Tyramide Signal Amplification coupled with multiple immunolabeling and RNAscope *in situ* hybridization in formaldehyde-fixed paraffin-embedded human fetal brain.

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Abstract

Several strategies have been recently introduced to improve the practicality of multiple immunolabelling and RNA *in situ* hybridization methods. We present a modified hybrid protocol of recently described complex detection strategies: (1) elution of antibodies prior to second round of staining (2) use of integrated polymers of HRP with secondary antibodies, and (3) tyramide signal amplification of multiple immunofluorescence labelling, to achieve a high sensitivity sequential multiple labeling using antibodies from the same species. A modified protocol of the novel RNAscope *in situ* hybridization method, including coupling with immunofluorescence on sections of early human fetal brain, has also been developed. These two techniques, when properly optimized, were highly compatible with routine formaldehyde-fixed paraffin-embedded tissue that preserves the best morphological characteristics of delicate fetal brain samples, allowing high power signal amplification for detection of protein and mRNA of genes that are sparsely expressed in the human fetal telencephalon.

Introduction

Immunohistochemistry (IHC) and RNA *in situ* hybridization (ISH) are very important techniques in the field of developmental neurobiology that allow us to assess expression of protein and mRNA for a wide variety of transcription factors and discrete signaling molecules (e.g. SHH and FGF). However, the technical complexity, insufficient sensitivity and specificity of the routine application of these two methods make their use very limited for some histological analyses, particularly post-mortem human embryonic and fetal tissue.

The development of appropriate multiple immunofluorescence labelling methods is one of the main obstacles in applying IHC. The selection of the appropriate combination of primary antibodies becomes problematical when the primary antibodies are raised in the same host species, which leads to cross-reactivity of secondary antibodies with each of the primary antibodies. Considering most of the available commercial primary antibodies are raised either in mouse or rabbit, matching the appropriate combination of primary antibodies from different host species becomes difficult and restrictive (Buchwalow et al. 2018). A second general problem is the insufficient sensitivity of primary antibodies to target antigens that present in small or barely detectable amounts, leading to poor signal visualization for these antigens (Van der Loos 2007; Warford et al. 2014). However, relatively novel detection strategies have been recently introduced to improve the practicality of multiple immunolabelling methods and circumvent some of these technical obstacles. Some of these strategies involve elution of antibodies between rounds of staining, using HRP polymer conjugated secondary antibodies and tyramide signal amplification (for more detailed information on the principle and history of these methods see Shan-Rong et al. 1999; Stack et al. 2014; Goto et al. 2015; Shojaeian et al. 2018).

Unlike other RNA analysis techniques (e.g., RT-PCR), *in situ* analysis of RNA biomarkers allows spatial descriptive analysis of RNA expression in the developing fetal brain and is important for verifying the results of single cell RNA transcriptomics. For example, investigating the graded and compartmented

expression of genes (transcription factors, morphogens, and receptors) in developing cerebral cortex permits us to understand how the dorsal pallium is stereotypically divided into functionally distinct areas even at the earliest stages (Alzu'bi et al. 2017; Clowry et al. 2018; Molnár et al. 2019). However, a long-standing criticism of routine RNA ISH techniques is the lack of sensitivity and specificity, especially for genes expressed at very low levels (e.g., genes for morphogens and their receptors); therefore, use of these techniques in the field of developmental neurobiology was challenging. Recently, Advanced Cell Diagnostics, Inc., (Hayward, California, USA) presented a novel RNA ISH technology (RNAscope *in situ* hybridization) that implement a unique strategy of probe formulation which ensured both signal amplification and background suppression (Wang et al. 2012).

This study presents detailed modified protocols of these recently introduced strategies for multiple immunolabeling and RNA *in situ* hybridization in formalin-fixed paraffin-embedded human fetal brain. We implemented a hybrid protocol of complex detection strategies for multiple immunofluorescence labelling. In addition, a modified protocol of RNAscope *in situ* hybridization technique, including coupling with immunofluorescence on sections of early human fetal brain is also presented.

