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Maria Rex · Richard Church · Kevin Tointon
Paul J. Scotting

Combination of non-isotopic in situ hybridisation with detection of enzyme activity, bromodeoxyuridine incorporation and immunohistochemical markers

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Abstract The advent of non-isotopic in situ hybridisation allows the possibility to detect the presence of both mRNAs and other markers in cells. We have established conditions for simultaneous analysis of gene expression and a variety of other immunohistochemical markers in tissue sections. We report the analysis of expression of a family of transcription factors (*Sox* genes) in combination with detection of: (1) protein antigens (using both monoclonal and polyclonal antibodies); (2) bromodeoxyuridine to mark cells which are proliferating; and (3) acetylcholinesterase activity. The approaches we describe, which demonstrate the compatibility of non-isotopic in situ hybridisation with a range of other treatments, should be generally applicable and open to many variations of probe, antibodies and colour detection systems.

Introduction

An important goal of many biologists is to establish which cells in a tissue or organ express their gene or protein of interest. In many cases it is also important to know whether the same cells express other specific markers. A variety of approaches are available to study such molecules but recent developments in the study of gene transcripts using non-radioactive in situ hybridisation (ISH) now enable detection of gene transcripts and proteins in the same sections and coexpression of multiple markers in single cells can therefore be studied.

Previous methods for simultaneous detection of gene transcripts and other histochemical markers in single tissue sections have relied on the use of histochemical techniques prior to radioactive ISH (Brahic and Ozden 1992; Lui and Salpeter 1994). In such experiments it was necessary to carry out the immunohistological analysis before ISH, since the radioactive ISH rendered the sample unsuitable for further studies. This approach has several drawbacks: one does not know the result of the ISH experiment before one carries out the immunohistochemistry; all reagents for the immunohistochemical analysis must be RNase-free to avoid degradation of the mRNA in the tissue; the radioactive ISH technique does not give very precise cellular resolution. Recent development of non-radioactive ISH methods now enable a variety of analyses to be carried out on the same tissue samples without the aforementioned difficulties. In particular, ISH can be carried out prior to immunohistochemical analyses.

We describe conditions under which sections can be stained to detect simultaneously: (1) gene expression (using ISH) and protein antigens (using immunohistochemical agents); (2) gene expression and proliferation [using immunohistochemical detection of bromodeoxyuridine (BrdU) incorporation]; and (3) gene expression and enyzme activity [in this case we have assayed acetylcholinesterase (AChE)]. These methods should be applicable to many different molecular markers with only minor modifications. Thus we show that one can now detect all of the above molecular components of the cell and ask questions concerning the molecular status of a cell expressing a particular gene.

Materials and methods

Fixation and sectioning

Tissue preparation

Chick embryo tissue was dissected and immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for several hours at room temperature or overnight at 4° C.

Frozen sections

Following fixation, the tissue was submerged in OCT compound (Miles) and snap frozen in iso-pentane cooled in liquid nitrogen

M. Rex · K. Tointon · P.J. Scotting (✉)
Department of Biochemistry,
University of Nottingham Medical School,
Queen's Medical Centre, Nottingham NG7 2UH, UK
Tel. +44 115 9709367; Fax: +44 115 9422225
e-mail paul.scotting @nottingham.ac.uk

R. Church
Department of Pathology,
University of Nottingham Medical School,
Queen's Medical Centre, Nottingham NG7 2UH, UK

onto a chuck for frozen sectioning. Tissue was allowed to equilibrate to approximately −25° C in the cryostat. Sections were cut at a thickness setting of 5 µm and picked up on silane (Sigma)-coated slides. The sections were then air dried for 20 min and stored frozen at −20° C before staining.

