies), combining FISH with IHC presents a cost and time effective strategy for increasing the number of targets that can be labelled simultaneously.

Fresh frozen versus fixed tissue preparation was a factor conferring successful IHC following FISH probe assay. We tested IHC using antibodies targeting nuclear, vesicular and cytoplasmic proteins and found reliable labelling of membrane-bound proteins (vAChT and Phox2b) on fresh frozen samples but not cytoplasmic proteins (TH and GFP). Coexpression of Phox2b protein and mRNA with flocculent Phox2b-GFP labelling validated that neurons with the transcript had the related protein expression and confirmed the identity of the flocculent neurons (**Figure 5**). In contrast, fixed frozen tissue preparations yielded reliable IHC labelling regardless of subcellular localization of the antigen. Previous studies have demonstrated that protease (e.g., pronase[8,20]) pretreatment can have a detrimental effect on IHC. The contents of the protease solution utilized in the RNAscope protocol is proprietary, and permeabilization by protease is recommended for probe access into cells. Labelling of cytoplasmic proteins using the antibodies described here has been previously verified on free floating 30 µm fixed frozen mouse brain sections [10,21,22]. We slide-mounted 30 µm thick fixed samples and performed the FISH-IHC protocol, as opposed to the 14 µm thick fresh frozen sections recommended by the manufacturer. In the absence of modifications, or the need to troubleshoot variables (antigen retrieval, higher antibody concentration, change of protease), reliable IHC was achieved on thick, fixed samples with demonstrated labeling of the cytoplasm and axonal processes together with FISH probe labelling (**Figure 6**). While similar approaches were employed by other research groups[7-9], the current study achieved combined ISH-IHC on neurons and in a fluorescent set-up.

There were a series of critical steps in the methods to take note of. For the fresh frozen preparation, fixation time should not exceed 15 minutes; longer fixation times elicited higher background labelling. The protease step was optimized since tissues of different thickness and from various organs require different types of protease to achieve permeabilization. Fixed frozen sections adhere less to glass slides and dislodge more easily during wash steps. Hence, extra care must be taken in manual handling of fixed frozen section, to avoid tissue loss or damage.

Although we found combined FISH and IHC to be an effective strategy, the disadvantages include cost and technically demanding assay when combining the two methods. One limitation of the study is that a side-to-side comparison of the two tissue preparation protocols was not performed. Also, our evaluation of results was limited by the number of channels the epifluorescent microscope could accommodate; the set-up allowed a maximum of 4 channels at a given time: 346, 488, 550 and 647 nm (excitation light). We were able to achieve multiplex labelling of 5 targets by labelling two proteins with different subcellular localizations using the same flurophore (**Figure 4, Table 1**). By using a confocal microscope, discrete excitation of many additional fluorophores may be used for individual protein labelling via IHC, or for imaging of fluorescent molecules expressed by transgenes.

Combined FISH and fluorescent IHC can reduce the reliability of each technique in isolation. In the future, we aim to improve cytoplasmic protein labeling on fresh frozen tissue with an antigen retrieval treatment[23]. Previous studies show that heat induced antigen retrieval increases accessibility of the protein epitope[24-26]. Heat treatment cleaves the crosslinks and methylol groups of the protein and unfolds the antigens in tissues, exposing epitopes which would otherwise be hidden in the tertiary protein structure under biological conditions. This

accessibility may improve the success of protein labelling[26,27]. Additionally, we will target different epitopes of the same cytoplasmic protein to determine if the success of protein-antibody labelling depends on the specific antibody clones used.

In conclusion, combined FISH and IHC is useful for neurochemical identification of heterogenous populations of cells in the brain, such as the NTS. This study presents two protocols assaying different mouse brainstem tissue preparations – fresh frozen, or fixed - for simultaneous multiplex fluorescent labelling of mRNA and proteins *in situ*. Both protocols may be widely applied to detect the expression pattern of low abundance mRNAs, such as GalR1. Thick (30 µm) fixed frozen preparations permeabilized with protease conferred more reliable cytoplasmic protein detection and more tissue handling challenges when, compared to thin (14 µm) fresh frozen preparations.

ACKNOWLEDGMENTS:

This work was funded by Australian Research Council Discovery Project grant DP180101890 and Rebecca L Cooper Medical Research Foundation project grant PG2018110

