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Abstract:	In the brain, tissue-specific mRNA variants of key synaptic genes are produced by precise removal of introns present in the precursor RNA. The spliced mRNA is then translated into specific protein isoforms required for normal brain function. Current techniques designed to study the expression of specific mRNA splice variants in the brain (and other tissues) rely on methods that use RNA extracted from brain tissue containing a mixture of cell types, a specific cell population or from single cells. None of these techniques provide information on the expression of the mRNA splice variants of a gene with cell-type specificity and subcellular precision, while maintaining the cyto-architecture of the studied brain region. Here we describe a modified protocol of in situ hybridization combined with an immunodetection method for the identification of alternative splice variants of a gene with high specificity and subcellular resolution in a specific neuronal cell type from neocortical brain slices. The specificity of this method is given by the use of short DNA oligo probes labeled with digoxigenin (DIG), a plant steroid that can be detected using high affinity and specificity antibodies. The ability to see where a desired RNA splice variant is expressed in a neuronal cell type allows a better interpretation of the role in brain function ~~in health~~ and disease.

TITLE:

Identification of alternative splice variants in cortical pyramidal neurons via an *in situ* hybridization and immunodetection method

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KEYWORDS:

Alternative splicing, *in situ* hybridization, brain, neocortex, pyramidal neuron, indirect immunofluorescence

SHORT ABSTRACT:

This protocol describes a method of *in situ* hybridization for detecting specific splice variants of a gene in a cell-specific manner and with subcellular resolution. This is achieved by using digoxigenin (DIG)-labeled PCR primers designed to amplify a particular alternative exon followed by a versatile immunodetection procedure.

LONG ABSTRACT:

In the brain, tissue-specific mRNA variants of key synaptic genes are produced by precise removal of introns present in the precursor RNA. The spliced mRNA is then translated into specific protein isoforms required for normal brain function. Current techniques designed to

study the expression of specific mRNA splice variants in the brain (and other tissues) rely on methods that use RNA extracted from brain tissue containing a mixture of cell types, a specific cell population or from single cells. None of these techniques provide information on the expression of the mRNA splice variants of a gene with cell-type specificity and subcellular precision, while maintaining the cyto-architecture of the studied brain region. Here we describe a modified protocol of *in situ* hybridization combined with an immunodetection method for the identification of alternative splice variants of a gene with high specificity and subcellular resolution in a specific neuronal cell type from neocortical brain slices. The specificity of this method is given by the use of short DNA oligo probes labeled with digoxigenin (DIG), a plant steroid that can be detected using high affinity and specificity antibodies. The ability to see where desired RNA splice variant is expressed in a neuronal cell type allows a better interpretation of their role in brain function in health and disease.

INTRODUCTION:

Alternative splicing is a widely used co-mechanism of gene regulation[1,2]. In humans most ~~gene~~ transcripts carry various exons undergo alternative splicing[3,4]. Alternative splicing factors act to catalyze the precise removal of introns and the selection of exons to generate a mature RNA that will encode a protein variant in a particular time-window during development, a certain physiological condition or in a cell- and/or tissue-specific manner required for normal tissue development and function [5].

In the brain alternative splicing is a widely used mechanism to produce specific protein isoforms required for neuronal development and physiology[2,5-7]. Defects in alternative splicing in the brain are linked with deficiencies in neuronal migration and function which are attributed to several neurological diseases [5].

The expression analyses of alternative splice variants rely mostly on the detection and quantification of mRNAs from bulk brain tissue containing a mixture of cell types[7,8]. More sophisticated methods can isolate RNA from a specific cell population using fluorescence-activated cell sorting (FACS)[9] or from the soma of a single neuron using patch pipette or laser capture microdissection[10]. However, none of these techniques are capable of combining the detection of specific mRNA splice variants in a single cell-type with subcellular precision. This problem is particularly important because alternative splicing can vary from cell to cell and be impossible to detect when, for example, those changes occur in an underrepresented type of neuronal population.

Hence, to overcome these technical limitations, we describe a modified protocol of *in situ* hybridization combined with an immunodetection method designed to localize specific mRNA splice variants of a gene in neocortical pyramidal neurons with subcellular resolution in fixed brain slices. This is achieved by using digoxigenin (DIG)-labeled DNA oligo probes (designed as PCR primers for the amplification of a particular alternative exon) and followed by specific binding of anti-DIG antibodies for subsequent immunodetection.

This method will allow researchers to determine the location and the relative expression levels

of specific splice variants of a gene, with the final goal to understand their physiological relevance for brain function in health and disease.

PROTOCOL:

Procedures involving animal subjects have been approved by Le Comité de déontologie de l'expérimentation sur les animaux (CDEA) at Univeristé de Montréal and adhere to the Canadian Council on animal care (CCAC) guide for the care and use of laboratory animals.

Note: This protocol describes two ~~optional~~ ways to treat brain slices; the first ~~one~~, the brain slices are placed on a glass slide; ~~and~~ in the second ~~option~~, slices are handled in solution. The latter is called "the floating sections protocol" and the modifications needed to implement this option are indicated as separate steps. Omit these modifications if the option of placing slices on glass slides is chosen.

1. Tissue fixation and sectioning

Note: All steps up to and including the hybridization step are sensitive to RNAse activity. Whenever possible use new plastic RNAse-free containers, clean glass and metal surfaces with absolute ethanol and prepare all solutions with RNAse-free molecular biology grade reagents and water.

1.1) Place the mouse in a closed container with a cotton ball saturated in isoflurane anesthesia for 3 minutes. ~~Take the~~ mouse ~~out~~ and maintain isoflurane inhalation by placing a tube with a saturated cotton ball over the nose. Confirm absence of pedal reflexes by toe pinching.

1.2) Open the abdominal cavity with a straight incision perpendicular to the body midline and as wide as the ribcage from left to right. Open the ribcage cutting through the diaphragm and sternum to expose the heart. Cut the sides of the ribs and pin them to the sides to expose the heart [11].

1.3) Introduce the 25 G needle of a vacutainer blood collection set into the posterior end of the left ventricle of heart and make an incision to the right atrium using iris scissors as an outlet for the excess fluid.

1.4) Slowly inject 5-10 mL 1x phosphate-buffered saline (1x PBS, Table 1) at 37 °C through the cannula using a syringe until the liver is light brown. Fix the mouse by injecting 10-20 mL of ice cold 4% paraformaldehyde (PFA) in 1x PBS using another syringe at an injection rate of approx. 2 mL/min until the body is stiff. Note: Volumes vary depending on the size of the mouse.

1.5) After perfusion, dissect the brain[12]. Place the dissected brain on ice in a vial containing 4 mL 4% PFA in 1x PBS. Keep the brain in this solution for 90 min at 4 °C with gentle agitation.

1.6) Wash the fixed brain in 4 mL 1x PBS 6 times (30 min each) and then dehydrate it with 4 mL 30% sucrose in 1x PBS overnight. Use the brain sections within 2 weeks of the fixation date.

