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# Detection of Stem Cell Populations Using in Situ Hybridisation

Virginie Sottile

*Wolfson STEM Centre, School of Clinical Sciences, The University of Nottingham, UK*

## 1. Introduction

RNA in situ hybridisation (RISH) on tissue sections allows the detection of mRNA expressed in discrete populations within a tissue. Based on the use of a specific riboprobe complementary to the target sequence, RISH enables the detection of individual cells present within an organ whose structure and organization are maintained.

Originally developed with radiolabelled probes, RISH now mostly relies on non-radioactive detection techniques, using nucleotide tags such as fluorescein or digoxigenin (DIG) as discussed below. Such non-radioactive labelling can both reduce the risks to health and the environment, and offer higher sensitivity with lower probe detection time (Wilkinson, 1995, Moorman et al., 2001).

The fact that RISH on tissue sections provides information on both cell number and cell distribution makes it an important technique for the study of stem cell populations, with a range of applications including identifying endogenous populations through the detection of stem cell markers' mRNAs, characterising the in vivo behaviour of endogenous stem cells, or following the transplantation of exogenous cells in animal studies. The analysis of stem cell populations after they have been injected into an animal model, whether embryonic or adult, is particularly amenable to RISH: using a probe specific for the injected cells, such as for example an anti-GFP probe for labelled stem cells, the number and distribution of injected cells in the host tissue can be analysed on tissue sections. Since probe specificity will directly affect the success of the experiment, the choice and design of the probe represent critical aspects of the preparation which should be determined before starting the RISH.

## 2. RISH technique for the detection of stem cells within a host organ

### 2.1 Principle and preparation

The detection of the target sequence involves 3 phases: (i) preparation of the sections, (ii) hybridisation of the probe, and (iii) post-hybridisation detection of the probe. The probe can be detected through fluorescence or enzymatic methods. Fluorescence methods can have more limited sensitivity than enzymatic methods.

Probes labelled by incorporation of DIG-labelled ribonucleotides can be detected by anti-DIG antibodies which are coupled with an enzyme such as alkaline phosphatase (fig. 1). The enzyme is then used to trigger a colour reaction in situ within the cells in which the DIG-

labelled probe has bound to the target RNA. Different methods and kits for probe labelling and preparation are available from commercial sources ([www.roche-applied-science.com/dig](http://www.roche-applied-science.com/dig), Gandrillon et al., 1996). One method routinely used relies on the prior sub-cloning of a partial or entire cDNA fragment of the gene of interest, and the *in vitro* transcription carried out in the presence of DIG-labelled nucleotides (fig. 2).