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Figure 1

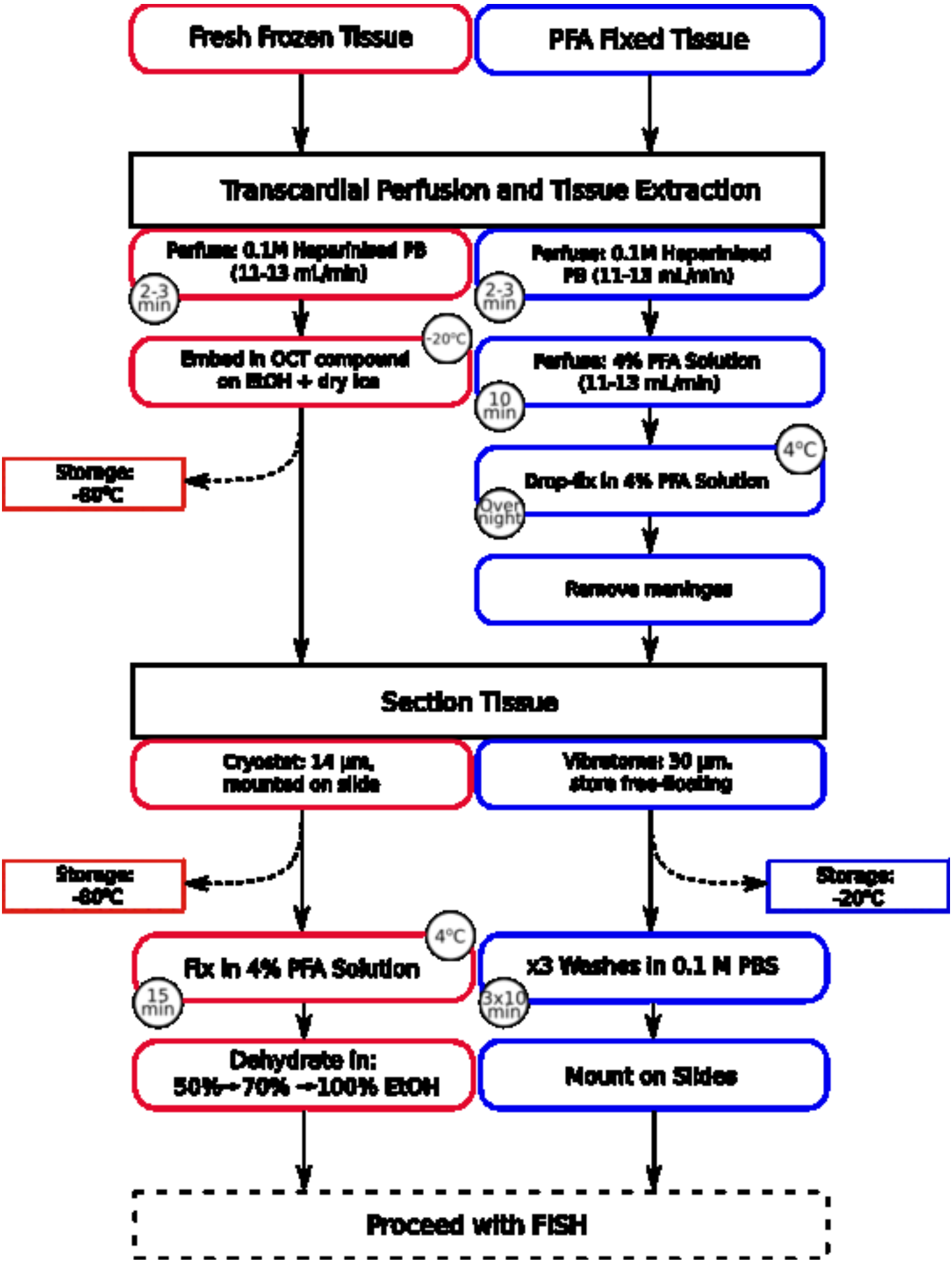

Figure 2

Click here to access/download;Figure;Figure2_combined protocol.png

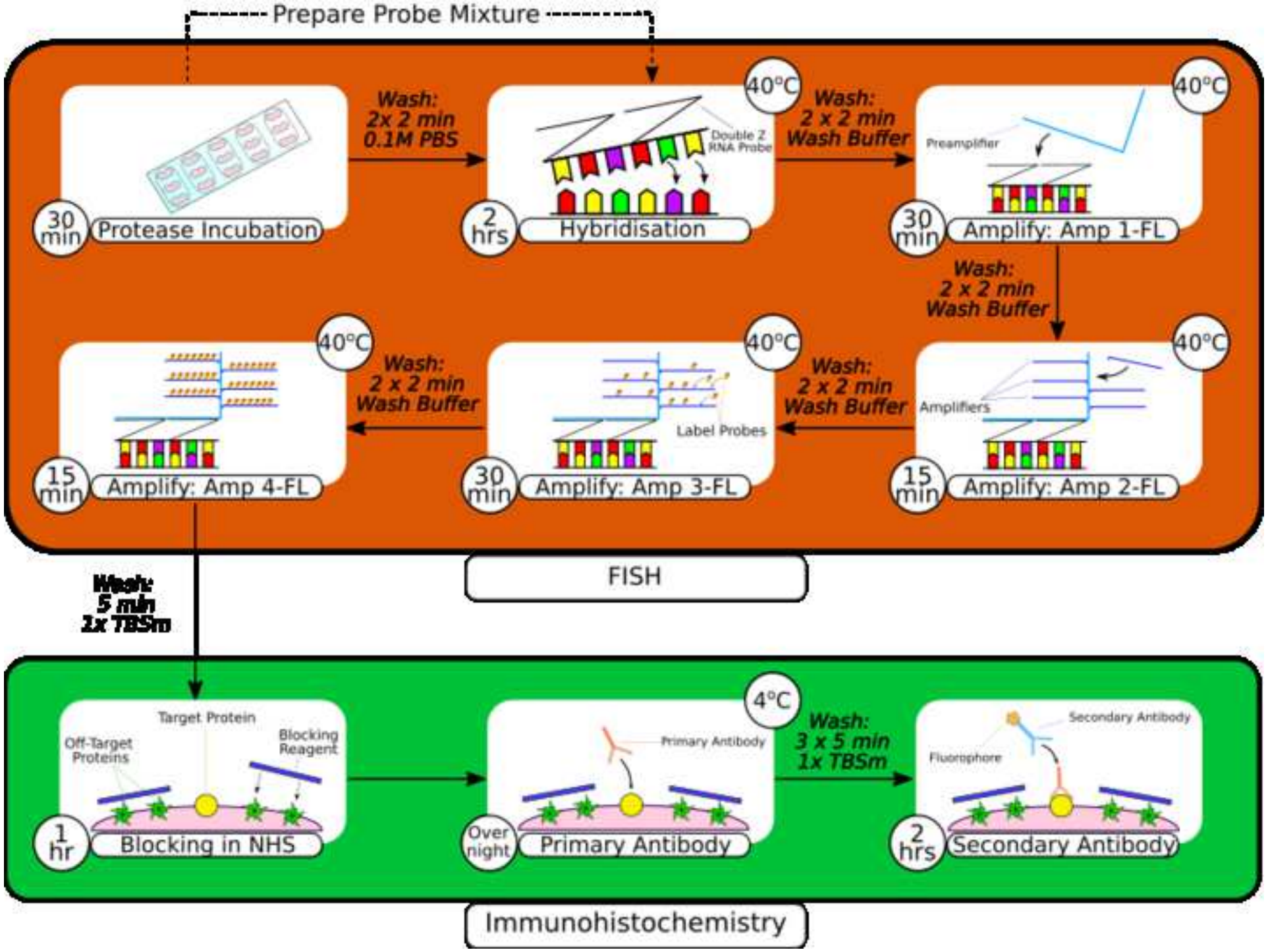

Figure 3

Click here to access/download;Figure;Results fig3.png

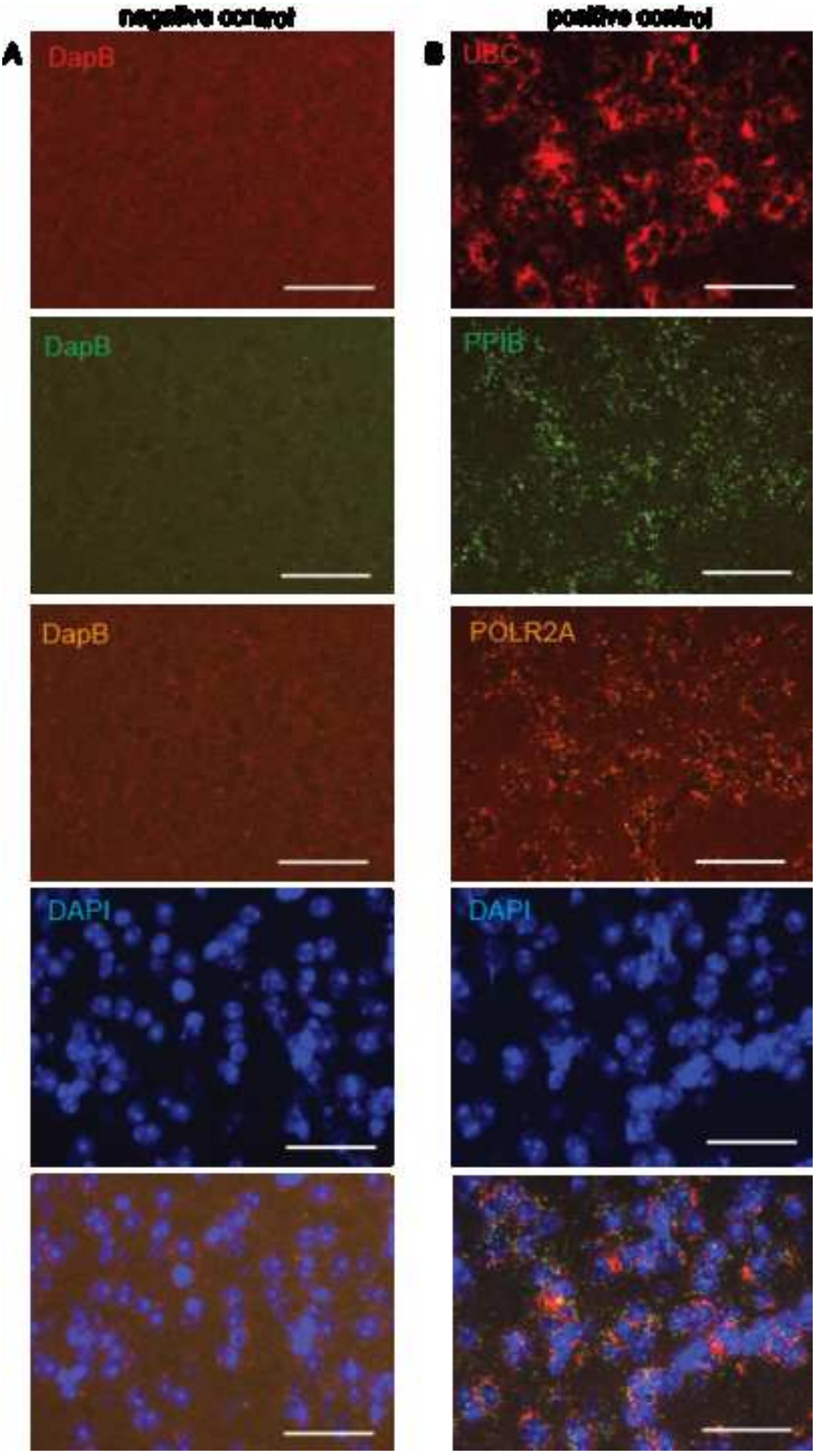

Figure 4

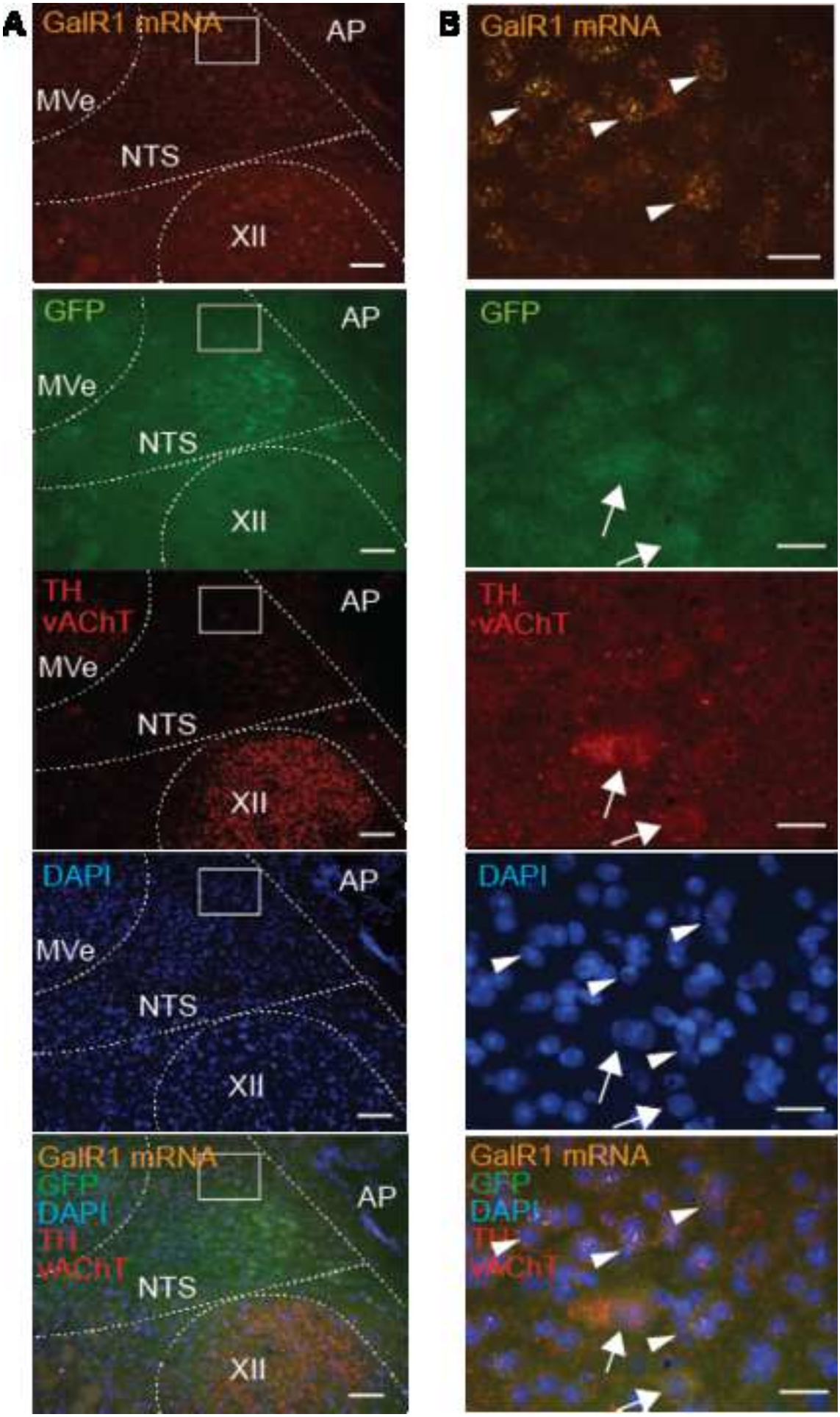

Figure 5

Click here to access/download;Figure;Results fig5.png

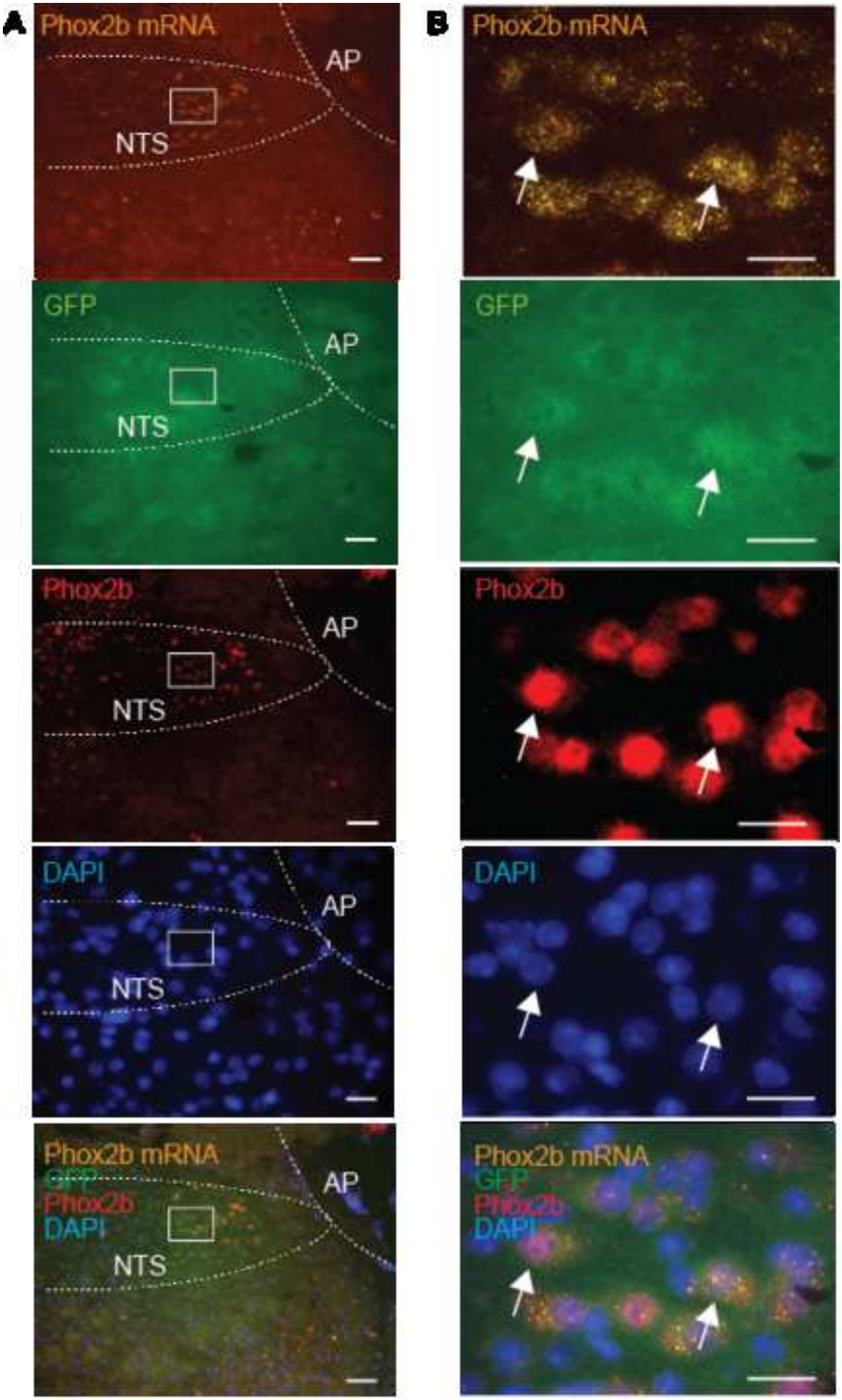

Figure 6

Click here to access/download;Figure;MAR21 Results fig6 (1).png

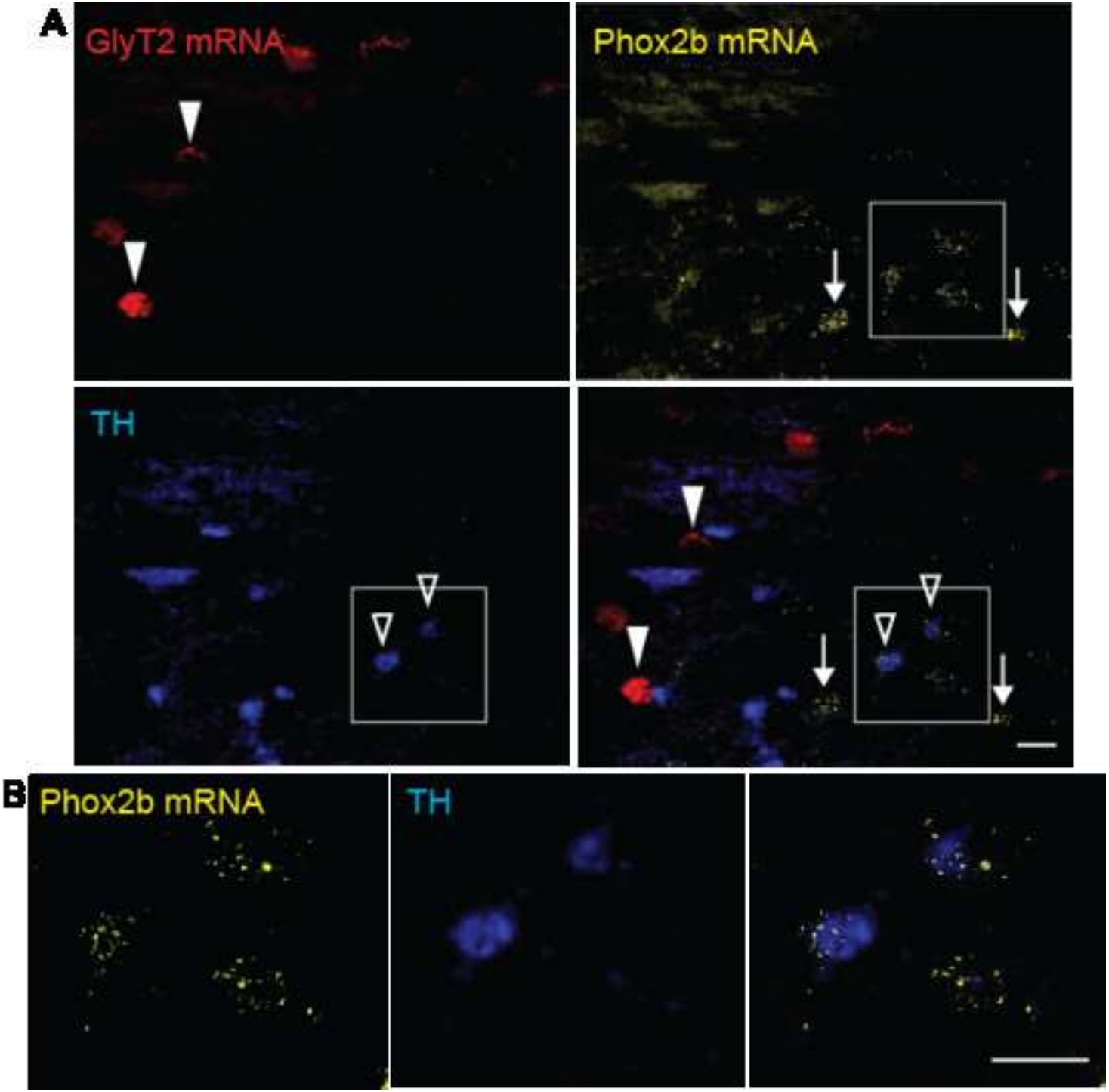

Table 1

		Primary antibody or RNAscope probe	Secondary antibody or Amp 4-FL-Alt Display module	Excitation (nm)	Tissue preparation
Figure 3	probe	POLR2A (C1)	Amp 4-FL-Alt B Display module	647	fresh frozen
	probe	PPIB (C2)	Amp 4-FL-Alt B Display module	488	
	probe	UBC (C3)	Amp 4-FL-Alt B Display module	550	
	probe	DapB (C1, C2, C3)	Amp 4-FL-Alt B Display module	647, 488, 550	
	DAPI			346	
Figure 4	antibody	rabbit-anti-GFP	donkey-anti-rabbit	488	fresh frozen
	antibody	sheep-anti-TH	donkey-anti-sheep	647	
	antibody	goat-anti-vAChT	donkey-anti-goat	647	
	probe	GalR1 (C1)	Amp 4-FL-Alt B Display module	550	
	DAPI			346	
Figure 5	antibody	rabbit-anti-GFP	donkey-anti-rabbit	488	fresh frozen
	antibody	mouse-anti-Phox2b	donkey-anti-mouse	647	
	probe	Phox2b (C2)	Amp 4-FL-Alt A Display module	550	
	DAPI			346	
Figure 6	antibody	mouse-anti-TH	donkey-anti-mouse	346	fixed
	probe	GlyT2	Amp 4-FL-Alt A Display module	647	
	probe	Phox2b	Amp 4-FL-Alt A Display module	550	

Name of Material/Equipment	Company	Catalog Number	Comments
ANIMALS			
C57BL/6 mouse	Australian BioResources, Moss Vale	MGI: 2159769	
Phox2b-eGFP mouse	Australian BioResources, Moss Vale	MGI: 5776545	
REAGENTS			
Cyanoacrylate	Loctite		
Ethylene Glycol	Sigma-Aldrich	324558	
Heparin-Sodium	Clifford Hallam Healthcare	1070760	Consult local veterinary supplier or pharmacy.