Wax sections

Following fixation, the tissue was soaked in two 30-min changes of saline at 4° C and then dehydrated through increasing concentrations of alcohol at room temperature (except where stated) for at least 30 min as follows: 1:1 saline:ethanol mix at 4° C; 70% ethanol twice; 85% ethanol; 95% ethanol and 100% ethanol twice. The tissue was then given two 30-min incubations in Histoclear (CellPath), followed by 20 min in 1:1 Histoclear: paraffin wax and three 20-min changes of wax at 60° C. Ribbons of 6 µm were cut on a microtome, collected on silane-coated slides and dried at 37° C overnight.

In situ hybridisation (ISH)

Probe preparation

RNA riboprobes were synthesised according to the method recommended by the suppliers of digoxigenin (DIG)-labelled nucleotides (Boehringer Mannheim). Approximately 1 µg of plasmid DNA (*cSox2*, *cSox3* and *cSox11* clones as described in Uwanogho et al. 1995, *cSox21* clone described in Rex et al. submitted) was linearised by digestion with a single restriction enzyme and was then incubated with either T3 or T7 RNA polymerase to generate sense or antisense riboprobe. Probes were not hydrolysed and were between 0.5 and 1.5 kb in length.

Hybridisation

The method for ISH was generally as described by Uwanogho et al. (1995). All steps were carried out at room temperature except where specified. Wax-embedded slides were dewaxed in Histoclear and hydrated through decreasing concentrations of alcohol; frozen sections were used directly following the protocol for detection of AChE (as described below). This was followed by incubation for 5 min in PBT (PBS, 0.1% Tween 20) and the slides were then overlaid with approximately 100 µl of proteinase K (10 mg/ml prewarmed to 37° C) and left at room temperature for 15 min. Proteinase K was removed by washing with three changes of PBT and slides were then fixed with 4% paraformaldhyde in PBS for 20 min. The DIG-labelled riboprobes were diluted in hybridisation solution [50% formamide, 5×SSC (pH 4.5 with citric acid), 1% SDS, 50 mg/ml yeast tRNA, 50 mg/ml heparin (Sigma)]. The dilution factor varied for different probes (the optimum was established empirically, usually 50–300 ng of probe in 100 µl volume was used on each slide). Probes were heated to 80° C for 5 min and cooled on ice prior to addition to slides. Slides were incubated at 70° C overnight with a cover slip, in a humidified chamber.

Posthybridisation washing

Slides were washed twice in 50% formamide, 5×SSC (pH 4.5), 1% SDS, 30 min each at 65° C; 3 times in 50% formamide, 2×SSC, 30 min each 65° C; and 3 times in TBST (140 mM NaCl, 25 mM TRIS-HCl pH 7.5, 300 mM levamisole, 0.1% Tween 20), 5 min each at room temperature.

DIG detection

Slides were submerged in 1% blocking reagent for nucleic acid hybridisation (Boehringer Mannheim) in TBST for 30 min at room temperature. They were then submerged in a 1:5000 dilution of preabsorbed (as described by Strähle et al. 1994) sheep anti-digoxigenin–alkaline phosphatase Fab fragments (Boehringer Mannheim) and incubated at 4° C overnight. Slides were then washed in three changes of PBT (20 min per wash) followed by two 5-min changes of freshly made NTMT buffer (0.1 M TRIS-HCl pH 9.5, 50 mM $MgCl_2$, 0.1 M NaCl, 0.1% Tween 20, 2 mM levamisole). Alkaline phosphatase activity was detected by incubating in an X-phosphate: NBT mixture [100 ml NTMT plus 45 µl of 75 mg/ml NBT (4-nitro blue tetrazolium chloride in 70% dimethylformamide) and 35 µl of 50 mg/ml X-phosphate (5-bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide)]. Colour was allowed to develop in the dark at room temperature. The reaction was stopped by incubating in TE (10 mM TRIS-HCl pH 7.8, 10 mM EDTA).