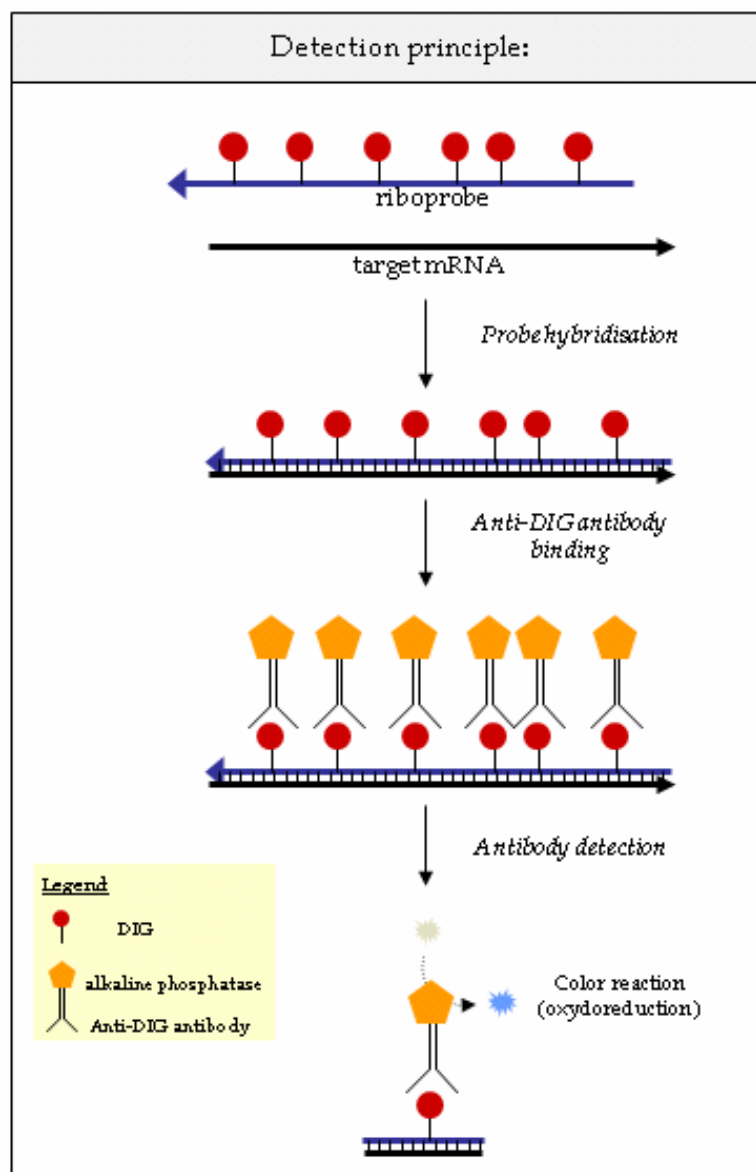


Fig. 1. Principle of the probe hybridisation-based detection of the target sequence using an enzymatic-based colour reaction.

Detection of the target sequence requires a stepwise protocol which can run over 3 to 6 days depending on the level of expression of the gene of interest and on the target tissue. The method described here is used for the successful detection of target mRNA in frozen sections using DIG labelled probe. However, several steps may need to be optimized by the user for optimal results, as indicated throughout. This technique requires organization in order to prepare all necessary supplies and solutions ahead of the procedure (see section 3), and care in order to avoid degradation of the sample's RNA by contaminating nucleases.

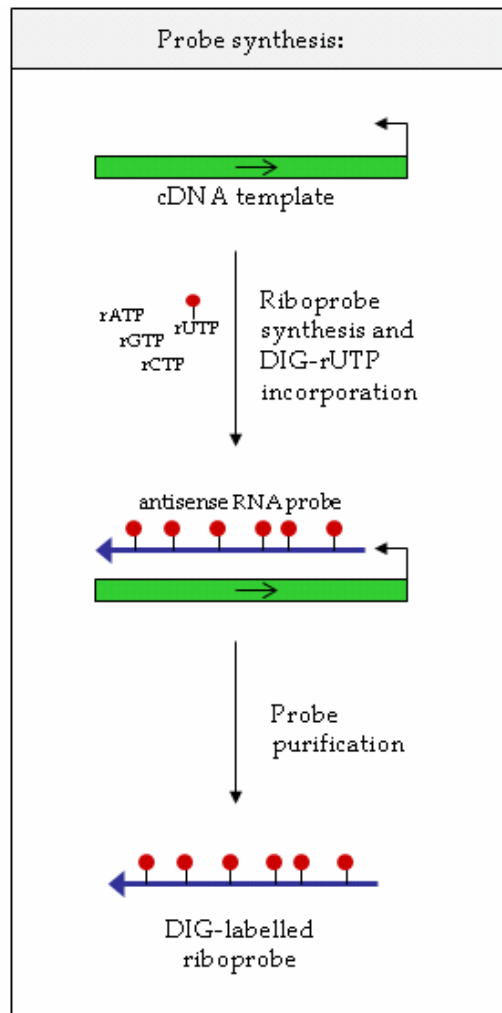


Fig. 2. Principle underlying the preparation of the DIG-labelled antisense probe used to detect a target sequence.

