CHAPTER 3

Validation of Sox Genes PCR data by *in-situ* hybridisation

Introduction

Having tested the mouse foetal cDNA panel by PCR with primers for members of the *Sox* gene family, a selection of *Sox* genes, which showed differing RTPCR expression patterns, was then taken for analysis by *in-situ* hybridization, in order to identify expression at the cellular level. Tissue sections were probed with radiolabelled oligonucleotides designed to *Sox 2, 4, 6, 15, 16, 17* and *LZ2*, together with control oligo probes for *Ribosomal protein S29* (*Rps29*), *RAR-related orphan receptor alpha* (*Rora*), *Epidermal growth factor* (*Egf*), and the sense oligo probe for *Protease 26S subunit ATPase,* (*Psmc1*).

3.1 Special Equipment and Suppliers

Sterilizing oven 180^{o}C	Scientific Laboratory Suppliers
Incubating oven 40-60^{o}C	Scientific Laboratory Suppliers
Cryostat	Leica
Autoradiography equipment and dark room facilities	
Safe light filter	Kodak 1 # 152 1509, Ilford 914 # CEA 4B, or Agfa-Gaevert # R1
X-ray film	Kodak BioMax MR # 871 5187, # XAR 5 or Amersham Hyperfilm β-max
Rapid fix	Kodak

X-ray cassette	Amersham Pharmacia
X-ray film developer	Kodak # D19 or Ilford#D19
X-ray envelope	Amersham Pharmacia
Liquid photographic emulsion	Kodak # NTB-2 or Ilford#K5
LC29	Ilfotec # LC29
Superfrost slides	BDH # 406/0169/02
Scotlab Easicounter 4000	
Hot dot: HCT.dot, sample range 0.5 – 2 □l	SL 8329 # B-QC-7001, 48
Nunc square petri dish	Nunc # 166508
Slide racks stainless steel or black plastic-style	BDH
Cryo-M bed	Bright Instruments
Nuclear research emulsion in gel form	Ilford
Variable light intensity illuminator, dual lamp precision	Northern Light
Microscope Leica DM RB	Leica
Lenses x5, X10, X20, X40	Leica
Lens, Nikon PN-11 52.5	Nikon
Lens, NIKKOR 105mm 1:2,8	Nikon #244228
Relay camera MTI CCD 72	MTI
Leica DC 200 version 2.51 windows NT application	Leica
MCID Image Analyser software, M4-Image version 3, Rev 1.4	Imaging Research Inc. Brock University, St. Catherines, Ontario, Canada L2S 3A1

3.2. Materials and Solutions

Chemicals and Suppliers

"(poly)L-lysine hydrobromide (mol. wt > 350,000) "	Sigma # P1524
[35S]deoxyadenosine ‘5 (a-thio)triphosphate 10 mCi/ml	Amersham # SJ1334
amberlite mixed bed resin MB-150	Sigma # A5710
ammonium acetate	BDH # 100134T
aluminium sulphate	Sigma # A 7523
bovine serum albumin fraction V	Sigma # A8022
C14 standards	Amersham # RPA 504
chromium potassium sulphate	Sigma # C 5926
dextran sulphate	Pharmacia # 17-0340-02
dithiothreitol	Sigma # D9779
DPX mountant	BDH # 360294H
Ethanol	BDH # L227007
ethylene diamine tetraacetic acid (EDTA)	Sigma # ED2SS
Eosin yellowish	BDH # 341972Q
ethylene glycol	Sigma # E-9129
ficoll 400	Sigma # F2637
formamide super pure	Gibco/BRL # 540-5515
glass wool	Sigma # G8389
glycerol	Sigma # G 7757
hematoxylin	Sigma # H 9627
heparin (sodium)	BDH/Merck # 28470
isopentane	Sigma # 27,041-5
paraformaldehyde	Merck # 21046205 438