Methods And Materials

Human fetal brains and ethical approval

Human fetal tissue from terminated pregnancies was obtained from the joint MRC/Wellcome Trust-funded Human Developmental Biology Resource (HDBR, <http://www.hdbbr.org>; Gerrelli et al. 2015) based in Newcastle University and the Institute of Child Health University College London. All tissue was collected with appropriate maternal consent and approval from the Newcastle and North Tyneside NHS Health Authority Research Ethics Committee (REC reference 18/NE/0290) and the London-Fulham NHS Health Authority Research Ethics Committee (Rec Reference 18/LO/0822). Both centres are licensed by the UK Human Tissue Authority (license numbers 12534 and 1220). Fetal samples from 8, 10, 12, and 19 post-conceptual weeks (PCW) were used in this study. Ages were estimated from foot, and heel to knee length measurements according to Hern (1984).

Tissue processing and sectioning

Fetal brains were rapidly isolated and fixed for at least 24 hours at 4°C in 4% paraformaldehyde (Sigma Aldrich, Poole, UK) dissolved in 0.1 M phosphate-buffered saline (PBS). The exact post-mortem interval to fixation is unknown but is in the range of 1-12 hours. After fixation, whole or half brains were dehydrated in graded ethanol (70% for 15 min, 100% for 45 min, 2 x 100% for 60 min) at room temperature (RT). The fixed brains were dissected into blocks of approximately equal size, with the number depending on the size of the brain. Blocks were then incubated in xylene for 2 hours before embedding in paraffin (Shandon Pathcentre Tissue Processor, Thermo Scientific, Epsom, UK). Brain tissue blocks were cut into 8 µm thick sections (Leica RM 2235 microtome) mounted onto Superfrost+

slides (Thermo Fisher Scientific, Cramlington, UK) and used for immunostaining and RNAscope *in situ* hybridization.

Multiple immunofluorescence: first round (For 1st primary antibody)

Paraffin sections were first dewaxed in xylene (2 x 5 min) and then rehydrated via four changes of graded ethanol (100%, 100%, 95%, and 70%). Endogenous peroxidase activity was blocked by treatment with methanol peroxide (3ml of 30% hydrogen peroxide + 180 ml methanol) for 10 minutes. For antigen retrieval, sections were rinsed under tap water and boiled in a microwave in 10mM citrate buffer pH6 for 10 minutes. Sections were then incubated with the appropriate normal 10% blocking serum (species in which secondary antibody was raised) in Tris-buffered saline pH 7.4 (TBS) for 10 minutes at RT before incubation with the primary antibody (diluted in 10% normal blocking serum) overnight at 4°C. Details of all the primary antibodies used in this study can be found in **Table 1**. Then, sections were washed 2 x 5 minutes in TBS and incubated with HRP polymer-conjugated secondary antibody for 30 minutes (ImmPRESS™ HRP IgG [Peroxidase] Polymer Detection Kit, Vector Labs, Peterborough, UK) at RT, washed 2 x 5 minutes in TBS and incubated in the dark for 10 minutes at RT with fluorescein tyramide diluted at 1/500 in 1X Amplification buffer (Tyramide Signal Amplification (TSA™) fluorescein plus system reagent, Perkin Elmer, Buckingham, UK). Tyramide reacts with HRP to leave fluorescent tags covalently bound to the section.

Second round (for 2nd primary antibody)

Prior to starting the second round of staining, sections were first washed in TBS (2 x 5 minutes) and boiled in 10mM citrate buffer (in microwave for 10 minutes) to remove all antibodies and unbound fluorescein from the first round. Sections were then incubated in 10% normal serum before incubating with the second primary antibody (Table 1) for 2 hours at RT. Following washing (2 x 5 minutes in TBS), sections were again incubated with ready to use HRP-conjugated secondary antibody for 30 minutes at RT, washed 2 x 5 minutes in TBS, then incubated in the dark for 10 minutes at RT with CY3 tyramide diluted at 1/500 in 1X Amplification buffer (Tyramide Signal Amplification [TSA™] CY3 plus system reagent, Perkin Elmer). The same steps can be repeated for a third round of staining (not reported here but see Alzu'bi et al 2017a) using CY5 Tyramide (Tyramide Signal Amplification (TSA™) CY5 plus system reagent, Perkin Elmer). Sections were washed (in three changes of TBS for 5 minutes each) before applying 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Thermo Fisher Scientific, Cramlington, UK) and mounted using Vectashield Hardset Mounting Medium (Vector Labs).