Immunohistochemistry

Slides were processed for immunohistochemistry to detect other markers immediately following colour detection of the ISH signal. Slides were removed from TE but were not allowed to dry. All treatments were at room temperature. Slides were blocked with PBS plus 20% serum and then incubated with primary antibody/antiserum at usual dilutions [we used 1:50 anti-neurofilament monoclonal (Dako), 1:10 HNK-1 monoclonal (recognizes a carbohydrate epitope present on N-CAM and neural crest cells, gift from C. Stern), 1:50 anti-desmin polyclonal (Euro-diagnostica), 1:50 anti-BrdU monoclonal (Dako)] in PBS plus 5% serum for 1–2 h. Following a brief rinse with PBS, slides were incubated in biotinylated secondary antibody [sheep anti-mouse (Amersham), goat anti-rabbit (Vector Laboratories)] diluted 1:100 in PBS for 30 min. Following a brief rinse with PBS, slides were incubated in alkaline phosphatase streptavidin (Vector Laboratories) diluted 1:500 in PBS for 30 min. Following a brief rinse with PBS, slides were incubated in Fast Red (Sigma, prepared according to supplier's instructions) and colour was allowed to develop for 15–120 min. Sections were mounted in Mowiol (Calbiochem).

BrdU detection

Embryos were labelled with BrdU (Sigma) by injection with approximately 5–30 µg/g body weight. Embryos were then processed for ISH as described above and detection of BrdU was carried out subsequently. Before slides could be screened for BrdU they first had to be pretreated to allow the antibody access to the DNA label. Firstly, the sections were digested in 0.4% pepsin (Sigma) in 0.01 M HCl for 20 min at 37° C. Slides were then washed in tap water and placed into 2 N HCl at 37° C for a further 10 min. Slides were then washed again and placed into Scott's water (0.04 M $NaHCO_3$, 0.08 M $MgSO_4$) for 2 min. After a further wash in tap water the sections were ready for BrdU immunohistochemistry (as described above).