phosphate buffered saline (PBS)	Oxoid #BR14
polyadenylic acid (potassium salts)	Sigma # P9403
polyvinylpyrrolidone	Sigma # P5288
sephadex G50 medium	Pharmacia # 17-00 45-01
"sheared salmon sperm DNA 10 mg/ml type III, sodium salt, "	Sigma # D-9156
sigmacote siliconising solution	Sigma # SL2
sodium azide 1% w/v	Severn Biotech
sodium chloride	BDH # 102415K
sodium hydroxide	BDH # 102525P
sodium dodecyl sulphate (SDS)	BDH # 442444H
sodium dihydrogen orthophosphate	BDH # 301324G
disodium hydrogen orthophosphate	BDH # 102494C
sodium iodate	Sigma # S 4007
sodium metabisulphite	Sigma # S 1516
sodium pyrophosphate	Sigma # S 6422
sodium thiosulphate 5 hydrate	BDH # 102684G
terminal deoxynucleotidyl transferase	Pharmacia # 27-0730-0
trisodium citrate	BDH # 102425M
xylene	BDH # 102936H

3.2.1. 10 X Phosphate-buffered saline (PBS) *fc*(final concentration)

75.972 g sodium chloride *1.3 M*

9.937g disodium hydrogen orthophosphate *70 mM*

4.14g sodium hydrogen orthophosphate *30 mM*

made up to 1 L with sterile water, treated with 1 ml depc and autoclaved.

1 X PBS was prepared by diluting the stock in depc-treated water.

3.2.2. (Poly)L-lysine *fc*

25 mg (poly)L-lysine hydrobromide (mol. wt > 350,000) *5 mg/ml*

5 ml of depc-treated water

Store frozen as 1 ml aliquots in sterile eppendorf tubes

3.2.3. Ethanol

Absolute (99.7%) ethanol is diluted with depc-treated water to give the required concentration (for example, 50%, 70%, 95%).

3.2.4. 4% Paraformaldehyde in PBS

Paraformaldehde powder is toxic and harmful to mucous membranes: thus always wear gloves and a facemask when handling it and perform the following procedure in a fume hood.

- 40 g paraformaldehyde, slowly added to 500 ml depc-treated water in a sterile 1 L beaker.
- Heated to 60^{o}C with continuous stirring, using an autoclaved magnetic stir bar: heat kept below 65^{o}C.
- Drops of 6 M NaOH gradually added to the milky suspension until it clears.

- 100 ml sterile 10 x PBS in depc-treated water, then added and mixed thoroughly.
- Cooled and made up to volume in a 1 L volumetric flask.
- PH checked as 7.0 with pH paper, and stored in a refrigerator/cold room at 4^{o}C.
- Paraformaldehyde solution is best used within a 2 week period, since it polymerises.

3.2.5. 20 x SSC *fc*

438.25 g sodium chloride *3.75 M*

220.5 g trisodium citrate *0.375 M*

2 L water

2 ml depc *0.1%*

treat as depc water, see section 1.2.1.

3.2.6. Dithiothreitol

Dithiothreitol (DTT) is a reducing agent and when using ^{35}S-labelled probes, it will reduce or prevent cross-linking of sulphur residues *fc*

3.09 g DTT *1 M*

29 ml depc-treated water.

Stored as 1 ml aliquots in sterile eppendorf tubes at -20^{o}C.

3.2.7. TENS buffer

To a sterile 500 ml bottle the following are added: *fc*

20 ml 1 M Tris-HCl (pH 7.5) *20 mM*

5 ml 0.5 M EDTA (pH 7) *5 mM*

14 ml 5 M sodium chloride *140 mM*

5 ml 10% sodium dodecyl sulphate *0.1%*

466 ml depc-treated water

Filter through a 0.2 μm filter into a sterile baked bottle.