RNAscope *in situ* hybridization

RNA *in situ* hybridization was performed using the RNAscope Reagent Kit (ACD Bio Techne, Abingdon, United Kingdom). The routine RNAscope *in situ* hybridization protocol includes four main steps: sections pre-treatment, probe hybridization, signal amplification, and signal detection. Each sample was quality controlled for RNA integrity with a probe specific to the housekeeping gene *GAPDH*. Negative control background staining was evaluated using a probe specific to the bacterial *DapB* gene.

Sections Pre-treatment

Paraffin sections were first baked on a heating pad for 10 minutes at 60⁰C, dewaxed in Xylene (2 x 5 minutes), then incubated in 100% ethanol (2 x 1 minute) and air dried for 5-10 minutes at room temperature (RT). Sections were then covered with drops of 30% hydrogen peroxide solution for 10 minutes at room temperature and washed in distilled water by moving the slides rack up and down 3–5 times. For target retrieval, sections were boiled with the manufacturer's target retrieval buffer (ACD Biotechne) for 20 minutes at 90-100⁰C using a steamer. Individual tissue sections were isolated on slides using a hydrophobic barrier pen. To increase target accessibility, protease digestion was then carried out by incubating sections with protease plus solution (ACD Biotechne) at 40⁰C for 30 minutes (any oven or incubator maintain a temperature between 37 – 40°C is also suitable). Sections were finally washed with distilled water before proceeding to probe hybridization.

Probe Hybridization and Signal Amplification

Sections were incubated with the target gene's probe for 120 minutes at 40⁰C. Details of all the probes used in this study are found in **Table 2**. The slides were washed 2 x 2 minutes in 1X wash buffer at RT, and the hybridized signals were then amplified by six consecutive signal amplification steps using six different solutions provided by the manufacturer (Hybridize Amp 1-6, ACD Biotechne). In detail, sections were incubated with Hybridize Amp 1 for 30 minutes at 40⁰C, Hybridize Amp 2 for 15 minutes at 40⁰C, Hybridize Amp 3 for 30 minutes at 40⁰C, Hybridize Amp 4 for 15 minutes at 40⁰C, Hybridize Amp 5 for 30 minutes at RT, Hybridize Amp 6 for 15 minutes at RT. Sections were washed 2 x 2 minutes in 1X wash buffer (ACD Biotechne) at RT after each signal amplification step.

Signal detection

For signal detection, sections were incubated for 10 minutes at RT in red solution (RNAscope 2.5 HD Assay – RED, ACD Biotechne) freshly prepared following the manufacturer instructions (mixing 1 volume of RED-B to 60 volumes of RED-A to make the total volume needed to cover the sections). Slides were then rinsed in distilled water for 2 minutes, counterstained with 10-20% hematoxylin (using a higher concentration of hematoxylin can obscure any positive signal). Slide should be dried for 10-15 minutes in 60⁰C dry oven and mounted using DPX (Sigma-Aldrich). Positive signals are indicated by red chromogenic dots in the cytoplasm or nucleus (Figures 3 and 4).

RNAscope fluorescent in-situ hybridization coupled with immunofluorescence

To avoid RNA degradation during the immunofluorescence steps, *in situ* hybridization was carried out first using RNAscope Multiplex Fluorescent Reagent Kit v2 Assay (ACD). Sections pre-treatment and probe hybridization steps were performed as described above. Unlike RNAscope chromogenic assay, the signal amplification in RNAscope fluorescent multiplex v2 assay only included 3 steps: AMP1 for 30 minutes at 40⁰C, AMP2 for 30 minutes at 40⁰C and AMP3 for 15 minutes at 40⁰C. The sections were then

incubated with C1-HRP (as the probe, *ZIC4* used in this case, was a channel 1 probe) at 40°C for 15 minutes and washed 2 x 2 minutes in 1X wash buffer at RT. The hybridized signals were detected by incubating the sections with Cy3 diluted in 1X amplification buffer (1/500, Tyramide Signal Amplification Cy3 plus system reagent, Perkin Elmer) at 40°C for 30 minutes. Sections were then washed and incubated with HRP blocker for 15 minutes at 40°C before proceeding to the immunofluorescence.