1.7) Place a cryostat specimen holder into the cryostat for 10 min. Use an embedding matrix bottle to apply diluted embedding matrix (1:6 in water) to the middle of the head of the specimen holder until covered. Wait until it is frozen.

1.8) Turn the holder handle and rub the frozen embedding matrix over the surface of a slide warmer to form an even layer. Place in the cryostat.

1.9) Freeze the brain by immersing it in 2-methylbutane at -50 °C. Remove using blunt forceps and glue it with a drop of diluted embedding matrix to the prepared specimen holder. Immediately cover the brain in a thin layer of embedding matrix by alternately immersing it into 2-methylbutane at -50 °C and diluted embedding matrix ~~for~~ 5 times. Place it in the cryostat.

1.10) Cut the brain into 40 µm thick cryosection [12]. Immediately after cutting, place each brain section over a clean glass slide treated with tissue capture pen to increase tissue attachment to the slide, and store it at -80 °C in air-tight plastic slide mailers [13].

1.11) Modification of step 1.10 required for the floating section protocol

1.11.1) Cut the brain into 40 µm thick cryosection [12]. Immediately after cutting, place each brain slice in a 6 well plate filled with 1x PBS. Store at 4 °C and use within 24 h.

2. Probe design

2.1) Design the probe using the following considerations:

2.1.1) Carefully study the sequence of the desired constitutive and alternative exons to be detected using the genome browser[14] and links therein (Figure 1).

2.1.2) Use a primer design program (such as Primer 3)[15,16] to design a pair of primers.

2.1.2.1) Design primers 20-22 mer in length for quantitative PCR (qPCR), with an amplicon (PCR product) of around 100 bp.

2.1.2.2) Design two pairs of primers to detect mutually exclusive splicing events (Figure 1A).

Note: The forward primers should be complementary to the constitutive exon adjacent to the alternative exon, and each reverse primer complementary to each mutually exclusive alternative exon (Figure 1A).

2.1.2.3) Design two pair of primers to detect exon inclusion/exclusion splicing events (Figure 1B).

Note: The forward primers should be complementary to the constitutive exon adjacent to the

alternative exon. Design one reverse primer complementary to the alternative exon, and one reverse primer spanning the junction between the constitutive exons flanking the alternative exon (Figure 1B).

2.1.3) Use BLAST [17] to check the specificity of the primers to the gene of interest.

2.1.4) Order the primers purified by desalting. Note: Further purification is not necessary.

2.2) Prepare and test the primers

2.2.1) Resuspend the primers in water to a final stock concentration of 100 pmol/µL (100 µM).

2.2.2) Perform Q-PCR[18] with the primers using reverse transcribed RNA[19] (cDNA) extracted from the same tissue (i.e. cortex) where the *in situ* hybridization will be performed.

2.2.3) Test the efficiency of the primers using serial dilutions of the cDNA. Confirm amplification efficiency between 90-110% [18]. Use the Q-PCR results as a complement to the *in situ* hybridization results.

3. Labeling of the probe with DIG

3.1) Mix the reagents listed in Table 2 to label the probe (reverse primer). Incubate for 15 min at 37 °C.

3.2) Stop the reaction by placing the tube on ice and adding 2 µL 0.2 M EDTA pH 8.0.

3.3) Precipitation of the probe

3.3.1) Add 2.5 µL 4 M LiCl and 75 µL absolute ethanol to the tube. Mix well and leave at -80 °C overnight.

3.3.2) After incubation, centrifuge at 20,000 x g for 30 min at 4 °C. Completely remove the supernatant and dry the pellet for 10 min at room temperature (RT).

3.3.3) Resuspend the pellet in 100 µL of water (final concentration ~ 2 pmol/µL). Use the labeled probe the same day and/or store it at -20 °C for a maximum period of 1 year.

3.3.4) To evaluate if the probe is labeled with DIG-dUTP, perform a dot blot according to the manufacturer's instructions using specific DIG antibody, followed by a horseradish peroxidase (HRP) conjugated antibody (Figure 2).

Note: The reaction of HRP with DIG forms a brown precipitate showing that the probe is labeled with DIG (Figure 2).

4. *In Situ* Hybridization

Note: To change solutions for floating slices (floating section protocol), transfer the slices to new wells filled with the solution specified for each step. To change solutions for slices placed on glass slides, use a Coplin jar or directly apply the specified solution over the brain slices as described.

4.1) Pre-Hybridization Treatment

4.1.1) Thaw the slides containing the brain sections (from step 1.10) at RT for 5-10 min.

4.1.2) Hydrate the slides in 1x PBS for 10 min at RT in a Coplin jar.

4.1.3) Modification of steps 4.1.1 and 4.1.2 required for the floating section protocol

4.1.3.1) After step 1.11, select the brain slices to be used for the *in situ* hybridization and transfer them to a 24 well plate filled with 1x PBS. To move the brain slices from well to well, use a clean brush or a custom-built glass hook. Place no more than 8 brain slices per well.

Note: To make a glass hook, first heat the tip of a Pasteur pipette in a Bunsen burner until the tip is sealed. Then hold the pipette perpendicularly to the burner (with the flame ~5 mm from the end) and wait for the glass to bend. Let it cool before use.

4.1.4) In the meantime, prepare pre-hybridization buffer according to the Table 3 (500 µL/slide).

4.1.5) Remove the slides from the Coplin jar and take off the excess fluid with an absorbent paper sheet. Add 500 µL of pre-hybridization buffer on each slide, and incubate for 1 hr at 60 °C in a humid chamber. Do not allow the slides to dry out in order to maintain the signal specificity.

4.1.6) Modification of step 4.1.5 required for the floating section protocol

4.1.6.1) Pre-hybridize the brain sections by carefully transferring them to a new well in a 24 well plate filled with 500 µL pre-hybridization buffer for 1 hr at 60 °C.

4.2) Hybridization with the DIG-labeled Probe

4.2.1) Remove the pre-hybridization buffer solution with a micropipette, and then gently add the hybridization buffer prepared as per Table 4. Incubate for 16-18 hr at 38 °C in a humid chamber.

4.2.2) Modification of step 4.2.1 required for the floating section protocol

4.2.2.1) Transfer the brain sections to a new well containing 500 µL of hybridization buffer. Incubate the brain section for 16-18 hr at 38°C.

Note: For floating sections, all further changes of solution, wash or incubation steps are

performed by transferring the sections to a new well in a 24-well plate filled with the corresponding solution.

4.2.3) To remove the non-hybridized probe, wash the slides twice (10 min each) at 42 °C with 2x Saline-Sodium Citrate (SSC) buffer (Table 1) in a Coplin jar.

4.2.4) Wash the sample once for 10 min at 42 °C with 1x SSC buffer (Table 1).

4.2.5) Wash the sample once for 10 min at 42 °C with Tris buffer A (TA buffer; Table 1).

5. Signal detection (Primary antibody detection)

5.1) Incubate the slides in permeabilization/blocking reagent for 30 min at RT.