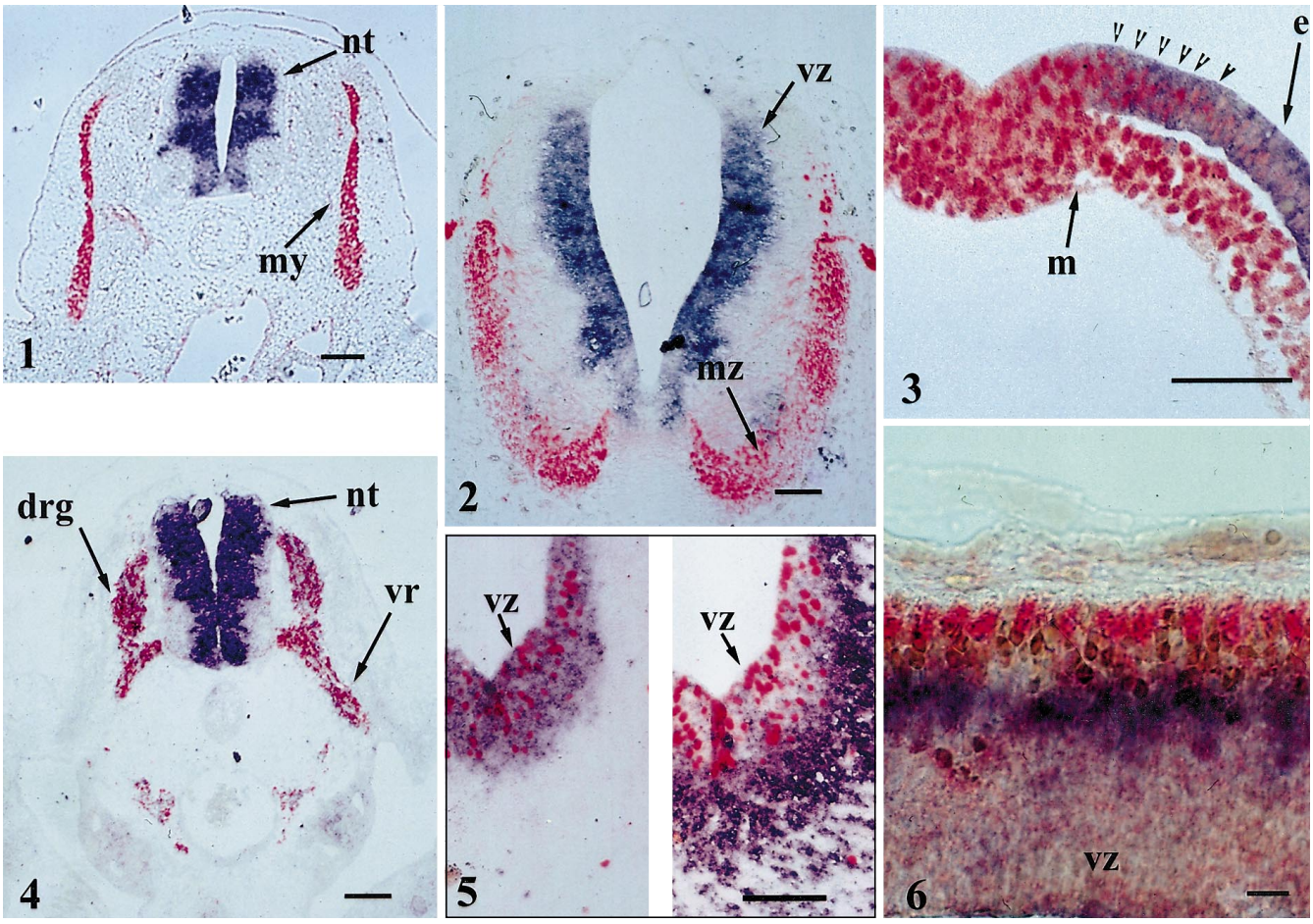
Enzyme histochemistry for AChE activity

Since this assay requires the enzyme in question to be active, this protocol was carried out on lightly fixed (4% paraformaldehyde, 4° C, 30 min) frozen tissue prior to the ISH protocol. Frozen sections of chick embryo were allowed to thaw and dry at room temperature. The sections were then incubated in the following solution for 1 h (Karnovsky and Roots 1964): 5 mg acetyl thiocholine iodine (Sigma), 6.5 ml 0.1 M sodium acetate buffer pH 6.0, 0.5 ml 0.1 M sodium citrate; 1 ml 30 mM copper sulphate, 1 ml distilled water, 1 ml 5 mM potassium ferricyanide. Sections were rinsed in distilled water and then incubated in the following solution for 1 min (Graham and Karnovsky 1966): 0,15 g 3´3 diaminobenzidine tetrahydrochloride (DAB), 5 ml 20 vol (6%) hydrogen peroxide, 300 ml 0.005 M TRIS-HCl, 0.8% saline buffer pH 7.6. Sections were then rinsed well in tap water and allowed to dry prior to further staining. Since this protocol preceded the ISH protocol, all steps were carried out with care to avoid skin contact and thus possible contamination with RNase. It was not found necessary, however, to sterilise all solutions.

Results

ISH plus immunohistochemical detection of a protein antigen

In this application, colour substrates for alkaline phosphatase are used to assay both binding of probe for target mRNA and antibody binding to a chosen protein. X-phosphate/NBT was used for detection of the ISH probe while Fast Red was used for detection of the protein antigen. We found this was possible since the Fast Red substrate used to detect the antibody binding to a protein antigen does not appear to be sufficiently sensitive to detect the probe used for ISH. Thus we first carried out ISH and assayed probe binding with the blue, X-phosphate/NBT colour substrate, and then carried out immunohistological detection of a protein antigen which was assayed using the Fast Red substrate. As a control,