## 2.2 Slide preparation

RISH on frozen tissue section provides reliable results and high sensitivity with minimal tissue damage. The tissue of interest should be dissected and processed with care and diligence following the protocol presented below (fig. 3) to avoid structural damage and degradation of the mRNA which will impair the experimental outcome.

Following the harvesting of the appropriate tissue, the sample is washed in PBS for 5min at room temperature ('RT'). The tissue is then fixed in 4% ice-cold paraformaldehyde (PFA), for a period ranging from 30min to overnight depending on the size and density of the tissue (this parameter needs to be optimised by the user for each type of tissue). The sample should remain at 4°C throughout. Following fixation, the PFA should be removed and the sample washed in PBS for 5min at RT.

Before cryoembedding, the sample should be transferred to a 15% sucrose solution kept at 4°C until it sinks to the bottom of the vessel (this may require overnight incubation depending on the size of the sample). This is repeated by transfer to a 30% sucrose solution at 4°C until the tissue sinks to the bottom of the vessel (this may also require overnight incubation, depending on the size of the sample).

Sucrose-equilibrated samples are then washed once with OCT for 5min at RT, before being transferred to an appropriate mould (plastic or foil) filled with OCT, the tissue is orientated according to the plane of sectioning to be used. The mould is placed in a plastic beaker containing cold isopentane, and the beaker dipped into a vessel containing liquid nitrogen. The mould content progressively becomes opaque as it freezes over a few seconds. Care should be taken to remove the sample as soon as its content is frozen to avoid cracks in the sample. Cryopreserved samples can be stored at  $-20^{\circ}\text{C}$  until sectioning. The cryostat will then be used to generate 10 to  $12\mu\text{m}$  thick tissue sections, following manufacturer's instructions, and the sections produced will be stored at  $-20^{\circ}\text{C}$  until in situ hybridisation.