For immunofluorescence, sections were first boiled in 10 mM citrate buffer pH6 (in microwave for 10 minutes) to remove unbound tyramide from the first round (RNAscope fluorescent *in situ* hybridization), and incubated in 10% normal serum before incubating with the primary antibody of the target antigen for 2 hours at RT. Sections were then washed 2 x 5 minutes in TBS and incubated with HRP polymer-conjugated secondary antibody for 30 minutes at RT. Signals were detected by incubating the sections with fluorescein tyramide diluted at 1/500 in 1X Amplification buffer (Tyramide Signal Amplification fluorescein plus system reagent, Perkin Elmer) in dark for 10 minutes. Sections were then washed (2 x 5 min) before applying 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Thermo Fisher Scientific) and mounted using Vectashield Hardset Mounting Medium (Vector Labs).

Image acquisition

RNAscope *in situ* Hybridization Images were captured using Leica SCN400 Slide Scanner. Fluorescent images were obtained with a Zeiss Axioimager Z2 apotome. Processing of images, which included only adjustment of brightness and sharpness, was achieved using the Adobe Photoshop CS6 software.

Results And Discussion

Multiple Immunofluorescence

In Figures 1 and 2, we present examples of applying combination of three complex detection strategies in multiple immunofluorescence labelling: elution of antibodies between rounds of immunostaining, HRP polymer-conjugated secondary antibodies, and tyramide signal amplification on sections from 8, 10, and 19 PCW brains. Compartmentalization and cellular identities of different parts of the fetal brain, identified by the expressions of specific antigens, were clearly visualized. Various combinations of primary antibodies from the same and different host species (mouse/ mouse, rat/rat, and mouse/goat) that detect either nuclear or cytoplasmic antigens were used here. A polymer-enhanced detection system (ImmPRESS™ HRP IgG [Peroxidase] Polymer Detection Kit, Vector Labs) that contains HRP-conjugated secondary antibody was applied. For signal detection, the TSA method was employed from Perkin Elmer according to the manufacturer's instructions. However, we further diluted the fluorescein tyramide in 1X Amplification buffer to 1/500 dilution instead of the recommended 1/50; this step was found to be critical for achieving a good balance of signal amplification and background suppression. Elution of the bound primary and secondary antibodies after the first round of staining was achieved by boiling the sections with the same buffer used for antigen retrieval (10 mM citrate buffer, pH 6.0) in microwave for 10 min. The heat treatment using the citrate buffer did not significantly reduce fluorescent signal from the

first round, confirming that HRP-activated tyramide binds covalently and efficiently to electron-rich amino acids of proteins at the site of the immunoreaction making it resistant to the citrate treatment (Bobrow et al. 1989; Hasui et al. 2005; Shojaeian et al. 2018).

The use of these three strategies represents a significant improvement in multiple immunofluorescence labelling, profoundly improving the practicality of this method by giving bright images of antigen labelling we previously struggled to detect using fluorescently tagged secondary antibodies. The development of compact HRP polymer-conjugated secondary antibodies, to introduce large number of peroxidase molecules, has remarkably enhanced the detection sensitivity of this method (Shan-Rong et al. 1999; Shojaeian et al. 2018). This system also significantly simplified the staining procedures; it is ready to use and provides faster staining steps compared with using biotinylated secondary antibodies followed by treatment with avidin-biotin complex (ABC). Additionally, this system lowers the cost by permitting further dilution of expensive primary antibodies.

The intermediary antibodies elution method in combination with TSA methods provided a remedy for one of the major problems in using multiple immunofluorescence labelling. These methods enable the researcher to choose the primary antibodies for the target antigen regardless of their host species and without the fear of cross-reactivity (Pirici et al. 2009; Zhang et al. 2017). In addition, TSA offers sensitivity 10–200 times that of standard IHC, which enhances the signal visualization of the target antigens (Shojaeian et al. 2018) even if they are expressed in barely detectable amounts. The commercially available TSA kits provide enough reagents for staining 50–150 slides, using 1/500 dilution and not 1/50 dilution as recommended, of fluorescein tyramide in 1X Amplification buffer. This allows us to use the kit for large number of slides, considering that the 1X Amplification buffer can be replaced in the lab with a solution of tris buffered saline pH=7.4 containing 0.003% hydrogen peroxide.