5.2) Dilute the mouse anti-digoxin antibody to 1:500 with the permeabilization/blocking reagent.

Note: Anti-digoxin antibody also recognizes digoxigenin (DIG), therefore it is used as primary antibody for the specific detection of the hybridized DIG-labeled probe

5.3) Remove the permeabilization/blocking reagent with a micropipette, and gently add the diluted anti-digoxin antibody. To additionally detect YFP-expressing layer V neurons[20], add goat anti-GFP antibody to the sample at a dilution of 1:350 (Figure 3). Incubate for 2 hr at RT, or overnight at 4 °C

Note: The fluorescence of the transgenic cellular marker (YFP) is lost during the protocol. Therefore it is necessary to use an antibody to detect it (anti-GFP).

5.4) Modification of step 5.3 required for the floating section protocol

5.4.1) Transfer the brain slices to a new well containing the antibody dilution. Incubate for 2 hr at RT, or overnight at 4 °C.

6. Signal Amplification and Development (Secondary antibody detection)

Note: To continue with colorimetric detection go directly to ~~the~~ step 6.2. For fluorescence detection continue with step 6.1 skipping step 6.2 (Figure 3).

6.1) Fluorescence detection

6.1.1) Following step 5.3 (step 5.4 for floating sections), wash the slides 3 times (10 min each) at RT in TA buffer.

6.1.2) Incubate the slides for 2 hr at RT with red fluorescent anti-mouse antibody at a dilution of 1:200 in permeabilization/blocking reagent and green fluorescent anti-goat antibody 1:500 (if a goat anti-GFP antibody is used). Keep slides in the dark during incubation periods from this step on.

6.1.3) If nuclear co-staining is desired, add a solution containing 0.4 µg/mL of nuclear stain in 1x PBS to the slides immediately after the secondary antibody incubation. Incubate the samples for 10 min at RT.

6.1.4) After the incubation period, wash the slides 3 times (10 minute each) in TA buffer at RT. For floating brain sections, continue to section 7.

6.1.5) Add 3 drops of antifade mounting medium to each slide and carefully place a glass coverslip on top. Remove excess liquid and let dry in the dark for one day at RT. Image the samples using a confocal microscope[21].

6.2) Colorimetric Detection

6.2.1) Following step 5.3 (or step 5.4 for floating sections), wash the slides 3 times (10 min each) at RT in TA buffer.

6.2.2) Incubate the brain slides with biotinylated anti-mouse antibody at a dilution of 1:1000 in permeabilization/blocking reagent for 2 hr at RT.

6.2.3) Wash the slides 4 times (10 min each) with PBS at RT. Prepare the avidin-biotin complex (ABC) solution at least 30 min before use as per Table 5.

6.2.4) Add the ABC solution over the slide. Incubate the brain samples in ABC solution for 1 hr at RT. A complex with the biotinylated antibody is formed[22].

6.2.5) Wash the slides 3 times (10 min each) at RT in TA buffer.

6.2.6) Wash once in Tris buffer B (TB buffer; Table 1) for 5 min at RT.

6.2.7) Develop the specific signal using metal enhanced DAB (3,3'-Diaminobenzidine) substrate kit.

Note: DAB is toxic and harmful to the environment. Discard following institutional guidelines.

6.2.7.1) According to the manufacturer, prepare 1x DAB in 1x stable peroxidase substrate solution and incubate until dark staining is evident.

Note: Staining can take more than 20 min.

6.2.8) Wash the slides twice with 1x PBS (10 min each) at RT. To continue with the floating section protocol, continue to step 7.

6.2.9) Add 3 drops of colorimetric mounting medium to each slide and carefully place a glass coverslip on top. Remove excess liquid and let dry in the dark for one day at RT. Image the samples using an optical microscope coupled to a digital camera.

7. Attachment of floating brain slices to the glass slide.

Note: These steps are exclusively for floating brain sections.

7.1) Fill a Petri dish with 20 mL of 0.1% gelatin diluted in 1x PBS.

7.2) Submerge one half of the glass slide in the gelatin solution supporting one end of the glass slide at the bottom of the Petri dish and the other end on the outer edge of the petri dish.

7.3) Place the brain sections in the solution and with the help of a brush, slide them on the glass slides while still floating. Dry the glass slides for 10 min at RT. Mount in antifade mounting medium (for fluorescent detection) or colorimetric mounting medium (for colorimetric detection) and carefully place a glass coverslip on top. Let dry for one day at RT in the dark.

REPRESENTATIVE RESULTS:

Probes to detect two mutually exclusive exons (probe 1 and probe 2) present in an alternative splice variant of a voltage-gated ion channel gene (splice variant 1 and 2) were used (Figure 1). The representative results show that probes 1 and 2 were successfully labeled with DIG as seen by the colorimetric precipitate formed on the PVDF membrane (Figure 2). The expression of the gene in mouse brain cortex had ~~been~~ already confirmed by searching the Allen Brain Atlas[23] w the images suggest expression in layer V pyramidal neurons (Figure 4, see black arrow towards a triangular shaped soma). However, with these data it is difficult to infer the subcellular distribution of the gene transcripts and there is no information on splice variants. By contrast, the *in situ* hybridization protocol described provides a much greater level of resolution that can be complemented by the general information provided by the Allan Brain Atlas. Low magnification pictures of a representative experiment (Figure 5 A-C, G-I) show expression of YFP (green) as a marker of layer V pyramidal neurons[20] and expression of the alternative exons of variant 1 and 2 of the gene (red). A clear difference in the relative expression of both variants is seen, showing that variant 2 has higher expression. This correlates with the Q-PCR data obtained from total cortical RNA using the same set of primers designed to use the reverse primer as a probe (not shown). High magnification pictures (Figure 5 D-F, J-L) show co-localization of the signal in the soma of YFP-positive layer V pyramidal neurons. The localization is probably nuclear but the lack of nuclear co-staining in this experiment doesn't allow us to reach a conclusion. Most interesting is the co-localization (yellow) of the splice variant expression with the apical dendrite of the neuron (arrows). Taken together these representative results demonstrate that the technique here described allows the specific detection of alternative exons of a gene that share a high degree of homology. In addition, with simultaneous immunodetection of a transgenic neuronal marker, the expression in a specific cortical region and in a particular neuronal type with subcellular resolution can be achieved.