Lethabarb (Sodium Pentabarbitol) Euthanasia Injection	Virbac (Australia) Pty Ltd	N/A	Consult a veterinarian for local pharmaceutical regulations regarding Sodium Pentabarbitol
Molecular grade agarose powder	Sigma Aldrich	5077	
OCT Compound, 118mL	Scigen Ltd	4586	
Paraformaldehyde, prilled, 95%	Sigma-Aldrich	441244-1KG	
Polyvinylpyrrolidone, average mol wt 40,000 (PVP-40)	Sigma-Aldrich	PVP40	
ProLong Gold Antifade Mountant	Invitrogen	P36930	With or without DAPI
RNAscope Multiplex Fluorescent Reagent Kit (up to 3-plex capability)	Advanced Cell Diagnostics, Inc. (ACD Bio)	ADV320850	Includes 50x Wash buffer and Protease III
RNase Away	Thermo-Fisher Scientific	7003	
Tris(hydroxymethyl)aminomethane	Sigma-Aldrich	252859	
Tween-20, for molecular biology	Sigma-Aldrich	P9416	
EQUIPMENT			
Benchtop incubator	Thermoline scientific micro incubator	Model: TEI-13G	
Brain Matrix, Mouse, 30g Adult, Coronal, 1mm	Ted Pella	15050	
Cryostat	Leica	CM1950	
Drawing-up needle (23 inch gauge)	BD	0288U07	

Hydrophobic Barrier Pen	Vector labs	H-4000	
Kimtech Science Kimwipes	Kimberley Clark		
Delicate Task Wipes	Professional	34120	
Olympus BX51	Olympus	BX-51	
Peristaltic pump	Coleparmer Masterflex	L/S Series	
Retiga 2000R Digital Camera	QImaging	RET-2000R-F-CLR	colour camera
SuperFrost Plus Glass Slides (White)	Thermo-Fisher Scientific	4951PLUS4	
Vibrating Microtome (Vibratome)	Leica	VT1200S	
Whatman qualitative filter paper, Grade 1, 110 mm diameter	Merck	WHA1001110	
SOFTWARES			
CorelDRAW	Corel Corporation	Version 7	
FIJI (ImageJ Distribution)	Open Source/GNU General Public Licence (GPL)	N/A	Rueden, C. T.; Schindelin, J. & Hiner, M. C. et al. (2017), "ImageJ2: ImageJ for the next generation of scientific image data", BMC Bioinformatics 18:529, PMID 29187165, doi:10.1186/s12859-017-1934-z and Fiji: Schindelin, J.; Arganda-Carreras, I. & Frise, E. et al. (2012), "Fiji: an open-source platform for biological-image analysis", Nature methods 9(7): 676-682, PMID 22743772,
PRIMARY ANTIBODIES			