RNAscope

Figure 3 A-C illustrates employing RNAscope RNA ISH for detection of expression of three nicotinic acetylcholine (ACh) receptor subunit genes in the cortical plate of a 12 PCW brain. These receptors were expressed in relatively different levels from high for *CHRNA4*, to moderate for *CHRNA5*, and low for *CHRNA7*. This was correlated with expression observed by RNAseq of age-matched samples from human fetal brain (Alzu'bi et al. 2020). Using previously described methods of probe manufacture and non-radioactive tissue *in situ* hybridization histology (Bayatti et al. 2008) we had been able to observe approximately similar patterns of expression, but the staining had been more diffuse, with higher background and was strictly a qualitative observation (Fig. 3F-K). RNAscope detection provides a punctate staining with low background that permits a quantitative analysis of expression levels (Chermahini et al. 2019).

Figure 4 illustrates the chromogenic RNA detection of mRNA for the diffusible morphogen Sonic Hedgehog (*SHH*), important in patterning the developing fetal forebrain (Kiecker and Lumsden, 2004; Bardet et al, 2010; Alvarez-Bolado et al, 2012). Very discrete expression in signaling centres, predicted from observations of other vertebrate brains, was observed.

In Figure 5, we present two examples of coupling RNAscope fluorescent in-situ hybridization with immunofluorescence. These RNA *in situ* hybridization experiments were performed according to the manufacturer's instructions (Wang et al. 2012) with a slight modification described in the methods. Expression of mRNA for the transcription factor *ZIC4*, for which there is currently no antibody available, is compared with immunofluorescence for transcription factors PAX6 (Fig. 5A) and FOXP2 (Fig 5B). PAX6 and *ZIC4* are largely expressed in different locations, whereas FOXP2 and *ZIC4* show extensive colocalisation in the thalamus, in particular (Fig 5C).

RNAscope represents a valuable addition to RNA ISH methodology in the field of developmental neurobiology. This technique has many advantages- it is highly compatible with routine formalin-fixed, paraffin-embedded fetal brains; sensitive enough, with remarkable background suppression, to allow detection of genes that are expressed at low levels like receptors and morphogens. Finally, it is a time saving method that can be performed in one day compared with routine RNA ISH which is time consuming and labor intensive (3-4 day protocol).

However, there is one drawback for RNAscope technology. the reagents and technology for making the probes are copyrighted and are expensive to purchase. However, the apparently guaranteed success of the method so far, in our hands, saves us a lot of time and money when considering the high failure rates we have encountered in using conventional methods. In addition, as described above, most of the incubation steps for protease digestion, probe hybridization, and signal amplification are required to be performed under strictly controlled conditions of temperature and humidity in a special oven sold by the manufacturer. Although we have found the oven easy to use and to give excellent results, any oven or incubator that can maintain a temperature between 37 – 40⁰C was found to work perfectly well.

Abbreviations

CHRNA, cholinergic nicotinic receptor; *DapB*, bacterial gene for dihydrodipicolinate reductase; FGF, Fibroblast growth factor; FOXP2, Forkhead box protein P2; GAD67, glutamate decarboxylase 67 kilodalton isoform; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; OTX2, orthodenticle homeobox 2; PAX6, paired box protein 6; ROBO1, roundabout homologue 1; RNA, ribonucleic acid; RT-PCR, real time polymerase chain reaction; SCGN, secretagogin; SHH, sonic hedgehog; *ZIC4*, zinc finger protein of the cerebellum 4.

Declarations

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hybridization histology. AA was funded by a grant from the Deanship of Scientific Research, Yarmouk University, Jordan.

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Tables

Table 1: Primary antibodies used in this study

Primary antibody to	Species	Dilution	Supplier	RRID number (where available)
FOXP2	Mouse monoclonal	1/50	Santa Cruz, Heidelberg, Germany.	AB_2721204
OTX2	Mouse monoclonal	1/200	Santa Cruz.	Catalogue No. SC-514195
ROBO1	Rabbit polyclonal	1/1500	Abcam, Cambridge, UK	AB_449561
SCGN	Rabbit polyclonal	1/500	Sigma-Aldrich, Poole, UK.	AB_1079874
DLX2	Mouse monoclonal	1/200	Santa Cruz.	Catalogue No. SC-393879
GAD67	Mouse polyclonal	1/1000	Merck Millipore, Watford, UK	AB_2278725
PAX6	Rabbit polyclonal	1/500	Cambridge Bioscience, Cambridge, UK.	AB_2565003