Figure Legends:

Figure 1: Primer/probe design: The figure shows the ~~rational to choose~~ the annealing/binding site of the forward (Fw) primers (only used for PCR) and the reverse (Rv) primers (used for PCR and as a probe). The figure represents the genomic sequence, and connecting lines on top indicate the splicing events that give ~~rice~~ to splice variants 1 and 2 of the gene. (A) To design primers for mutually exclusive alternative splicing choose a sequence in the upstream

constitutive exon as annealing site for the forward primer and a sequence in the alternative exon (A in red or B in blue) for the reverse primer/probe (probe 1 and 2). (B) To design primers for alternative exon inclusion/exclusion splicing choose a sequence in the upstream constitutive exon as annealing site for the forward primer, a sequence in the alternative exon for the exon inclusion reverse primer/probe (red) and a sequence in the constitutive exon junction for the exon exclusion reverse primer/probe (green). The size of the amplicon for all sets of primers is only shown once in A. Alternative primers are shown as red or blue rectangles and constitutive exons as black ones. Primers are shown as arrows. (C) Example showing Homo sapiens microtubule associated protein tau (MAPT), transcript variant 6, mRNA (NM_001123066.3). The upper panel shows a screen shot from the genome browser (http://genome.ucsc.edu) were exon 7 is included in the upper transcript and excluded in the lower transcript. Exon 6 and 8 are constitutive exons. The lower panel shows the gene sequence from exon 6 to 8. Alternative exon 7 in blue characters and exon 6 and 8 encased in a black rectangle. In this example one set of primers (highlighted sequence) was designed to detect exon inclusion (presence of exon 7 in the transcript): Fw primer (black arrow) and Rv primer (red arrow) and a second set to detect exon 7 exclusion: Fw primer' (black arrow, partially shown) and Rv primer (green arrow) complementary to the exon6/exon8 junction.

Figure 2: Test to evaluate DIG-labeling of the probe. 50 µl sample of each DIG-labeled probe (Probe 1 and Probe 2) are analyzed by dot blot on a polyvinylidene fluoride (PVDF) membrane. The membrane is then incubated with anti-DIG antibody followed by a secondary antibody labeled with horseradish peroxidase (HRP) and colorimetric signal development with DAB as a substrate. The brown precipitate demonstrates that the probes are labeled with DIG.

Figure 3: Workflow chart: The chart shows each protocol step represented by a box. The steps to label the probe are shown in purple boxes. The steps for fluorescent and colorimetric detection are shown in red and green boxes, respectively, and the common steps in light orange boxes. Washing steps are represented with lighter colors boxes. Numbers in a blue box correspond to the protocol step numbers in the text. DIG: Digoxigenin, dUTP: 2'-Deoxyuridine 5'-Triphosphate, ON: Overnight PBS: Phosphate buffered saline, RT: Room temperature, SSC: Saline-Sodium Citrate, TA: Tris buffer A, TB: Tris buffer B.

Figure 4: Expression of ion channel subunit mRNA in the mouse cortex: Images obtained from the Allen Institute for Brain Science [23] show expression of a voltage activated channel subunit in adult mouse detected by colorimetric *in situ* hybridization. The approximate location of cortical layers is shown (dotted line and labels). Inset shows the maximum amplification available of the square area in layer V/VI and arrows indicate pyramidal shaped cells. A low amplification image of the complete sagittal brain slice is shown on the lower right (B); a reference key based on Nissl staining is also shown, with layer V highlighted in blue. The inset on the lower right corner shows the corresponding sagittal section before enlargement (A). Bar in panel A: 349 µm, bar in B 350 µm and bar in inset: 52 µm.

Figure 5: Specific detection of alternative splice variants of a gene in neocortical pyramidal neurons. Two alternative exons of a gene (Variant 1 and 2) are detected using a modified *in situ*

hybridization protocol performed ~~in~~ floating section. Fixed brain slices (40 µm thick) from a 2 month old (P76) thy1-YFP line H mouse strain[20] were used to detect neocortical pyramidal neurons. (A, D, G, J) Antibodies were used to detect YFP-positive neurons (YFP). (B, E, H, K) Splice variants were detected by *in situ* hybridization followed by fluorescent immunodetection ~~was (ISH)~~ in YFP-positive neurons. Images in D, E, F, J, K and L are magnified views of the white box area in A, B, C, G, H and I, respectively. (C, F, I, L) Merge shows colocalization of *in situ* hybridization and YFP-positive neurons. Arrows indicate dendritic expression in Layer V YPF-positive neurons.

Table 1. Buffers ~~Composition~~. This table lists the composition of buffers and reagents bought (*), diluted from a stock solution (**) or prepared from ~~its~~ components (***), that were used in the protocol.

Table 2: Labeling of the probe with DIG. List of reagents used to label the probe. This reaction adds ~~a tail of~~ adenine, including the modified nucleotide Digoxigenin-11-2'-deoxyuridine-5'-triphosphate (DIG-dUTP) to the reverse primer (the probe). The indicated reagents (*) are included with the terminal transferase enzyme (Reagent Table). The total volume indicates the final volume of the labeling mix.

Table 3. Pre-Hybridization buffer. This table lists the reagents used to prepare the pre-hybridization buffer. The composition of 20x SSC and 50x Denhardt's can be found in the Table 1. Volumes are listed to prepare 1 mL of buffer.

Table 4. Hybridization buffer. This table lists the reagents used to prepare the hybridization buffer. (*) See composition of 5x Buffer in Table 1. (**) See step 3 of the protocol. Volumes are listed to prepare 1 mL of buffer.

Table 5. ABC Solution. This table lists the reagents used to prepare the ABC solution. The indicated reagents (*) are included in the ABC staining kit (Reagent Table). Volumes are listed to prepare 1 mL of buffer.

DISCUSSION:

Alternative splicing is a highly regulated process by which a single gene can encode multiple protein isoforms. This is a particularly important process in the brain ~~were~~ neuronal-specific variants of a gene are required for normal development and function[7]. In fact, the study of alternative splicing has emerged as an important target for the understanding of several neurological diseases[24]. Hence, the identification of mRNA splice variants of a gene and the specialized function of the encoded protein isoforms is vital for the understanding of brain physiology.

To study the expression of specific mRNA splice variants in the brain and other tissues, total RNA is extracted from dissected brain tissue and their average expression in this mixture of cell types is assessed by PCR, exon-array and/or RNA-seq. These methods have a ~~big~~ limitation, especially in a tissue like the brain, ~~were~~ the extracted RNA comes from a mix of different cell

types (neurons, glia, etc.) with different functions. To overcome this limitation, it is possible to study the expression of splice variants with cell-type resolution using fluorescence-activated cell sorting (FACS)[9], a method that separates fluorescently labeled cell subpopulation but only preserves the neuronal soma. Alternatively, to study single-cell mRNA splice variants it is possible to extract RNA from a single cell soma using a patch pipette or laser capture microdissection[10]. These last techniques are technically challenging and require training and equipment not commonly available. Most importantly, with these techniques it is impossible to uncover at the same time in a cell-type specific manner the subcellular distribution of alternative spliced variants of a gene. Cell-type specificity can be achieved by the design and production of custom antibodies to detect each protein isoform encoded by a specific mRNA splice variant[25]. However, this approach is long, expensive and not useful for screening.