Anti-Tyrosine Hydroxylase Antibody	Millipore Sigma	AB1542	Sheep polyclonal (1:1000 dilution), RRID: AB_90755

Anti-Tyrosine Hydroxylase Antibody, clone LNC1	Millipore Sigma	MAB318	Mouse monoclonal (1:1000 dilution), RRID: AB_2201528
Anti-Vesicular Acetylcholine Transporter (VAchT) Antibody	Sigma-Aldrich	ABN100	Goat polyclonal (1:1000 dilution), RRID: AB_2630394
GFP Antibody	Novus Biologicals	NB600-308	Rabbit polyclonal (1:1000 dilution), RRID: AB_10003058
Phox2b Antibody (B-11)	Santa Cruz Biotechnology	sc-376997	Mouse monoclonal (1:1000 dilution), RRID: AB_2813765
SECONDARY ANTIBODIES			
Alexa Fluor 488 AffiniPure Donkey Anti-Rabbit IgG (H+L) (min X Bov, Ck, Gt, GP, Sy Hms, Hrs, Hu, Ms, Rat, Shp Sr Prot)	Jackson ImmunoResearch	711-545-152	Donkey anti-Rabbit (1:400 dilution), RRID: AB_2313584
AMCA AffiniPure Donkey Anti-Sheep IgG (H+L) (min X Ck, GP, Sy Hms, Hrs, Hu, Ms, Rb, Rat Sr Prot)	Jackson ImmunoResearch	713-155-147	Donkey anti-Sheep (1:400 dilution), RRID: AB_AB_2340725
Cy5 AffiniPure Donkey Anti-Goat IgG (H+L) (min X Ck, GP, Sy Hms, Hrs, Hu, Ms, Rb, Rat Sr Prot)	Jackson ImmunoResearch	705-175-147	Donkey anti-Goat (1:400 dilution), RRID: AB_2340415
Cy5 AffiniPure Donkey Anti-Mouse IgG (H+L) (min X Bov, Ck, Gt, GP, Sy Hms, Hrs, Hu, Rb, Rat, Shp Sr Prot)	Jackson ImmunoResearch	715-175-151	Donkey anti-Mouse (1:400 dilution), RRID: AB_2619678
Cy5 AffiniPure Donkey Anti-Sheep IgG (H+L) (min X Ck, GP, Sy Hms, Hrs, Hu, Ms, Rb, Rat Sr Prot)	Jackson ImmunoResearch	713-175-147	Donkey anti-Sheep (1:400 dilution), RRID: AB_2340730
RNASCOPE PROBES			
Galanin Receptor 1 oligonucleotide probe	ACDBio	448821-C1	targets bp 482 - 1669 (Genebank ref: NM_008082.2)

Glycine transporter 2 oligonucleotide probe	ACDBio	409741-C3	targets bp 925 - 2153 (Genebank ref: NM_148931.3)
Phox2b oligonucleotide probe	ACDBio	407861-C2	targets bp 1617 - 2790 (Genebank ref: NM_008888.3)

Dear reviewers,
Please our response to your comments below, in italics.