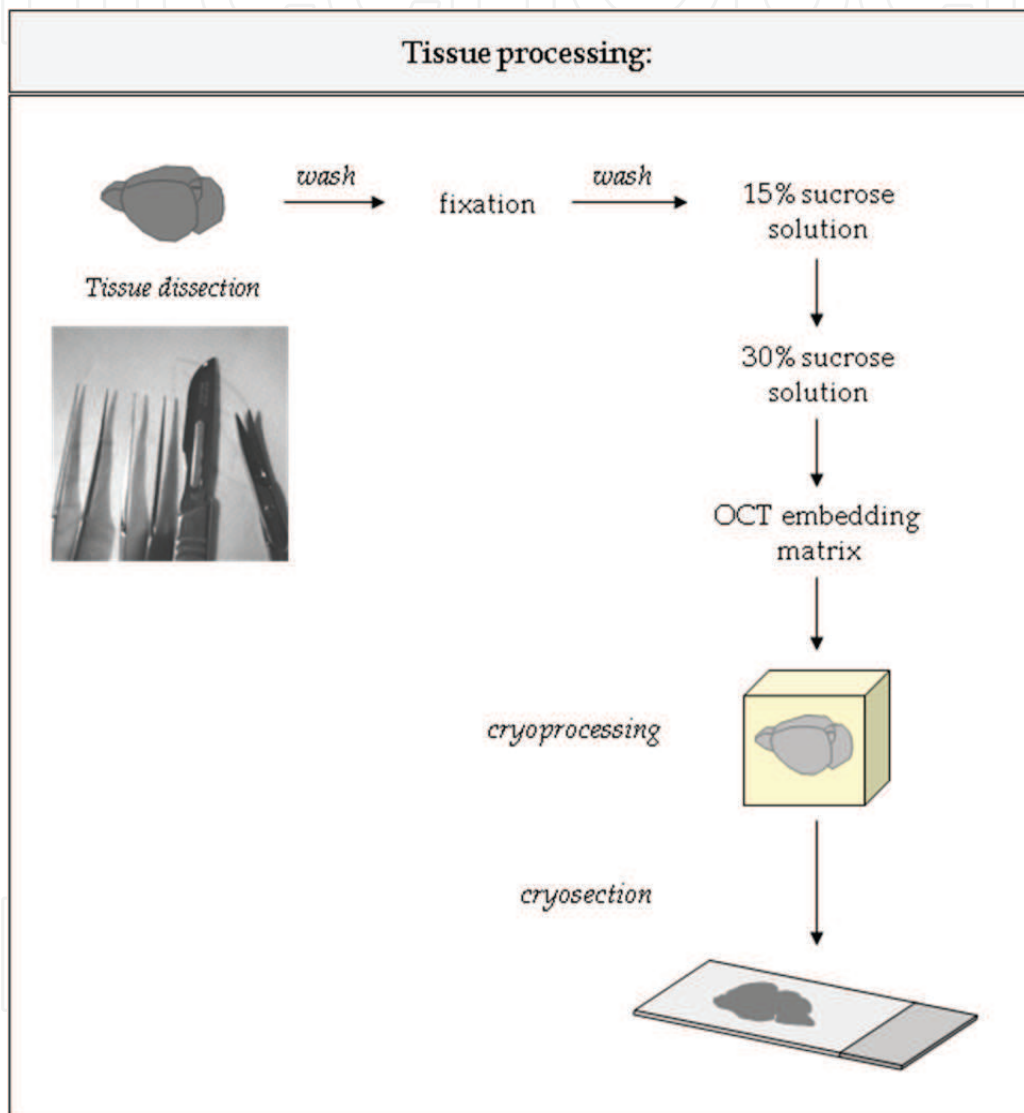


Fig. 3. Preparation of tissue cryosections for RISH.

### 2.3 Probe hybridization

Work with riboprobes requires significant precautions to minimize exposure of the slides to contaminating RNases, which can degrade both the probe and target sequence before the hybridisation step is completed. All steps described below (fig. 4) must therefore be carried out in a clean working area, using gloves and RNase-free solutions.

Before starting with the pre-hybridisation treatment, the slides need to be immersed in PBS for 3min at RT in order to remove the residual OCT matrix. Each slide is then re-fixed with 1ml of PFA and incubated 10min at RT (*NB: this step needs to be carried out in a fume cupboard to minimise exposure to fixative*). During this incubation step, the Proteinase K solution should be pre-warmed at 37°C.

After 3 brief washes with PBST (for 5min each), each slide is then covered with 500µl of prewarmed Proteinase K solution for 5min at RT to permeabilise the sample. After a series of 3 PBST washes for 3min each, sections are PFA-fixed again for 20min to maintain their integrity following permeabilisation, and washed again 3 times in PBST.

Before adding the riboprobe to the samples, the probe solution is pre-heated at 68°C for 5min in Hybridisation solution to reduce secondary structure. The probe solution is added to each slide, covered with a hybrid-slip. The sections are placed in a hermetic box humidified with a solution made of 50% formamide and 5x SSC and incubated overnight in the hybridisation oven at 70°C. The temperature and length of hybridisation need to be optimized by the user, as the nature of the sequence and the tissue can affect the efficiency of this procedure.

## 2.4 Probe detection

### 2.4.1 Posthybridisation washes and antibody binding:

Following the hybridisation step, extensive washing is crucial to ensure signal specificity and low background. The wash Solutions 1 and 2 need to be pre-warmed before the start of these post-hybridisation washes. Slides will then undergo (i) 3 washes in Wash Solution 1 at 70°C, for 30min each ; (ii) 3 washes in Wash Solution 2 at 65°C, for 30min each ; (iii) 3 washes in Wash Solution 3 at RT, for 5min each (fig.4).

Once the unbound probe has been removed by serial washes, slides are prepared for the probe detection, which is carried out using an anti-DIG antibody. Before the antibody is applied, slides are exposed to 500µl of Block solution for 1 hour to decrease unspecific signal. Slides are then covered with 500µl anti-DIG antibody solution, covered with parafilm and placed in a humidified box placed overnight at 4°C.