Here we devise a protocol of *in situ* hybridization that allows the detection of alternative splice variants of a gene in a specific neuronal cell type with subcellular resolution. This protocol is an extensive modification of previously described antisense DIG-labeled deoxyoligonucleotides techniques for the detection of mRNA (for example see Andres et al., 1996 [26]) that we combined with currently available immuno-detection tools for DIG-labeled probes. This protocol uses short DIG-labeled DNA oligo probes of approximately 20 mer for the specific detection in cortical pyramidal neurons of the two alternative splice variants of a gene differing in a few base pairs. Importantly, the use of short DNA oligo probes provides high specificity and reduces cost and time when compared with the use of longer probes of at least 150 mer generated by PCR and checked by sequencing[23], the use of in-vitro transcribed RNA probes[27-30] or 1000 > mer long probes synthetized by a company[31]. Furthermore, avoiding the use of an *in situ* hybridization kit[31] increases versatility and further reduces costs. The results obtained with this technique can be used as a complement to screening techniques like RT-PCR or RNA-seq or genome-wide *in situ* hybridization databases[23,32].

The versatility of the method allows detection of the DIG-labeled probe by colorimetric or fluorescent methods. Each immuno-detection method has its advantages and disadvantages. The colorimetric detection method does not provide the same degree of subcellular detail as that provided with the fluorescence detection method (Compare Figure 4 and 5), but the staining of alternative exons is appreciable at low magnification (which is useful to detect high levels of expression over a ~~big~~ area of the brain) and remains unchanged for years. The fluorescent detection method is easily combined with antibody detection or transgenic markers for specific cell types and subcellular structures. The technique can also be adapted to the usual immunostaining methods used in a particular laboratory and therefore to the available equipment by choosing to use floating brain sections or brain sections on slides, this last option is also especially useful for small tissue like mouse spinal cord cryosections.

A critical step in this protocol is the design of probes that can distinguish two highly homologous splicing isoforms of a gene. Since different mechanisms of alternative splicing can be recognized[33], it is important to identify the type alternative exon to be analyzed. For example, if the preRNA of the gene of interest undergoes an alternative splicing with mutually exclusive exons, then this means that one of the exons in the preRNA is retained in the mRNA,

but not both. In this case, a probe for each exon must be design for the detection of each alternative splice variant, and one probe becomes the control for the other. If instead, the gene undergoes either intron retention, or exon skipping then only one primer would be needed to detect its presence and a probe complementary to the junction between the constitutive exons flanking the alternative exon can be designed as a control. In all cases, the design of more than one probe is necessary for the proper detection and analysis of alternative splice variants of a gene.

Another critical step of the protocol is the integrity of the tissue. The fixation step preserves the RNA and the structure of the tissue, nevertheless excess post-fixation time can modify the molecular structure of the RNA and protein affecting their detection. The preservation of the tissue is also increased with the glass slide protocol, were the brain slices are subject to less handling and fewer skills are required than when floating sections are used. We further improved this protocol by increasing the attachment of the brain tissue to the glass, especially of small tissue slices, with the use of a commercial compound that can be "painted" over the glass slide before use.

Lastly, a critical factor for the success of this technique is the actual expression of the alternative exon in the tissue studied. The higher the expression, the better the results, but the expression levels of alternatively spliced exons vary by definition and can be affected for example by the developmental age[7]. Therefore it is highly recommendable to verify the expression of the gene of interest in The Allen Brain Atlas and to determine the expression of the alternative exon(s) by Q-PCR before attempting the *in situ* hybridization. In the example shown in Figure 5 the variant 2, for example, has low expression (consistent with previous Q-PCR analysis; Not shown) making its detection more challenging.

The ability to see were the splice variant is expressed in the tissue with subcellular resolution allows a better interpretation of its role in normal brain function and can be used to study cell-specific expression changes in neurological disease. In addition, further developments in tissue clearing techniques[34], which enables the visualization and RNA labeling of intact neuronal networks, can be combined with this protocol for the screening of alternative splice variants of selected genes in neurons, glia, etc. from intact mammalian brains. These technical improvements together with big data reservoirs like the 2015 Allen Institute for Brain Science[23], could provide more effective and realistic experimental approaches to characterize at the molecular level intact neuronal circuits in health and disease.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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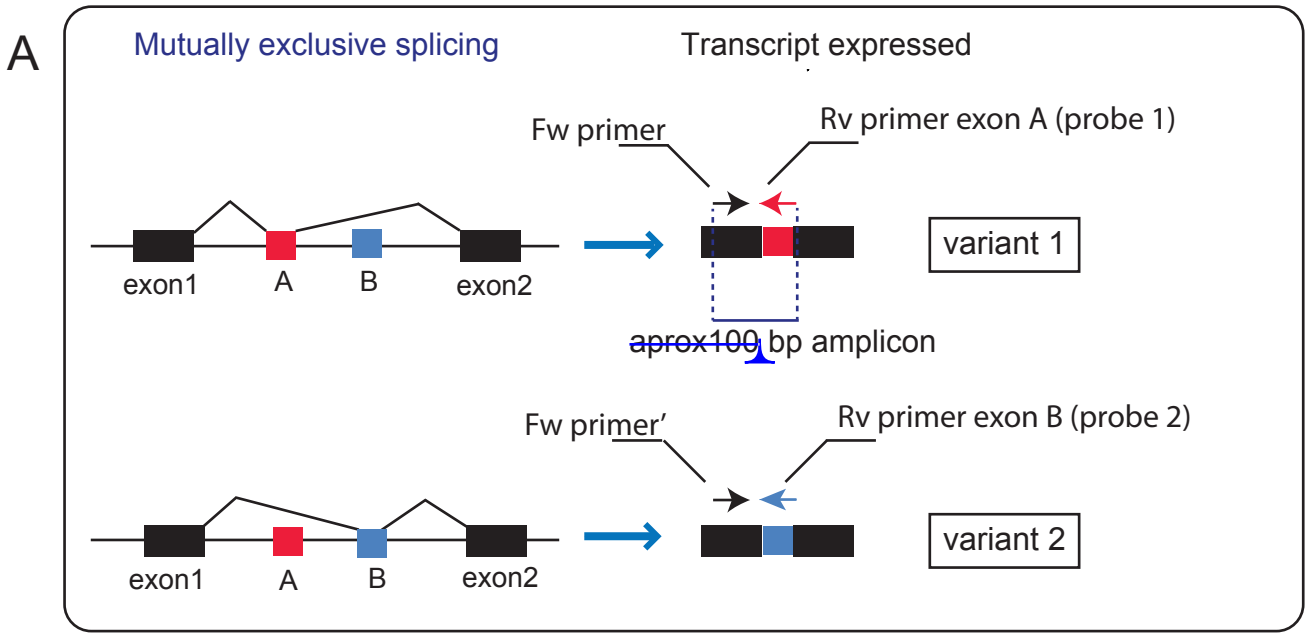