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Editorial comments:

NOTE: Please read this entire email before making edits to your manuscript. Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• Avoid pronouns “you” and “your” throughout.

• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write the text in protocol steps 5.2-6.4 to avoid this overlap.

Protocol steps 5.2 - 6.4 have been updated (now 4.2 - 5.6) to avoid textual overlap

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

Ethics statement is included before numbered protocol

2) 1.1.3: mention needle gauge. Which organ do you check for blood clearance? Cite a reference for transcardial perfusion.

A 23 inch gauge drawing-up needle was used to cannulate the heart during transcardial perfusion (Section 1.1.3) and a reference given for transcardial perfusion of mice. 27.5 gauge needle included at 1.1.2 for i.p injection. Details regarding blood clearance (specifically, monitoring of the liver and the effusate exiting the heart) is included at 1.1.3

3) 1.1.4: Cite a reference for brain extraction.

A reference was given (PMID: 22871843; Gage GJ et al 2012) to describe the process of isolating the brain from the skull cavity

4) 2.2.3: Unclear. What is meant by "blocking" the brain sections?
"Blocking" is a commonly used term to describe dissection of the fixed brain tissue into smaller 'blocks' which are then cut on a vibratome or cryostat.
Usage of this word was also queried by Reviewer 3. The terminology has been changed accordingly in the Protocol/Method section 2.2.3 *the manuscript.*

- **Protocol Numbering:**

1) All steps should be lined up at the left margin with no indentations.
All steps aligned left with no indentation

2) Add a one-line space between each protocol step.
A one-line space has been added between each protocol step

3) Please ensure that the protocol section does not exceed 10 pages.
The protocol is now less than 10 pages long

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The discussion is now restructured into paragraphs separately addressing the points numbered by the editor.

• **Figures/Tables:**
1. Please remove the embedded figures from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.

Embedded figures have been removed and Figure legends remain below the Representative Results text.

2. Please remove the embedded Tables from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends.
Embedded tables have been removed from the manuscript. Antibodies and oligonucleotide probe details have been relocated to the Table of Materials. In addition, a new table (Table 1) has been created which will be submitted separately in the Editorial Manager, specifying the antibody combinations for each experiment as requested by Reviewer 1 comment 3. A description of Table 1 is included with the Figure legends.

3. Please remove all products references from the tables. These can be specified in the table of materials instead.
Product references are removed from tables

• References: Please spell out journal names.
Journal names in the reference list are spelled out

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are RNAscope, cryomold, Whatman, ACD catalogue number,. ACDBio.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

All commercial language removed from manuscript e.g .at 4.2.2 "KimWipes" has been replaced with "laboratory wipes (see Table of Materials)" . However, whilst the editor states 'Examples of commercial sounding language in your manuscript are RNAscope...', we found many other JoVE publications in which 'RNAscope' is used and in some cases, 'RNAScope' is a key word (PMID: 24637627, PMID: 26131922, PMID: 30176002). As such we have left references to RNAscope in the text as using FISH as a descriptor is not sufficiently specific enough in the context of this protocol.

• **Table of Materials:**
1) Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as antibody RRIDs.

Antibody RRIDs have been added to the Table of Materials in the Comments section. All antibodies and oligonucleotide probes which were in the embedded tables have now been moved to the Table of Materials

2) Sort the list alphabetically.

We have done this.

3) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

Registered trademark symbols have been removed from the material names in the Table of Materials

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

N/A; all figures are original

_____

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
This paper describes a method to combine RNA FISH and protein IHC to detect up to 3 mRNAs and 3 proteins on a single section using two types of tissue preparations, fresh frozen and fixed frozen. This "multi-omic" approach is gaining popularity among researchers of different areas from neuroscience to cancer research. It provides detailed protocols for tissue preparations, FISH and IHC. In particular, the authors compared the two tissue preparations and found that IHC for cytoplasmic proteins was more successful with the fixed frozen samples. Overall, the manuscript is well written and provides a starting point and useful tips for researchers starting to take this 'multi-omic' approach.

Major Concerns:
none

Minor Concerns:
1. The authors concluded that compared to membrane proteins cytoplasmic proteins can not be successfully detected by IHC following FISH. This was based on only a few examples from the authors' experience and may not hold true generally. The different success rates of different proteins may also depend on the specific antibody clones used. This needs to be discussed.
We acknowledge that lack of cytoplasmic protein labelling could be troubleshooted further. We have changed the Discussion text to convey that whilst detection of IHC for membrane proteins was met with a successful outcome, further optimisation for non-membrane bound proteins is required.
We have also added the following sentence to the Discussion 'Additionally, we will target different epitopes of the same cytoplasmic protein to determine if the success of protein-antibody labelling depends on the specific antibody clones used.' Thank you for the suggestion.

Optimization with HIER, Tris-EDTA or other common antigen retrieval methods may improve success of membrane protein labelling.

2. This manuscript refers to RNAscope Z probes as riboprobes. This can be misleading since riboprobes refer to traditional FISH probes made from in vitro transcribed RNA whereas RNAscope Z probes are DNA oligos.
Thank you for pointing this out. We have removed references to 'riboprobes' throughout the manuscript. RNAscope Z probes are now referred to as 'RNAscope probe' or 'FISH probe'. Whilst the editor states above 'Examples of commercial sounding language in your manuscript are RNAscope...', we found many other JoVE publications in which 'RNAscope' is used to described the riboprobes and in some cases, 'RNAScope' is a key word (PMID: 24637627, Baleriola et al 2015 PMID: 26131922, PMID: 30176002)

3. Please indicate the fluorophore label used for each RNA or protein target in the figure legends. In Fig. 5B, TH and Phox2b were both shown in Red. Were the same dye used for both targets?
The manuscript now includes a table specifying the flurophore used for each antibody or RNA target (Table 1). In Fig. 5B (of the original manuscript), the same dye was used to label Phox2b and TH. Differing subcellular localisation of each protein - Phox2b is present in nuclei and absent in the cytoplasm, TH enzyme is present in the cytoplasm and absent in nuclei - justified labeling the tissue in this way, thus increasing the number of proteins labeled in a single tissue section. In the revised manuscript, we replaced Fig 5B with a different image.

Similarly, in Fig 4 (of the original manuscript) the same secondary fluorophore dye (647) was used to label vAChT and TH, because the two proteins have different subcellular localisation

Overall, where two antibodies were targeted with the same secondary flurophore, we sought to leverage the different subcellular compartmentalization of these proteins, and the number of microscope fluorescent filtersets available to us, to maximise the number of labels possible in a single tissue section.

4. The 'benchtop incubator' used for hybridization needs to be listed in the equipment table.
This equipment is now in the Table of Materials.

Reviewer #2:
Manuscript Summary:
The authors present a protocol for combining the popular RNAscope RNA in situ hybridization platform with immunohistochemistry to simultaneously demonstrate protein and RNA, and compare two tissue processing methods (fresh frozen with fixation of tissue on slides and perfusion fixation with 4% PFA). They then demonstrate the utility of this protocol using a series of combined IHC and RNAscope stains in mouse brain processed according to their protocol. They conclude that both processing methods are viable, and that staining for cytoplasmic markers by IHC using this method is problematic.

Major Concerns:
(1) This manuscript would benefit from having fewer, higher quality images in Figure 4 (e.g., more like figure 5), ideally with both low and higher power images to show positive staining. I

viewed the images in both the pdf and as Tiffs and in some images the dot-like RNAscope staining is hard to appreciate.
We thank the reviewer for the opportunity to provide higher quality images. Fig 4 has been revamped – it is now split into 2 different figures; Figure 4 and Figure 5 to present low power images (LHS column) and higher power images in which the resolution of the puncta-like RNAscope staining is much improved.