### 2.4.2 Antibody washes and colour detection:

After the antibody has been in contact with the tissue sections, extensive washing is required to remove the unbound antibody. Slides are washed 3 times in Wash Solution 3, for 20min each time, and twice in Detection buffer, for 5min each time.

The final step in the detection of the DIG-labelled probe is carried out through an enzymatic reaction which will highlight the cells containing the target sequence (fig. 1). To initiate the detection of the bound antibody in the tissue sections, each slide is overlaid with 300µl Colour solution, covered with a coverslip and incubated in the dark until the desired staining intensity is achieved. This step can take minutes to days, depending on the level of expression of the target RNA, and the quality of the sample. As a rule, fresh colour solution can be replaced every 8-10hours to prolong the reaction and minimise unspecific darkening of the tissue.

When the coloration is satisfactory (fig.5), the reaction is stopped by incubating the slide in TE buffer for 5min at RT. Slides can then be mounted with a coverslip for microscope examination, using a mounting medium such as Vectashield (following manufacturer's instructions).

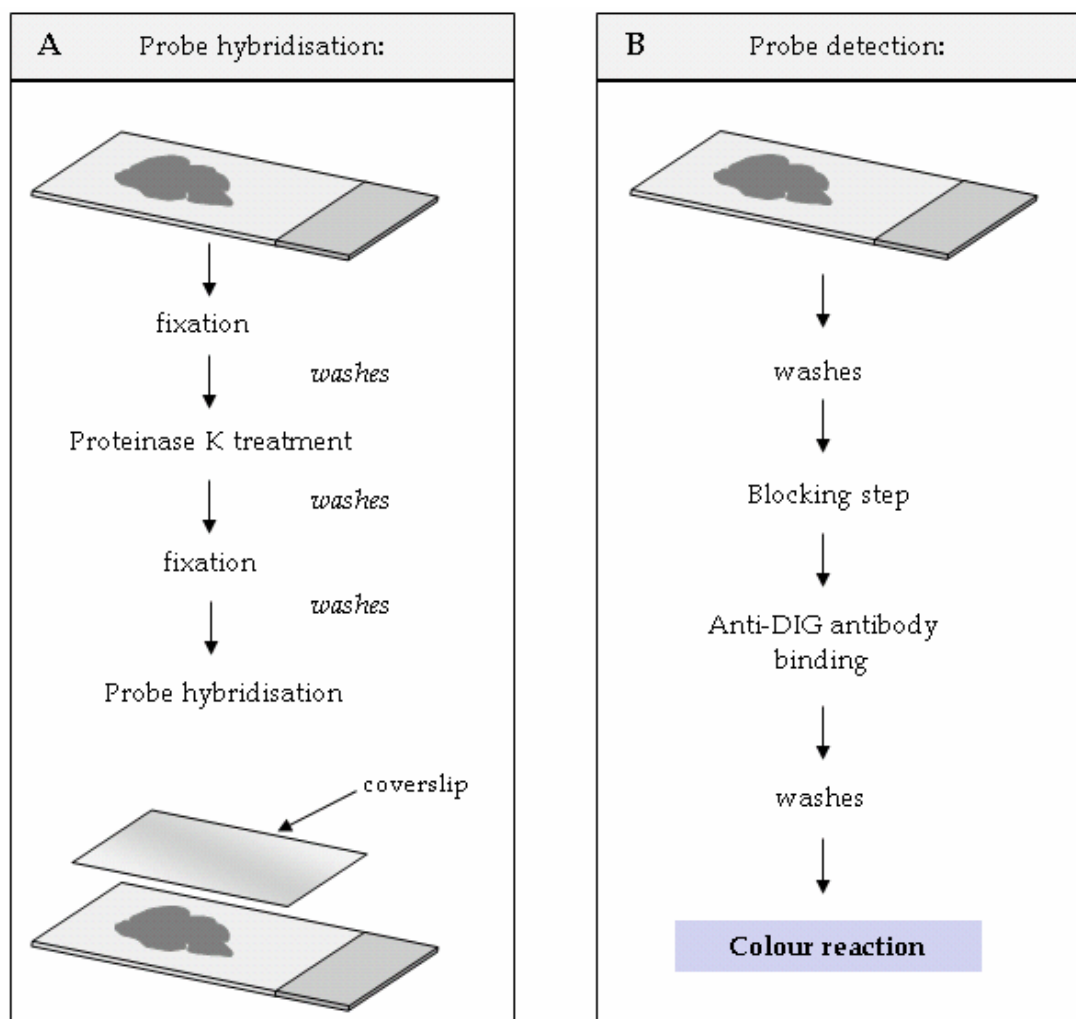


Fig. 4. Experimental steps for signal detection. (A) Preparation for the probe hybridisation phase. (B) Principle of signal detection after hybridisation of the probe.

### 3. Reagents and solutions

The section below describes the equipment and supplies used to carry out RISH on frozen tissue sections (Table1). A description of the different buffers and solutions used as described in the previous sections is also provided (Table2).

Supplies:	Equipment:
Hybrid-slips	Cryostat
Coplin jars	Microscope
Superfrost slides	Waterbath
Forceps	Hybridisation oven
Scissors	Heatblock
Glass coverslips	Hermetic slide box
Parafilm	Liquid nitrogen vessel

Table 1. Equipment and supplies used in the protocol.

<b>Solution:</b>	<b>Composition:</b>
Paraformaldehyde	Dilute 4g of paraformaldehyde in 100ml PBS, and incubate in 65°C until dissolved. Bring pH to 7.4 and aliquote. Store at -20°C