Figs. 1–6 Wax sections (**Figs. 1–5**), *scale bars* approximately 100 µm and Frozen section (**Fig. 6**), scale bar approximately 10 µm. Blue staining represents in situ hybridisation (ISH) signal and red staining is detection of antigens by immunohistochemistry. **Fig. 1** Transverse section through the trunk of a 5-day chick embryo. ISH detection of *cSox21* and immunohistochemical detection of desmin using a polyclonal antiserum. ISH is restricted to the neural tube (*nt*) whilst desmin staining is restricted to the developing muscles of the myotome (*my*). **Fig. 2** Transverse sections through the trunk of a 5-day chick embryo. ISH detection of *cSox21* and immunohistochemical detection of neurofilament protein using a monoclonal antibody. ISH is generally restricted to the proliferative ventricular zone (*vz*) of the neural tube whilst neurofilament staining is seen only in the more lateral marginal zone (*mz*) where more mature cellular structures are located. **Fig. 3** Transverse sections through a 12-h embryo. ISH detection of *cSox3* and immunohistochemical detection of the brachyury antigen using a polyclonal antiserum. ISH is restricted to the upper ectoderm (*e*) whilst brachyury is detected in the medial region of the ectoderm and in the lower cells of the mesoderm (*m*). Note that some cells are positive for both ISH and immunohistochemical staining of their nuclei (*arrowheads*). **Fig. 4** Transverse section through the trunk of a 5-day chick embryo. ISH detection of *cSox3* and immunohistochemical detection of HNK-1 using a monoclonal antibody. ISH is seen in the neural tube (*nt*) and at a lower level in the forming dorsal root ganglia (*drg*). HNK-1 is detected in the forming dorsal root ganglia and in glial cells associated with the ventral nerve roots (*vr*). **Fig. 5** Sagittal sections through the optic tectum of an 8-day embryo. ISH detection of *cSox2* (*left*) and *cSox11* (*right*) with immunohistochemical detection of bromodeoxyuridine (BrdU). Note the correlation of BrdU and *cSox2* expression in cells of the ventricular zone (*vz*). **Fig. 6** Sagittal section through the optic tectum of a 5-day chick embryo. ISH detection of *cSox11*, immunohistochemical detection of neurofilament protein using a monoclonal antibody, and acetylcholinesterase (AChE) activity detection (yellow/brown). Cells migrate from the lower ventricular zone (*vz*) towards the upper layers such that cells of increasing maturity are seen towards the top of the sections. Thus a progression from *cSox11*, through AChE to neurofilament staining is seen

when we added Fast Red substrate after the ISH alone with or without prior treatment with X-phosphate/NBT, we did not see any red substrate labelling (data not shown).

We have used ISH probes specific for *Sox* genes which encode putative transcription factors (Coriat et al. 1993; Uwanogho et al. 1995). These genes are expressed in the developing central and peripheral nervous system, so the markers analysed here are generally central nervous system related. All sections presented here are from chick embryo, but the techniques should be suitable for most tissue types. Use of 'sense-strand' control probes never produced an ISH signal.

Using this approach we were able to distinguish cells expressing a *Sox* gene but not the protein antigen in question (Fig. 1–4), cells expressing the protein antigen but no *Sox* gene (Figs. 1–3) and cells expressing both (Figs. 3, 4).

ISH plus immunohistochemical detection of BrdU incorporation

BrdU is often used to label cells which are actively synthesising DNA (BrdU being incorporated in place of dTTP) and therefore undergoing mitosis. In the nervous system, BrdU incorporation is also used to 'birth date' neurons and therefore follow specific populations of neurons through development. The method involves infiltration of BrdU into the animal by injection. The tissue is subsequently fixed and an immunohistological approach is used to identify those nuclei which have incorporated BrdU. Most often, antibodies against BrdU are used in conjunction with horseradish peroxidase (HRP) to produce a brown colour with diaminobenzidine tetrahydrochloride (DAB) as a substrate for the HRP. In this study we used alkaline phosphatase-conjugated antibodies (Fig. 5). We find that both methods work well, though the alkaline phosphatase plus Fast Red substrate gives clearer results.