Table 2: RNAscope probes used in this study

Probe	Catalogue number
Hs-CHRNA4	498331
Hs-CHRNA5	482401
Hs-CHRNA7	310101
Hs-CHRNA2	498351
Hs-SHH	600951
Hs-ZIC4	525661
Hs-GAPDH	310321
Negative Control Probe- DapB	310043

Figures

Alzu'bi et al Figure 1

Figure 1

Double immunofluorescence labeling in formaldehyde-fixed paraffin-embedded human 8 and 10 PCW fetal brain sections. (A) Double labelling with two antibodies originating from the same host species (mouse): FOXP2 (Fluorescein, green) and OTX2 (Cy3, red). The two transcription factors show largely complementary expression in the ganglionic eminences (GE) and thalamus (Th) although with some overlap (yellow) in the dorsal thalamic subventricular zone (B) Double labelling with two antibodies originating from rat; anti-SCGN (Fluorescein, green) and anti-ROBO1 (Cy3, red) adapted from Alzu'bi et al (2019) under a creative commons licence. The two cytoplasmic markers show extensive colocalisation in thalamic neurons and their afferents in the internal capsule (IC) and cortical subplate and Intermediate zone (IZ, yellow). Nuclei counterstained with DAPI (blue). ChP, choroid plexus. Scale bars = 1mm

Alzu'bi et al Figure 2

Figure 2

Double labelling with two antibodies originating from the same host species (mouse): GAD67 (Fluorescein, green) and DLX2 (Cy3, red) in the cortical plate of 19 PCW human fetal brain. Colocalisation of the cytoplasmic enzyme GAD67 with the nuclear transcription factor DLX2 in GABAergic neurons is clearly demonstrated. Nuclei counterstained with DAPI (blue). Adapted from Alzu'bi and Clowry (2019) under a creative commons licence. Scale bar = 200 μ m

12 PCW RNA Scope

10 PCW Conventional in situ

Alzu'bi et al Figure 3

Figure 3

(A-C) Chromogenic RNAScope in situ hybridization for nAChR subunits mRNA in the cortical plate of 12 PCW fetal brain. CHRNA4 was relatively highly expressed compared with moderate expression for CHRNA5 and low expression for CHRNA7. (D) Very strong expression was detected for the positive control housekeeping gene GAPDH. (E) No expression was detected for the negative control dapB gene. F-K show results from conventional in situ hybridization. Comparison of sense/anti-sense staining (F, G)

suggests expression of CHRNA4, particularly in the CP and VZ but it would not be easy to localize expression to individual cells as staining is diffuse. A similar result was obtained for CHRNA7 (J, K) whereas for CHRNA5, antisense stained more strongly than sense suggesting the experiment has failed or is difficult to interpret. MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. A-C adapted from Alzu'bi et al (2020) under a creative commons licence. Scale bars = 100 μ m

8 PCW

SHH

Alzu'bi et al Figure 4

Figure 4

(A) Chromogenic RNAScope in situ hybridization for SHH mRNA in sagittal section of 8 PCW fetal brain. SHH expression was detected in ganglionic eminences (GE) and preoptic area (POA). Distinctive expression was also detected at the zona limitans intrathalamica (ZLI) a signaling center and a restrictive border between the thalamus and the prethalamus. (B) Coronal section of 8 PCW fetal brain counterstained with H&E, SHH was also highly expressed in population of cells in the globus pallidus (GP). Scale bars = A, 200 μ m; A inset, 50 μ m; B, 300 μ m; B inset, 100 μ m

Alzu'bi et al Figure 5

Figure 5

RNAscope fluorescent in situ hybridization coupled with Immunofluorescence on sections from 10 PCW human fetal brain. (A) Double labelling for ZIC4 mRNA (Cy3, red) and PAX6 protein (Fluorescein, green) showing largely complementary patterns of expression. (B) Double labelling for ZIC4 mRNA (Cy3, red) and FOXP2 protein (Fluorescein, green) showing colocalisation of expression in the thalamus (C, yellow). Scale bars = B (and for A) 2mm; C 300 μ m.