Sucrose solutions	Dilute the necessary amount of Sucrose (7.5g for 15%, 15g for 30%) in 50ml PBS.
PBST	Add 1µl/ml of Tween20 to PBS and mix well.
Proteinase K solution:	Dilute Proteinase K powder in PBST to a final concentration of 1µg/ml.
20x SSC solution:	Dilute 175.3g/l NaCl and 88.2g/l NaCitrate in H <sub>2</sub> O, adjust pH to 4.5 and autoclave.
10% SDS	Dilute 100g/l SDS powder (follow manufacturer's instructions) in H <sub>2</sub> O, and adjust pH to 7.2.
Wash solution 1	Prepare the following solution in H <sub>2</sub> O: 0.5x Formamide, 1% SDS, 5x SSC
Hybridisation solution	Prepare solution as described for Wash solution 1 and add tRNA (50µg/ml) and Heparin (50µg/ml).
Wash solution 2	Prepare the following solution in H <sub>2</sub> O: 0.5x Formamide, 2x SSC
Wash solution 3	Prepare the following solution in H <sub>2</sub> O: 8g/l NaCl, 0.1M Tris (pH7.5), 10µl/ml Tween20.
Block solution	Dilute 100µl/ml of Sheep serum in Wash solution 3.
Anti-DIG solution	Dilute 10µl/ml of Sheep serum and 0.2µl/ml anti-DIG antibody in Wash solution 3.
Detection buffer	Prepare the following solution in H <sub>2</sub> O: 0.1M NaCl, 50mM MgCl <sub>2</sub> , 0.1M Tris (pH9.5), 10µl/ml Tween20.
Colour solution	Prepare the following solution in Alkaline phosphatase buffer: 4.5µl/ml NBT, 3.5µl/ml BCIP.
TE buffer	Prepare the following solution in H <sub>2</sub> O: 10mM Tris (pH7.5), 1mM EDTA (pH8).

Table 2. Solutions required for the protocol.

## 4. Optimisation and general advice

### 4.1 Integrity of the sample:

The quality of the results obtained will depend on the preservation of the sample integrity throughout the procedure, from tissue harvest to target detection. Because the technique relies on detection of mRNA, careful handling of the samples in RNase free conditions is essential for the 1st part of the procedure (until hybridisation). Gloves must be worn at all times, bench surfaces and equipment must be cleaned with RNase zap. It is also preferable to use a dedicated set of glassware and pipettes for RISH, and to use filter tips until the post-hybridisation stages.