3.2.8. Sephadex G50 solution

In a sterile 250 ml glass bottle the following are added: *fc*

5 g Sephadex G50 medium *24%*

120 ml filtered TENS buffer

- left to swell for 1-2 hours.
- Washed several times with TENS buffer to remove soluble dextran and 'fines'.
- Autoclaved at 10 lb/sq. inch (note the lower pressure) for 20 mins.
- TENS supernatant removed and sephadex store at + 4^{o}C with 5 mg of sodium azide (this should last a year, however, always check for evidence of microbial growth).

3.2.9. Tailing buffer

10 x Tailing buffer is obtained from Pharmacia. It is supplied free of charge with purchase of terminal deoxynucleotidyl transferase enzyme. 10 x Tailing buffer is referenced Deng, G, R and Wu, R., Methods in Enzymology, 100, 96 (1983).

3.2.10. Heparin *fc*

120 mg heparin (sodium) *120 mg/ml*

1 ml depc-treated water.

Store frozen in 50 μl aliquots in sterile eppendorf tubes.

3.2.11. Polyadenylic acid *fc*

100 mg polyadenylic acid (potassium salts) *10 mg/ml*

10 ml depc-treated water.

Stored frozen in 1 ml aliquots in sterile eppendorf tubes.

3.2.12. 50 X Denhardt's Solution *fc*

5 g Ficoll 400 *1%*

5 g polyvinylpyrrolidone *1%*

5 g bovine serum albumin fraction V *1%*

500 ml depc-treated water

Stir until all are dissolved completely. *Stored frozen as 25 ml aliquots.*

3.2.13. 0.5 M diSodium Hydrogen Orthophosphate

3.559 g disodium hydrogen orthophospate

50 ml depc-treated water

3.2.14. 0.5 M Sodium diHydrogen Orthophosphate

3.45 g sodium dihydrogen orthophosphate

50 ml depc-treated water

3.2.15. 0.5 M Sodium Phosphate pH 7 *fc*

7.8 ml depc-treated 0.5 M disodium hydrogen orthophosphate *0.5 M*

12.2 ml depc-treated 0.5 M sodium dihydrogen orthophosphate *0.5 M*

pH checked as 7

3.2.16. 0.1 M Sodium Pyrophosphate

2.23 g sodium pyrophosphate

50 ml depc-treated water

3.2.17. Oligonucleotide Hybridization Buffer

		fc
25 ml	thawed deionized formamide	*50%*
5 g	Dextran Sulphate	*10%*
10 ml	20 x SSC	*4 x SSC*
5 ml	Denhardt's solution	*5 x*
10 mg	Salmon sperm DNA	*0.02%*
1 ml	5 mg/ml polyadenylic acid	*0.01%*
50 µl	120 mg/ml heparin	*0.012%*
2.5 ml	0.5 M Sodium Phosphate pH 7	*0.025 M*
0.5 ml	0.1 M Sodium Pyrophosphate	*0.01 M*

- Left overnight on a rotary mixer, at room temp.
- Made up to 50 ml with depc-treated water, wrapped in foil and stored at 4^{o}C.
- On use, left at room temp for 30-60 mins and inverted several times, to allow bubbles to rise. Hybridization buffer should be okay for up to a year.

3.2.18. Deionized Formamide

Formamide is a potential carcinogen, wear gloves and do all procedures in the fume hood.

2 x 100 ml thawed super pure formamide (stored -20^{o}C)

200 g mixed bed resin Amberlite MB-150

stirred for 30mins

pH checked as approx 7

filtered and dispensed as 25 ml aliquots in 50 ml falcon tubes, store at -20^{o}C.