(2) Using two antibodies with the same secondary is problematic. While my experience with mouse tissue is limited, this seems suboptimal, and if at all possible, I would have preferred to see images separating these two antibodies into separate channels or different sections.
Thank you for this remark that was common with Reviewer 1. Please refer to the response to Reviewer 1, Comment 3.

(3) If this protocol is truly capable of staining for up to 5 separate stains, it would be nice to see at least one figure that shows, for example, 3 RNA scope and 1 or ideally 2 IHC channels.
Figure 4 has now been updated to improve the clarity of the image showing the labelling of 1 mRNA target (RNAscope; GalR1) with 3 protein targets (IHC; TH, vAChT, GFP) and DAPI. And Figure 6 contains an image with 2 mRNAs (GlyT2, Phox2b) and 1 protein target (TH). Hence we were able to label 5 targets in the same tissue.

(4) The authors state in the introduction "Labeling of cytoplasmic proteins using the antibodies described have been previously verified on free floating 30 micron fixed frozen mouse brain sections...." and report that this showed good staining without the artefacts reported in the protocol. No reference or image is offered. If this method works, then why not modify the protocol accordingly and demonstrate this method.
Standalone IHC worked previously when not combined with RNAscope conditions. Here, we are providing a method of combining IHC with RNAscope. The RNAscope manufacturer recommends the use of fresh frozen preparations, however, this approach did not offer best conditions for successful cytoplasmic protein labelling with the antibodies that has worked previously on our fixed tissue. This is the reason why we have introduced modification of the RNAscope protocol to conduct FISH on fixed frozen preparations.

(5) The advantages and disadvantages of the two proposed tissue preparation protocols should be outlined more clearly, and a side-to-side comparison of the two would be more useful than separate figures as shown.
The manuscript title and relevant sections of the manuscript have been changed to highlight that we did not compare fresh frozen vs fixed frozen preparations in parallel. Instead, we provided a method of combining RNAscope (FISH) with fluorescent IHC. As stated in the response to the reviewers previous comment, we initially used fresh frozen preparations as this was recommended for mRNA labelling by RNAscope manufacturer. When the RNAscope protocol was applied on fixed frozen preparations we identified improved cytoplasmic protein labelling.
We have included this limitation in the Discussion section: 'One limitation of the study is that a side-to-side comparison of the two tissue preparation protocols was not performed.'

Minor Concerns:
(1) the references to the figures are out of order

Thank you for pointing this out. In-text references to the figures have been revised and updated.

(2) It needs to made clearer which parts of the protocol are alternatives and which apply to all methods used
The subheadings in the Method have been modified to state which preparation they apply to. Furthermore, a statement has been included after the heading '3. FISH assay' to indicate that the rest of the protocol applies to both fixed and fresh frozen preparations.

We have also deleted the antigen retrieval step which only applied to fresh frozen preparations (please see the response to the next comment) as we are not presenting any associated figures in the results.
Parts of the protocol that are alternative and don't apply to results presented are indicated such as in '5. Optional:'

(3) It should be made clear that (presumably) use or non-use of antigen retrieval is dependent on the IHC protocol chosen.

Application of antigen retrieval step on fresh frozen preparations did not improve the assay, the results were not different. Since we didn't presentg any figures of the outcome of this step, we have now removed antigen retrieval from the methods section altogether.

(4) Part 5 on image analysis needs to either be presented in more detail or removed entirely
Further detail is now provided for the image analysis and quantification of cell profiles, encompassing signal thresholding, counting and calculations.

(5) Why do the authors use PHOX2B-GFP mice rather than just to IHC for this protein?
GFP is overexpressed in the Phox2b locus of the Phox2b-GFP mouse. We used GFP as another example of a cytoplasmic protein which, when combined with RNAscope, was not detected immunohistochemically, even though the protein is overexpressed in Phox2b neurons and robustly detected using standalone IHC. We successfully targeted Phox2b mRNA (Fig 4, Fig 5 and Fig 6 RNAscope), Phox2b protein (Fig 5 IHC for the Phox2b protein expressed in the nucleus) in the same tissue sample.

Reviewer #3:

The authors present what is ostensibly "a comparison of fresh frozen and fixed mouse brain preparations for multiplex fluorescence in situ hybridisation combined with immunohistochemistry".
The title has now been updated to fit the content of the manuscript.

This seems like a straightforward process that should be relatively easy to explain clearly and concisely. The authors have unfortunately not done this, and have instead produced a manuscript that is at points almost impenetrably difficult to follow, and which gives every impression it has been bolted together from random data left over from other experiments. Things which seem to be discussion are found in results. Things which seem to be results are found in discussion. Things which should be in methods are not present at all.

The methods, results and discussion have been totally revised and improved based on the comments from the reviewers. Please specify if there is a particular section that needs further work.

Having read through the manuscript multiple times, I am still unclear as to exactly which labels were and were not combined, and which fluorophores were used for the images collected.
Thank you for pointing this out. The same issue was brought up by reviewer 1 and 2. Please refer to reviewer 1 comment 3 for clarification. We have created a table that associates the antibody-flurophore associated with each figure.

Major Concerns:
It is important to note that while combining ISH and IHC is very useful, it is also not novel. The website itself features advice on how to combine IHC with RNAscope ISH, and many groups are already doing this routinely (the authors acknowledge this in their introduction). The authors need to be very precise about what they are and are not claiming to be novel, here, and focus their investigations and discussions accordingly.
Whilst we acknowledge the reviewers note that combining ISH and IHC is not novel, we have been advised by the editor that reviewer comments questioning the novelty of the article can be disregarded.

A key determinant of success is not tissue treatment (RNAscope ISH absolutely requires fixation regardless of initial tissue treatment) but antibody robustness. Antibodies that only work in fresh frozen tissue will almost invariably not work alongside ISH (where fixation and protease treatment are necessary). The authors have used a lot of antibodies (very few of which are shown) but have not indicated whether they used the same antibodies in both fresh and fixed tissue for direct comparison, so it is very difficult to determine whether their claims that "soluble proteins are lost while membrane bound proteins are not" is correct. It could simply be that some antibodies are better under some conditions than others. In some cases they appear to have used two antibodies with the same secondary fluorophore, rendering comparison of specific antibodies literally impossible.
We agree with the reviewers comments and have now included Table 1 for clarity. Also please refer to Review 1 comment #3

The authors also introduce a GFP-Phox2b mouse at one point, with almost no explanation as to why, and it is not entirely clear which images concern this mouse (I assume the images with GFP in them do, but do all the images?).
GFP is overexpressed in the Phox2b locus of the Phox2b-GFP mouse. We used GFP as another example of a cytoplasmic protein which, when combined with RNAscope, was not detected immunohistochemically, even though the protein is overexpressed in Phox2b neurons and robustly detected using standalone IHC. We successfully targeted Phox2b mRNA (Fig 4, Fig 5 and Fig 6 RNAscope), Phox2b protein (Fig 5 IHC for the Phox2b protein expressed in the nucleus) in the same tissue sample.

We hypothesised that overexpressed GFP antibody labelling to be more robust than native Phox2b protein expression, since it is overexpressed, however, we did not see GFP labelling with clarity; this supports our general observations when labelling cytoplasmic proteins.

In lighter news, the protocol sections are well-written and clear (with specific reservations listed

below). From a strictly methods-based perspective, the manuscript is fairly robust.

The manuscript needs to be restructured: the authors need to state exactly what they are proposing to investigate/demonstrate, and lay out their experiments in a clear and logical fashion so that this comparison (if a comparison remains their goal) is made obvious. State exactly what fluorophores were used, and what antibodies/probes were used, and which were assigned to which (and in which combinations). State exactly how many fluorophores were used per section/image: the abstract implies three antibodies AND three probes, but this is very, very difficult to achieve, and is impossible with the fluorophores listed in the manuscript. Three fluorophores plus DAPI is most common, though four plus DAPI is achievable with the right filters.
Did the authors mean "any combination of three markers, either mRNA or protein, plus DAPI"? Use a table if necessary.

We value and appreciate this comment. As mentioned above, the manuscript now includes a table specifying the fluorophore label used for each antibody or RNAscope probe target (Table 1).

The results need to explain the findings of these clearly defined comparisons, and the discussion can then address the underlying implications. If the authors want to present particular combinations of antibodies & probes that work really well for specific questions, this is all to the good: these could form a dedicated results/discussion element.