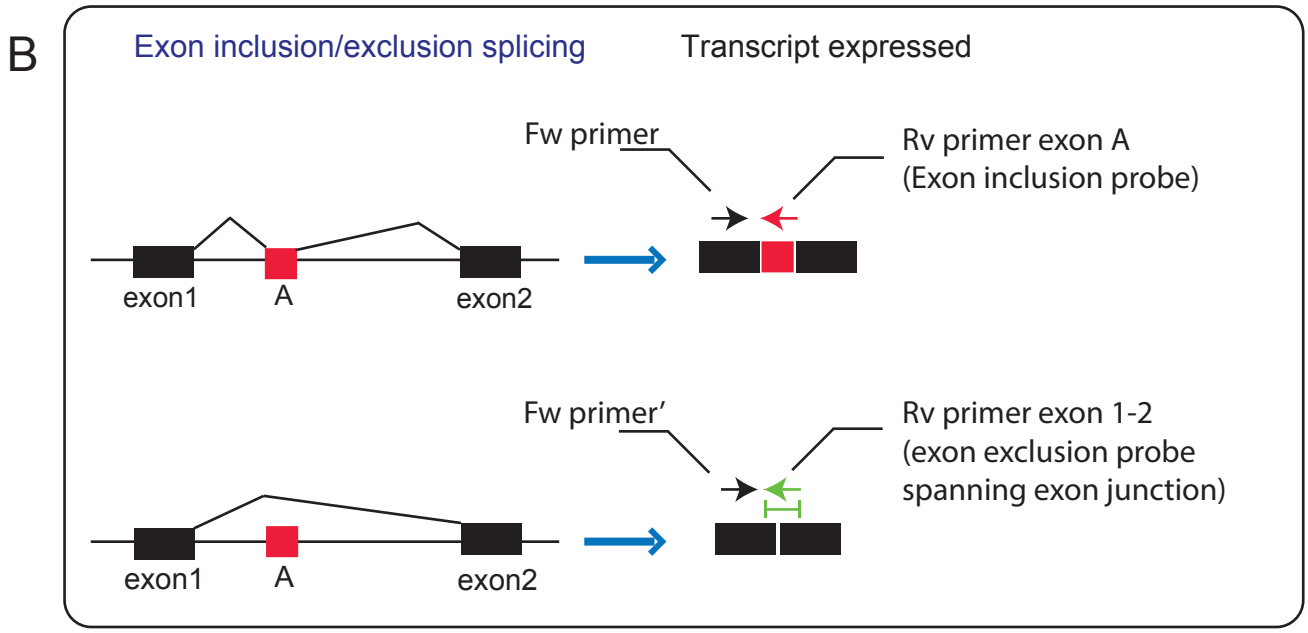

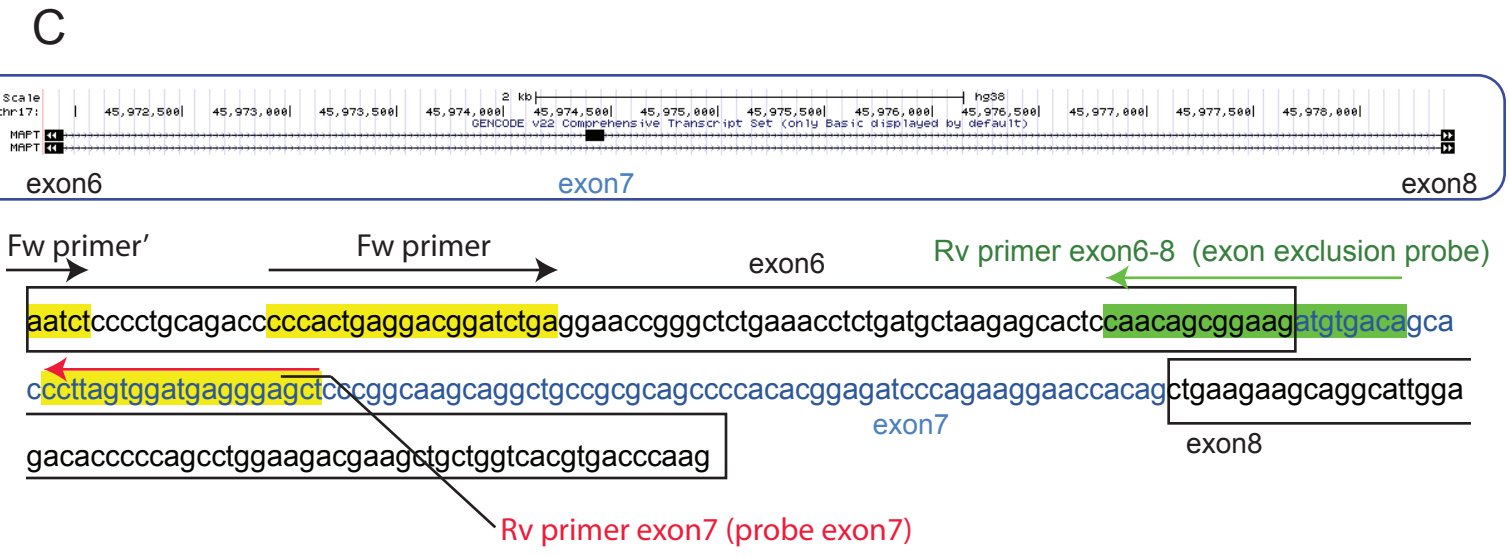

Figure 2

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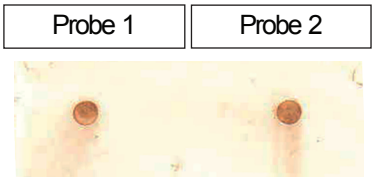