3.2.19. *In-situ* wetting solution	*fc*
50 ml 20 x SSC	*4 x SSC*
125 ml formamide	50%
75 ml double deionied water	

3.2.20. Wash solution	*fc*
50 ml 20 X SSC	*1 x SSC*
2 g sodium thiosulphate 5 hydrate	0.2%
1 L double deionised water	

3.2.21. Ammonium acetate solution	*fc*
11.56g ammonium acetate	*600 mM*
50 ml glycerol	*0.5%*
950 ml double deionised water	

3.2.22. Developer

10 ml Ilford LC29

290 ml double distilled water

3.2.23. Stop solution *fc*

6 g sodium metabisulphate *2%*

6 g chromium (III) potassium sulphate *2%*

300 ml double distilled water

3.2.24. Fix solution

100 ml B&W fixer

200 ml double distilled water

3.2.25. Hematoxylin *fc*

6 g haematoxylin *0.6%*

4.2 g aluminium sulphate *0.42%*

1.4 g trisodium citrate *0.14%*

0.6 g sodium iodate *0.06%*

269 ml ethylene glycol 35%

680 ml double distilled water

3.2.26. 1% acid ethanol

1% conc. Hydrochloric acid in 100% ethanol

3.2.27. 1% Eosin

1 g Eosin Y

100 ml double distilled water

3.2.28. Siliconizes glass wool

- Place glass wool in an autoclavable bottle.
- Pour in sigmacote
- Invert bottle a few times and remove the sigmacote.
- Autoclave.

3.3. Methods

General notes: RNA, unlike DNA, is highly susceptible to degradation, the most common cause being RNases found in abundance in bodily secretions (sweat and saliva). RNA is also susceptible to heat denaturation. Therefore it is strongly recommended that all glassware is baked, plastics autoclaved, solutions treated with depc where possible and gloves worn throughout.

All solutions must be free of RNase and stored in sterile (RNase-free) containers. Gloves must be worn at all times when handling containers. Also, all solutions must be treated with depc (0.1%) and then autoclaved: stock solutions are diluted with depc-treated water. Diethylpyrocarbonate (depc) is an alkylating agent and inactivates any protein present in the solutions.

3.3.1. Preparation of Glass Slides

Proper pre-treatment of glass slides is necessary to avoid:

- *sticking of labelled probe to glass and causing undesired background,*
- *degradation of mRNA,*
- *sections falling off during the hybridisation and post-hybridisation procedures.*
  - Good quality, precleaned slides, such as BDH Superfrost. 0.1mm thick were used. Wearing gloves throughout, the cellophane was removed and wrapping them in batches of 50, in aluminium foil, and baked in an oven at 180^{o}C for 4 hours, on a shelf, to destroy the ribonucleases.
  - Slides were removed from the oven and allowed to reach room temperature slowly.
  - 1 ml (poly)L-lysine hydrobromide (5mg/ml) was allowed to thaw and diluted in 50 ml with depc-treated water (to obtain 0.01% (poly)L-lysine) in a sterile 50

ml Falcon propylene; mixed well and poured into a square sterile Sterilin petri dish.

- Completely immersing each glass slide (one at a time) into the (poly)L-lysine solution for approximately 5 seconds; removed with another glass slide, taking care to only handle from the edge, the excess (poly)L-lysine solution was drained by blotting the bottom of the slide with a paper towel, placing in a sterile slide rack, and allowed to air-dry in a dust-free area, covered loosely with foil.
- When the slides were dry, they were placed in a slide box with silica gel and stored at 4^{o}C until used. Usable up to 1 month after coating with (poly)L-lysine, but is advisable to coat slides immediately prior to use, and not store for prolonged periods.
- 300 are an optimal number to process.

3.3.2. Collection of Tissues

Procedures must be adopted to minimize the effects of stress on the animal both in terms of animal welfare and the possible changes in gene expression, in addition to the possible degradation of mRNA. Therefore, animals must be killed quickly and the tissues removed and frozen as rapidly as possible without damage.

Tissues of interest were removed directly into isopentane (5 secs) held on cardice. Small samples were embedded directly in mounting medium e.g. DPX mountant or Cryo-M Bed, Bright Instruments. Samples were then stored in liquid nitrogen or at –70^{o}C.