Another critical factor comes from the fact that the hybridisation step takes place in a limited volume, over a long time and at high temperature. Evaporation is therefore a potential problem which could cause the sections to dry and become unusable. To ensure the slides do not dry up during this step, it is critical to maintain the slides in a humidified atmosphere in the hybridisation box, by including soaked tissue and carefully sealing the box.

As the length and relative complexity of the RISH procedure can be detrimental to the tissue, which is exposed to serial incubations and washes in stringent conditions, the quality, reproducibility and sensitivity of RISH can be significantly enhanced by the use of an automated slide processor (such as the BioLane™ HTI). The use of such automation can significantly reduce variability and preserve the integrity of precious samples, while allowing higher throughput for the batch processing of sections by RISH.

#### **4.2 Signal intensity:**

The length of the colour development phase can vary significantly (from minutes to several days) depending on the level of expression of the gene of interest, the nature of the probe, and the quality of the tissue. The optimal length of development needs to be determined on a case by case basis. It is crucial to regularly monitor the slides during this phase, and replace the colour solution to avoid drying of the slide as well as unspecific deposition of the substrate over time.

Some detection problems may also arise if the target tissue is likely to exhibit endogenous alkaline phosphatase activity, as this would cause background staining during the colour reaction. This can be avoided by adding levamisole to the colour reaction, which can inhibit endogenous activity (Ponder & Wilkinson, 1981).

#### **4.3 Analysis and quality control methods:**

**Positive controls:** Although it can be useful to use a probe for a housekeeping gene as a control for RISH detection, the interpretation can sometimes be difficult between ubiquitous staining and unspecific background staining. Therefore, using a control probe and tissue previously shown to give specific staining is usually the best way to validate the technique in the lab. Comparison with the expression pattern obtained by immunostaining, whenever possible, can provide assurances that the RISH signal obtained is specific. Above all, reproducibility of the staining in independent RISH runs is essential.

**Negative controls:** The 2 sets of controls described below are required to enable the evaluation of possible false positive staining on the tissue sections:

(i) **Sense control:** The parallel handling of a slide hybridised with a sense probe is a common method to ascertain the specificity of the probe. The sequence of the sense probe is identical to that of the target mRNA (whereas the antisense probe is complementary to it), thus the sense probe does not hybridise with the target transcript. Any signal detected in the sense control is therefore considered to result from unspecific binding of the probe or antibody, and allows the relative assessment of the antisense results (fig. 5).

(ii) **Alkaline phosphatase control:** A slide processed in parallel but not exposed to the anti-DIG antibody can provide another type of negative control, by measuring the contribution of endogenous alkaline phosphatase signal in the target tissue. Based on the results of this test, levamisole may be added to the Detection buffer to decrease background alkaline phosphatase activity in the tissue section (see section 4.2).