We treated embryos with standard concentrations of BrdU for 1 h whilst still alive. These embryos were then prepared for wax (or frozen) sectioning and sections processed for ISH. Following ISH and colour development using X-phosphate/NBT, we treated for detection of the BrdU incorporation using a slightly modified version of a standard protocol. Treatment with HCl and protease were reduced to a minimum time (best established empirically for the tissue being studied). Final Fast Red detection was as described for general immunohistochemistry.

We were thus able to correlate cell proliferation with expression of our gene of interest, *Sox2*, in the ventricular zone of the developing brain (Fig. 5).

Enzymatic detection of acetyl-cholinestense (AChE) activity plus ISH

The method for detection of AChE was developed some years ago by Karnovsky and Roots (1964). The method was modified by Liu and Salpeter (1994) in order to carry out subsequent radioactive ISH on the same tissue sample. We have applied a slightly modified version of this latter method. In order to detect such enzymatic activity it is necessary that the enzyme is still functional so tissues could be either unfixed or (in this study) lightly fixed (4% paraformaldehyde, 4° C, 30 min). Since it was necessary to carry out the enzyme detection prior to ISH, all steps were carried out with care to avoid contamination with RNase, especially via contact with skin. Following the initial enzymatic detection, DAB was used to produce a more permanent colour which will withstand the subsequent ISH protocol (as described in Liu and Salpeter 1994). The ISH was then carried out according to our routine protocol. The result is a yellow-brown stain for AChE and blue ISH result. Prior to the ISH, the AChE labelling is a darker brown, but this fades to yellow during the ISH protocol. We have found, however, that over a period of time, staining for AChE activity, when mounted in Mowiol, reverts to a brown stain.

We have thus shown that some cells exhibiting AChE activity do express *cSox11* whilst others do not (Fig. 6).

Simultaneous detection of multiple molecular markers

As a demonstration of the power of these combinatorial staining protocols we have carried out multiple staining of a single section for a combination of AChE activity, *Sox* gene ISH and immunohistochemical detection of neurofilament protein (Fig. 6). Cells expressing a variety of the different markers can be distinguished. The analysis of BrdU, immunohistochemical staining and ISH can likewise be carried out simultaneously.

Discussion

The results presented here demonstrate that simultaneous detection of mRNA, protein products, BrdU and AChE enzymatic activity can be achieved in single tissue sections. The only requirement is that the transcripts are sufficiently abundant to be detected using non-radioactive ISH and assays for protein or enzyme activity are also available. We have set out to demonstrate that standard techniques for detection of each type of marker can be carried out sequentially on the same sections with minor modifications to improve the overall results. Whilst others have reported ISH and immunohistochemical detection of protein antigens (Lloyd et al. 1995) or BrdU (Henrique et al. 1995), this report demonstrates that proteins can still be detected immunohistochemically after the ISH protocol, including a proteinase K treatment, and that the non-radioactive ISH protocol can still be successfully carried out even after a relatively harsh treatment such as that used to assay AChE.

We have used alkaline phosphatase and Fast Red to assay the presence of protein antigens immunohistochemically, but other reagents should be equally effec-

tive. Thus the use of HRP (which we have found works relatively well for immunohistochemistry following ISH) and fluorescent markers, should allow most combinations of detection systems to be successfully achieved simultaneously.

In no case have we found that the immunohistochemical detection of protein antigens was negatively affected by the prior ISH protocol (in some cases the result was actually improved, possibly due to better exposure of the antigen by proteinase K treatment). For proteins antigens which are particularly sensitive to proteinase, the proteinase K step of the ISH protocol can be replaced by pressure cooking (Norton et al. 1994) which yields quite acceptable ISH results in our hands.

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