My final major criticism of the manuscript in that the authors have, despite promoting this work as a multiplex technique, made so little effort to present any multiplex images, or give any indication they are aware of how to do this.
If you can stain and image multiple RNA and protein targets within a tissue, with multiple fluorophores, then you can also present that data in a single image, with different fluorophore channels assigned to different colours. This is a key strength of these approaches, allowing the reader to see exactly how different targets interact spatially, and yet of all the images presented, only three show more than a single fluorescence channel, and only two even feature DAPI signal (something that is almost always included by default in multiplex approaches, given the paucity of alternative fluorophores in the blue wavelength range).
The original Figure 4 has now been split into 2 figures, Figure 4 and Figure 5. The original Figure 5 is now Figure 6. We hope that different combinations of multiple mRNA, protein targets are now clearly showcased. Please refer to Table 1 for further detail about the combination and what the fluorophores represent in each figure. A similar explanation is provided above for Reviewer 1 comment 3.

I assume that the images presented in, for example, figure 5A are the same imaging field, but this is not immediately apparent, and nor is it easy to compare the two images presented, especially without a corresponding nuclear signal to provide a reference. These two images could easily be merged into a single image, with DAPI included, freeing up space on the page to include magnified regions as insets if necessary.
As it stands, the author is left to guess whether any given images are the same imaging field, and the interaction between markers (something the authors are keen to stress this approach allows) becomes a matter of squinting and supposition.
All Results figures have been revised such that images provided within a figure belong to the same field of view, except for Figure 3 where images from panel A are from a different tissue section to images from panel B. To make this clear, we have included a merged image (all channels merged) in each Figure.

The colours shown are moreover perplexing: not only do the authors document their fluorophore choices haphazardly (often referring to a channel as 'orange', or 'red' rather than the fluorophore used), but the images also appear to have been captured with a colour camera, and presented as they would appear to the eye. GFP signal, for example, is not shown as pure green, but as the washed-out blue-green that GFP fluorescence actually exhibits. DAPI signal (in the rare instances it appears at all) appears as washed-out blue-violet colour. This is…bizarre, to me, and is also an approach that hugely weakens the utility of their method. Cy3 ('orange') and Cy5 ('far red') are spectrally separable, with almost no fluorescence cross-talk: the two channels can be collected independently without any bleedthrough. If both are shown in a single image in their 'real' colours, however, it will be difficult for the reader to discern the individual fluorescence contributions purely because (to the human eye) red and orange shades are difficult to neatly distinguish. Conventionally, fluorescence channels should be captured not with a colour camera (as these often use a biased set of capture CCDs intended specifically to collect images satisfying to the human eye), but by using a high-sensitivity monochrome camera, with the individual monochrome channels subsequently false-coloured using colours the human eye can readily distinguish. Blue, green and red are popular assignments for DAPI/GFP/Cy5 (or blue, green, magenta for colour blind compatibility), but really, these colours can be anything: the presented colour need not reflect the fluorophore used for any reason other than convention.

We apologise for the confusion caused, since the colour is arbitrary and can be converted to black and white or other colours, using image processing software such as FIJI or ImageJ. We refer to colours to aid the reader, and only in the figure legends. Many publications describe the colour of the fluorophore in the figure legends. We have now included detail of the fluorophore in the figure legend. We trust Table 1 will communicate the relevant fluorophore information to readers.

The authors claim the images were post-processed in Fiji, but I see minimal evidence of this. In most instances the images either appear 'washed out' with high background, or 'overexposed'. To illustrate my point, I have submitted to the editors a (slightly awkwardly superimposed) version of the figure shown in 5A to illustrate this. Assuming the editors are prepared to forward this on to you: GlyT2 is assigned to green, while GalR1 is assigned to red. As shown (provided one ignores the relevant arrows/arrowheads), specific signals are very clearly either green or red, while shared non-specific background becomes orange, and can easily be noted and ignored.
Remember: the colours are arbitrary. This image is an RGB .tif image, with the blue channel completely empty: DAPI could easily be assigned to this channel (and this would have made the images far easier to align). It is more difficult, but not impossible, to represent more than three fluorophores within a single image (particularly if they label discrete regions, with minimal overlap). For example, DAPI and three mRNA/protein targets could be represented in CYMK format as
DAPI: greyscale
Target1: cyan
Target2: yellow
Target3: magenta
And for regions of overlap where it is important to show unique channels unambiguously, you can still resort to inset panels with individual fluorophores.

I hope this is helpful, but really: I should not be required to explain how best to present fluorescence multiplex data to authors attempting to publish a fluorescent multiplex methods paper. The authors have what I believe could be some truly beautiful images here, but they have not presented them as such. I would like to offer them the opportunity to try again.
We thank the reviewer for this constructive feedback and the effort taken to provide us with the superimposed image. All the figures have now been revamped and reorganised– Figure 4 is now split into 2 different figures; Figure 4 and Figure 5 – presenting low power images (LHS column) along with high quality higher power images in which the resolution of the puncta-like RNAscope staining is much improved. We have also included a superimposed/merged image of all the channels at the end of each figure.
After considering the cyan, magenta and yellow colour combination, we chose to keep red, blue, green and orange.

I suggest the authors take all their data and carefully document exactly what has been stained with what, and use only images that are strictly comparable for actual comparisons. Detail precisely how images were captured, using what microscope and what kind of camera, and how they were post-processed. Most of the commercial microscopy packages I am familiar with readily assemble images into multi-channel format, leaving the unique colours used for each channel to the whims of the investigators. These can be exported as multichannel images for imageJ/Fiji analysis/procession, or exported as RGB tiffs.
This should give the authors a head-start in arranging their multiplex images to actually show the multiplexing, hopefully.
For details of the microscope and camera used please refer to the Table of Materials.
Point 5 and 5 of the results section have been updated for more details on image acquisition and analysis.

Minor Concerns:
Title/Abstract:
The first half of the abstract ignores the 'comparison' element entirely and instead focusses on discussing the need for combining (RNAscope) ISH and IHC, followed by claims that their method allows three proteins AND three mRNA species to be resolved (something I do not believe to be the case). Following a treatise on specific neural markers and the authors' results, only then do they actually describe, almost in passing, their comparison of fresh frozen and fixed tissues. It almost seems as if the authors only remembered what they were discussing half-way through. Either that, or the title was decided post-hoc.

I would suggest either the title or the abstract (or both) be changed for consistency: mention the specific reasons to compare fresh/fixed tissues, the need for multiplex, and then describe the specific examples used within the manuscript.
The title has now been amended.

I also do not feel it is necessary to point out that "RNAse free conditions" and the like are not discussed within the manuscript: anyone working with RNA will understand the need for RNAse free conditions.
The sentence pointing out the RNAse free conditions has been removed from the abstract.

Introduction:

For the most part this is well-written, though (as with the abstract) I would suggest the authors restructure it. The very first paragraph is a firestorm of neuronal terminology and acronyms: for something that is supposed to be describing multiplex ISH/IHC, this is very jarring, leaving the reader wondering whether they have been supplied the wrong manuscript. Either introduce this section later, or break the reader into it more gradually, beginning with something that actually pertains to multiplex ISH/IHC, then only subsequently explaining the need for markers in the brain.
Also use this section to clearly lay out the experimental design, and the specific questions to be addressed. Here would be an excellent place to also introduce the GFP-Phox2b mouse, because otherwise it just appears out of nowhere.
We have restructured the Introduction – it is now focused and the experimental design is clearly laid out. A large amount of the neuronal terminology and acronyms has been removed.We now introduce the GFP mouse in the Introduction, and explain that we used the mouse because it overexpresses a cytoplasmic protein (GFP) in the Phox2b locus.

Protocol:
2.2 Tissue sectioning:
I do not like the use of "blocked" here. I assume you mean 'cut into blocks' for vibratome? Since blocking is most commonly used to refer to blocking of non-specific epitopes (something that also is performed in this manuscript), I would suggest another term is used here.
Thank you for the suggestion. The terminology has been changed accordingly in the Protocol/Method section of the manuscript.

3.5 "Dehydrate the tissue sections by , washing the slides with absolute ethanol by moving the rack up and down for 3-5 times."
Rephrase this. Perhaps "Dehydrate the tissue sections by immersion in absolute ethanol (moving the rack up and down for 3-5 times to ensure complete dehydration)"
Thank you for pointing out the awkward sentence structure. We have rephrased sentences referring to dehydration of tissue sections.