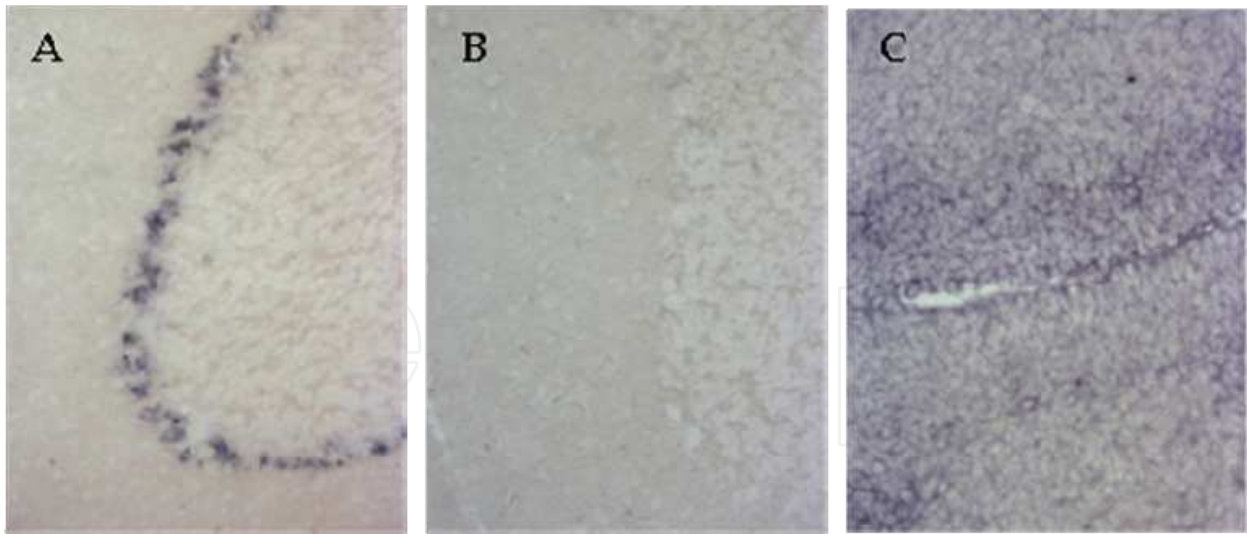


Fig. 5. Examples of RISH detection on mouse brain tissue. (A) Specific signal obtained with an antisense probe shows clear cell labelling on the tissue section with little background. (B) Negative control using a sense probe shows no unspecific signal in the tissue. (C) Example of unspecific background coloration observed throughout the tissue.

## 5. Related procedures and future research

### 5.1 Related methods:

The detection method described here is amenable to several variations and amendments. In addition to cryopreserved tissues, RISH can be performed on wax-embedded samples (Moorman et al., 2001). This can help preserve the tissue if its structure is likely to be damaged by freezing, however the signal obtained by RISH can be lower than when using cryosections.

RISH as described here can be adapted to detect more than one target using probes labelled with different tags. The detection of target sequences also recently benefited for the availability of custom made locked nucleic acid ('LNA') oligonucleotides which can provide increased sensitivity and improved hybridisation affinity (Thomsen et al., 2005). LNAs are particularly used to study miRNA expression in cells and tissues (Kloosterman et al., 2006).

The RISH approach described in this chapter is also compatible with subsequent immunodetection of specific antigens by immunofluorescence for instance (Sottile et al., 2006). The combination of multiple markers, at the RNA and protein levels, enables a more elaborated characterisation of target cell populations and their interaction with other cell types within the tissue of interest.

### 5.2 Stem cell research:

As discussed in the introduction, RISH can give access to a range of information for the study of stem cell-specific transcripts: whether there are any cells in the tissue expressing these markers, how many cells are positive, where they are located within the tissue (Alcock & Sottile, 2009).

In addition to the identification of stem cell populations in a tissue through the use of probes specific for stem cell markers, RISH offers another prime application: the study of exogenous stem cell populations in xenograft models. Careful design of the probe can offer

species-specificity, allowing donor-derived transcripts to be specifically detected using differences in sequence.

RISH is also useful to study stem cells in *ex vivo* models. As described here for the analysis of stem cells within tissue sections, RISH is a technique which is also effective for the analysis of stem cell-derived organoids generated *in vitro* such as embryoid bodies, neurospheres or mammospheres. Cell aggregates can be embedded in agarose first for easier manipulation at the cryopreservation stage, and the agarose mass can then be processed as a piece of tissue following to the protocol presented above. RISH can equally be used to study gene expression changes in cellularised scaffolds loaded with stem cell populations. Although the sample sectioning steps may require optimisation depending on the nature of the scaffold and properties of the biomaterial used, the RISH technique can be expected to occupy a significant place in the study of novel stem cell-based tissue engineering approaches.

## 6. Acknowledgements:

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