Figure 3

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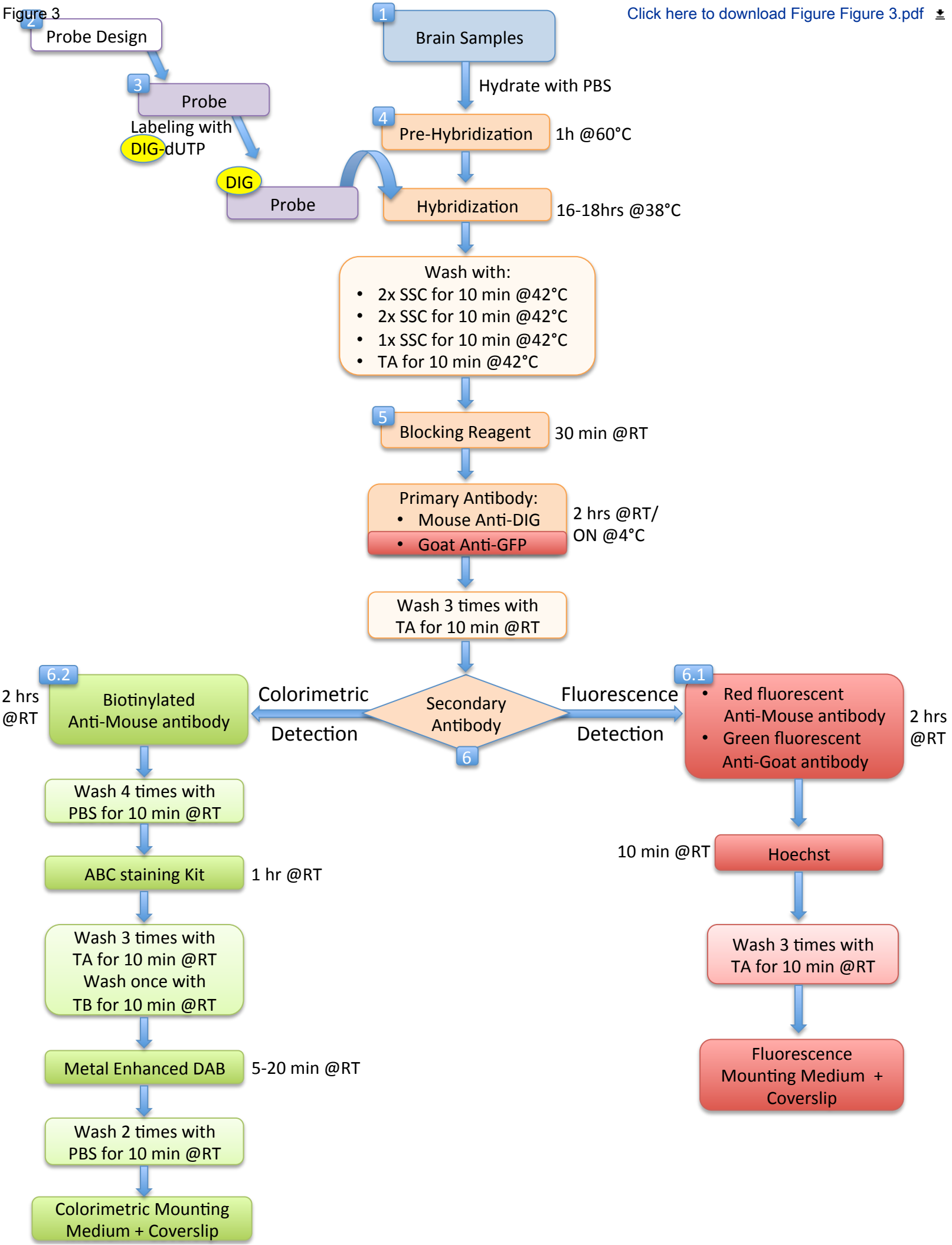