RNAScope signal step: please state exactly which RNAscope fluorescence kit was used (there are several), and explain in more detail which fluorophores were used. The mention of AMP4 implies that the kit used is one that does not allow the user to manually assign their own choice of fluorophores: state which AMP4 was used, and what fluorophores this thus assigns to which channels. Critically, note that use of this method (preassigned fluorophores) limits the choices for subsequent IHC.
The Editor specifically asked that we do not refer to the commercial aspects of the kit used. This information can be found in the Table of Materials, and Table 2. We have indicated this and notee that use of this method (preassigned fluorophores) limits the choices for subsequent IHC, in Section 3.3 of the protocol,

Bacterial DapB (methods and results page 11): the authors are incorrect in their reasoning here. DapB is not included as a control for bacterial contamination: the assumption is that any investigator competent enough to prepare fixed mouse brain tissue for ISH is also competent enough to not cover it in bacteria.
DapB is a negative control, and Polr2A, Ppib and UBC are positive controls. The latter three are used to confirm that the sample does indeed contain RNA that is robustly labelled, while the former is used to confirm that signal from probes that should not bind to anything (because

they're to bacterial genes) do not produce any non-specific labelling. No staining with DapB indicates that non-specific labelling is low/zero.

DapB labels bacteria and was used in the experiment as a negative control, indicating non-specific labelling and background signals. We have rephrased the relevant sections in the main text to clarify this (Please refer to 3.3.1, second paragraph of Results and Figure 2 legend).

5: imaging and analysis:
Add the requested detail (above) re microscope, camera, and objectives used. What magnification were images collected at? 20x is common for RNAscope, but this is not stated within the manuscript.
All the images were acquired using a 20x objective. The figure legends are now updated to include this information.

6: quantitative analysis of target transcripts
Remove this entire section? At no point in the rest of the manuscript are any quantitative methods used, so there seems little merit in including this here.
We do not provide quantitative results in this paper. To make it clear, we have included a sentence at the beginning of this section highlighting that no quantitative data was presented. However, we endeavoured to provide a method for quantitation based on our recent paper published (doi: 10.3389/fnins.2019.01343).

Results:
Page 11: introduction of GFP mouse here. Introduce it earlier, and explain why you used it. Did you perform negative (secondary antibody only) controls to establish whether the 'flocculant' staining was even specific or not? You could also stain a serial section that has not been subjected to FISH to show what staining in the absence of the FISH protocol looks like.
Thank you for your guidance; we apologise for the abrupt entry of the GFP mouse in this manuscript. In the revised manuscript, we introduce the GFP mouse in the final paragraph of the Introduction, and explain that we used the mouse because it overexpresses a cytoplasmic protein (GFP) in the Phox2b locus.
Please refer to the response to the minor comment 5 of reviewer 2 for further rationale explaining why we used the GFP mouse.

"The LPB was Phox2b, TH and vAChT negative, therefore anatomical landmarks were used to define the region"
With the greatest respect, the entire purpose of this paper is to reveal methods that allow brain regions to be identified via ISH/IHC. If you are going to just resort of anatomical landmarks ANYWAY, then what is the point of any of this?
We acknowledge that investigationg of the lateral parabrachial nucleus (LPB) travelled too far from the purpose of the paper. Results related to LPB have been removed from the manuscript.

Page 12:
"in the nucleus of neurons, in contrast to GFP which is over-expressed in the cytoplasm. Our results show the expected overlap of Phox2b riboprobe…"
This is almost impossible to tell without a DAPI counterstain. A single image, showing DAPI, Phox2b mRNA and Phox2b protein would be beautiful, and would illustrate this nuclear/cytoplasmic localisation perfectly.

This figure (Figure 5) has been revised and now illustrates DAPI, Phox2b mRNA and Phox2b protein. Inclusion of the DAPI counterstain makes the nuclear/cytoplamic localisation clear.
We thank the reviewer for the suggestion which has improved the manuscript.

The authors also in this section finally explain they can only visualise 4 fluorophores (one of which must be DAPI, given the 346 excitation), but go on to explain that "co- of up to 5 targets was achieved". You cannot actually show 'co-localisation' of five targets using only three fluorophores. You can make the argument that some targets are only expressed in different locations, so those cannot co-localise and can thus share the same fluorophore (indeed the authors do exactly this: "Note that TH and vAChT (Figure 4 A-B) were labelled using the same colour fluorophore since the subcellular location is different for these two proteins"), but this is an assumption, and a dangerous one: what, for instance, would the authors see if a mutation being investigated led to TH being aberrantly expressed in vAChT locations? Nothing at all unusual, because they make the explicit assumption this never happens. If the authors had made some effort to show, empirically, that the same two antibodies gave wholly separable staining patterns when used with different fluorophores, I would be more convinced, but they haven't. For a paper purporting to show a novel multiplex means of identifying brain regions, their method relies on a lot of underlying assumptions about brain anatomy and gene expression.
The '5 targets' alluded to are illustrated in revised Fig 4. Please refer to major comment 3 of reviewer 2 for more explanation. We have reworded the statement; the word 'colocalisation' is now excluded. We have made changes to say 5 targets processed at the same time in the same tissue section.
Please refer to comment 3 of reviewer 1 for clarification of TH and VAChT staining.

Figure 2 legend: "As the secondary antibodies target the host species of the primary antibody, the more rapid secondary antibody incubation should not affect background or off-target labelling except for where species crossreactivity occurs."This doesn't need to be here. Nobody will question your choice of primary/secondary incubation times, and everyone will assume you optimised the times for exactly these reasons.
The text in question has been removed from the Figure 2 legend whilst leaving in place: "The secondary antibody incubation (2 hours) was carried out at room temperature."

Figure 3: these could be shown as multiplex images, as well as in their respective specific channels. I assume the same imaging field is shown in each instance (i.e. the same imaging field is used in all +ctrl probe images). Include DAPI.
Make the scalebars thicker, consider adding "50" to them to make it really obvious.
Figure 3 has been updated to include the DAPI channel and a multiplex/merged image. The scale bars are now thicker. The Figure legend states 'The same imaging field is used in panel A and panel B.'

Figure 4: This figure is a mess. Much of this could be simplified by merging images into multiplex format, which would allow individual images to be larger. Somatostatin and GlyT2 are used together at one point: one is a C2 and the other a C3 probe: why is GalR1 (C1 probe) not also included here? Why does only a single full image have any DAPI staining? Why are no fluorophores listed? Why does SCP have its own scalebar in 4B (centre)? Why do the figures go A, A2, B…? Is…is A2 a magnified version of A? That's….that is not clear in the slightest.
The scalebars change their dimensions almost at random, and without constantly switching between the legend and the figure, this is really hard to follow.

We thank the reviewer for this critical appraisal. Changes have been applied to Figure 4 include:
-division of the figure into 2 separate figures (now Fig 4 and Fig 5) to provide clarity and simplify the Results.
- images with information straying from the aims of the study have been removed, such as the ones that belong to the LPB and VRC.
-DAPI images have been incorporated.
-A merged image with overlay of all the channels has been added.
-The 20x image and enlarged inset were provided to illustrate anatomical landmarks and the intensity of fluorescent signals in cellular profiles, respectively.
-The scale bars are thicker.

Figure 5:
See my version of how to present figure 5A, which I assume are the same imaging field. For figure 5B, the implication is that the two images are the same imaging field, but if this is the case, why are the scale bars different? Using the same fluorophore for Phox2b and TH might be acceptable, perhaps (see above), but I really question the authors' confidence in separating the two here: the staining indicated by the lower 'arrow' is almost identical to that of the upper 'arrowhead'. If one is supposed to be nuclear while the other is cytoplasmic, inclusion of a nuclear DAPI signal would really, really help clarify this distinction (as would inclusion of DAPI literally everywhere else).
Thank you, we have removed the original Figure 5B. Now Figure 5B illustrates the magnified insets of Figure 5A. Figure 5A includes the nuclear DAPI signal
Discussion:
As my above comments might suggest, I am not entirely convinced by the authors' conclusions. Much of the discussion concerns their assertion re: cytoplasmic vs membrane bound proteins, supported by a host of additional IHC experiments which they choose to note…only now? If your hypothesis is that cytoplasmic proteins, specifically, are affected here, then choosing not to show, or even mention until now, additional experiments that specifically test that hypothesis is just a baffling approach. These experiments should be in the results.
Similarly, the following paragraph details a number of studies that are only mentioned here, all of which would have been interesting, and all of which should have been included in the methods and results.
The methods, results and discussion have been rewritten. All the experimental outcomes presented in the discussion are now first introduced in the results section.

In summary, there is the core of a very nice paper here, but it really, really needs to be rewritten. The authors need to first decide exactly what they are trying to show, and then rework the manuscript to present their process in a clear and logical fashion. They need to include the necessary controls, and make it very clear what questions their technique can and cannot address, and how many targets they can label. Fluorophores need to be correctly identified throughout, whereas colours used for figures (which are arbitrary) should be reassigned to best show the multiplexing, which the authors should definitely show.
And if you're going to include it, please actually introduce and explain the GFP mouse.
We thank Reviewer 3 for their comprehensive feedback – the revised manuscript incorporates all the suggested revisions.