Figure 4

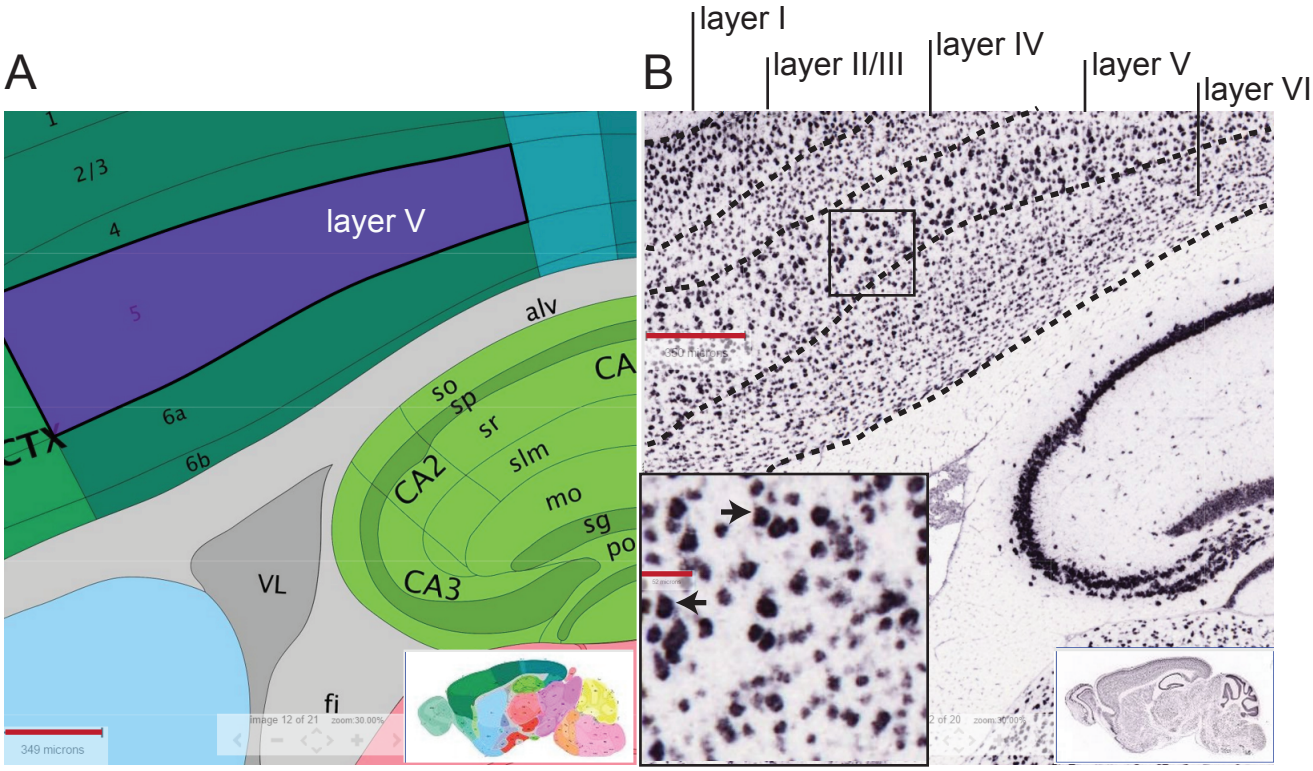

Figure 5

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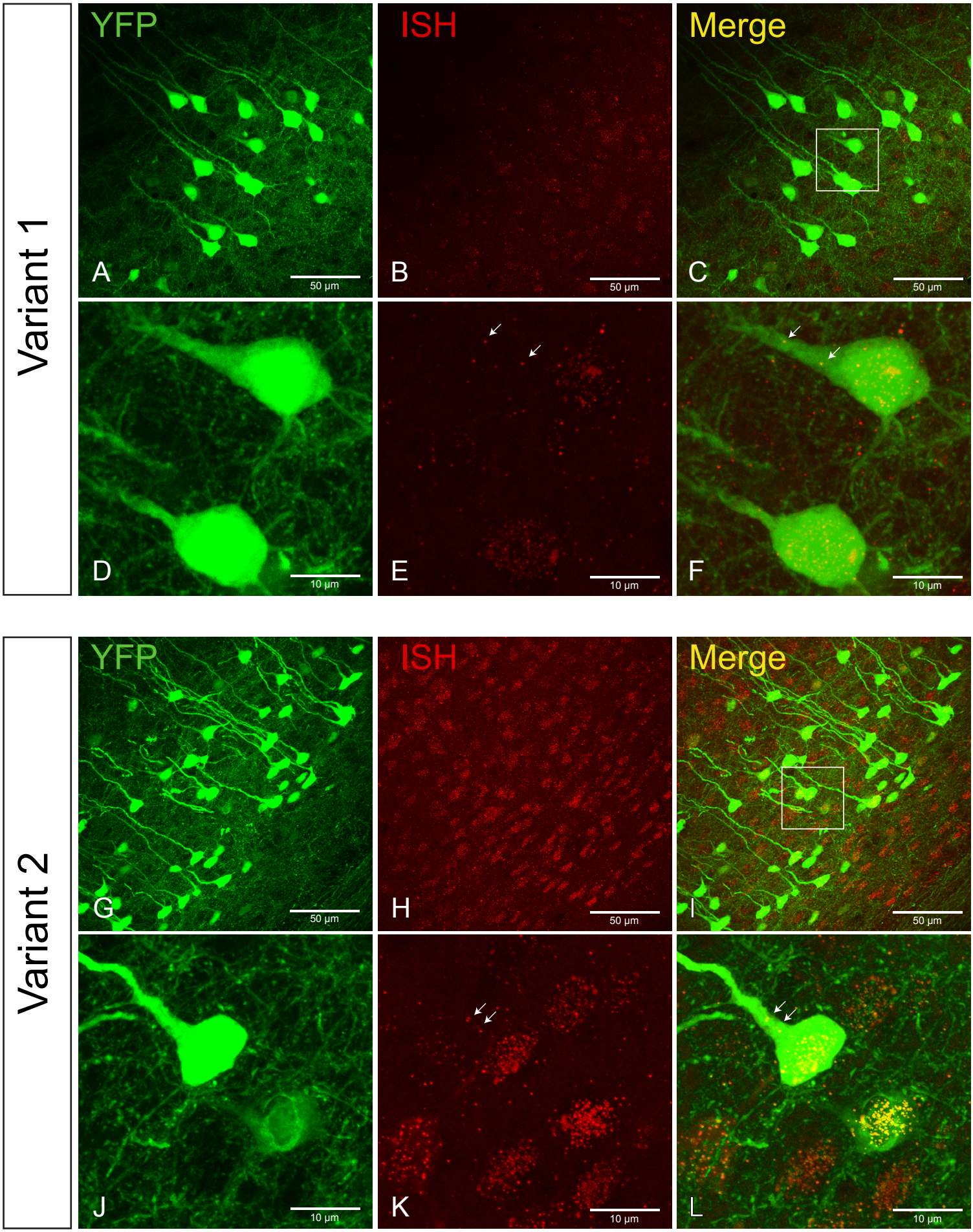

Table 1. Buffers Composition

Ingredient	Concentration	pH
20x SSC (stock)*	3 M NaCl 300 mM Sodium Citrate	7.0
2x SSC**	0,3 M NaCl 30 mM Sodium Citrate pH 7.0	7.0
1x SSC**	0,15 M NaCl 15 mM Sodium Citrate pH 7.0	7.0
10x PBS (stock)*	1,37 M NaCl 119 mM Phosphates 27 mM KCl	7.4
1x PBS**	137 mM NaCl 11,9 mM Phosphates 2.7 mM KCl	7.4
50x Denhardt's reagent*	1% Ficoll 1% Polyvinylpyrrolidone 1% Bovine serum albumin	5-7
TA buffer***	0.1 M Tris 1 M NaCl 50 mM MgCl2	7.5
TB buffer***	0.1 M Tris 0.5 M NaCl 50 mM MgCl2	9.5
5x Buffer***	0.3 M Tris 20 mM EDTA 3 M NaCl	7.5

Table 2. Labeling of the probe with DIG.

Component	**Volume**
100 µM primer stock (probe)	2 µL
Water	8 µL
TdT 5X buffer*	4 µL
25 mM $CoCL_2$*	4 µL
1 mM DIG-dUTP	1 µL
10 mM dATP	1 µL
Terminal Transferase*	1 µL (400 U)
Total volume	21 µL

Table 3. Pre-Hybridization buffer

Ingredient	**Volume**
20x SSC	200 µL
50x Denhardt's reagent	40 µL
Water	760 µL
Total volume	1000 µL

Table 4. Hybridization buffer

Ingredient	**Volume**
Formamide	500 µL
100 mM DTT	10 µL
5x Buffer*	200 µL
50% Dextran Sulfate	100 µL
Water	180 µL
2 pmol/µL Labeled Probe**	10 µL
Total Volume	1000 µL

Table 5. ABC Solution

Ingredient	**Volume**
Reagent A* (specially-purified Avidin)	10 µL
Reagent B* (Biotinylated Peroxidase)	10 µL
10% Triton X-100	30 µL
1x PBS	950 µL
Total volume	1000 µL

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment Reagent Table.xlsx

Name Used

Green fluorescent antibody
Red fluorescent antibody

Colorimetric mounting medium

Gelatin

Nuclear stain

Antifade mounting medium

Embedding matrix

Permeabilization/blocking reagent

Name Used

6 well plate
24 well plate
Cryostat
Confocal microscope
Coverslip
Digital camera coupled to Microscope
Dot blot apparatus
Mouse YFP-H
Plastic slide mailers
Primer 3
Optical Microscope
Slides
Slide Warmer
Tissue capture pen
Vacutainer blood collection set

Name Reagent	Concentration
2-methylbutane	
ABC Staining Kit	
Alexa Fluor 488 anti-goat antibody	
Alexa Fluor 568 anti-mouse antibody	
Biotinylated anti-mouse antibody	
Clear Mount mounting media	
$CoCl_2$	25 mM
Denhardt's Reagent	50 x
Deoxyadenosine triphosphate (dATP)	10 mM
Dextran Sulfate, sodium salt	
Digoxigenin-11-dUTP (DIG-dUTP)	1 mM
Dithiothreitol (DTT)	
Ethanol, anhydrous	
Ethylenediaminetetraacetic acid (EDTA)	0,5 M
Formamide	
Gelatin from porcine skin,Type A	
Goat anti-GFP antibody	
Hoechst 33342	
Horseradish Peroxidase (HRP) conjugated anti-mouse antibody	0.8 mg/mL
Isoflurane	
LiCl	
Metal Enhanced DAB Substrate kit	10 x
$MgCl_2$	1 M
Mouse anti-Digoxin unconjugated antibody	1.3 mg/mL
NaCl	5 M
Paraformaldehyde aqueous solution (PFA)	16%
Phosphate buffered saline (PBS)	10 x
ProLong Diamond Antifade	
Saline-Sodium Citrate (SSC)	20 x
Stable peroxidase buffer	1 x
Sucrose	
TdT 5X buffer	
Terminal transferase enzyme	400 U/µL
Tissue-Tek O.C.T. Compound	
Tris-HCl pH 7.5	1 M
Triton X-100	
Tyramide Signal Amplification (TSA) blocking buffer	
Water, molecular biology grade	

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We use #1.5 for confocal microscopy.
as/DXM-1200C/
link to the instruction manual explaining the Dot Blot protocol: http://www.bio-rad.com/webroot/web

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