3.3.2.1. Sectioning of Tissues

- Prior to cutting sections, tissues were transferred to the cryostat and allowed to equilibrate to the temperature of the cryostat.
- The cryostat microtome knife was sterilised with a piece of tissue soaked in absolute alcohol.
- Tissue was mounted on to the block using mounting medium, and the block held in the chuck ensuring the block was orientated to give the desired plane of sectioning.
- The block was trimmed until the area of interest was reached.
- The temperature of the chuck and the chamber may be altered depending on the tissue, and this is largely trial and error. In general, however, a temperature of between -15^{o}C and -20^{o}C is fine for most tissues. Sections may require cutting slowly or quickly, depending on tissue and temperatures. After cutting, minimal manipulation was used (merely teasing out with a fine paint brush), prior to placing a slide close enough for the section to 'jump' onto the coated slide in the orientation required to display a fully stretched section.
- Sections were cut at 10 μm thickness precisely. Multiple sections of various stages to a slide. Developmental stages 8.5d, 10.5d, 12.5d, 13.5d, 15.5d, and 17.5d were cut over a period of a few weeks, processing a day's worth of cutting at any one time.
- Once the slide had a full complement of sections on, it was placed on a rack, face away to thoroughly air dry for 1–2 hrs prior to fixing.
- Sections were fixed on the day of sectioning and this step required at least 1 hr. All sections were fixed on the day of cutting and stored at –70^{o}C until used.

- If it is impossible to fix on the day of sectioning, it is acceptable to place the slides in a slide box with silica gel, seal with tape and leave at –70^{o}C overnight. Next day remove the slide box from the -70^{o}C, allow to reach room temperature, then fix the sections in 4% paraformaldehyde.

3.3.2.2. Fixation of sections

Efficient fixation procedures are important for maintaining good tissue morphology, for stabilization and retention of tissue mRNAs, and for destroying residual RNase. Over-fixation may inhibit accessibility of probe to mRNA. Several pre- and post-fixation procedures are available.

The slides were placed (with mounted sections) in sterilized RNase-free autoclaved stainless steel or black plastic-style racks and treated as follows:

- 5 mins in 4% paraformaldehyde in 1 x PBS, chilled on ice.
- 2.5 mins in 1 X PBS.
- 2.5 mins in 1 X PBS.
- 5 mins in 70 % ethanol/depc-treated water.
- 5 mins in 95 % ethanol/depc-treated water.

3.3.2.3. Storage of fixed sections

After fixation, the sections were stored in clean slide boxes either at -70^{o}C or in 95 % ethanol/ depc-treated water at 4^{o}C in a spark-proof cold room or refrigerator. Sections held at -70^{o}C can lead to tissue desiccation and thus poor histology and mRNA signal, moisture condensation may occur on the sections on removal from -70^{o}C if sections are not allowed to come to room temperature properly. Moisture may

cause leakage of cellular RNase and thus mRNA degradation. These problems are not encountered when sections are stored in alcohol at 4^{o}C. Storage in alcohol in addition to the easy access and visualization of the appropriate sections needed for *in situ* hybridization also helps to de-fat the sections. This latter point is important since some probes bind to the white matter and may give increased background/non-specific labelling. Under these conditions, mRNA is stable for years in 95% ethanol/depc-treated water at 4^{o}C.

3.3.3. Design, synthesis and purification of oligonucleotide probes

Various types of nucleic acid probes have been used to detect mRNAs in tissues by *in-situ* hybridization, for example, ss (single stranded) c (complementary) DNA, ds cDNA, cRNA and synthetic oligodeoxyribonucleotide probes. The advantage of using oligoprobes are that they can be synthesised in large quantities to an accurately defined sequence, are more stable than other probes, can differentiate single base differences and being small molecules, have good tissue penetration properties. However, they are less sensitive than RNA probes and the hybridisation conditions are critical to a successful result. As the expertise to work with oligo probes existed in the group, it was decided to follow this route. Several factors must be considered when designing oligonucleotide probes. Probe length and GC/AT ratio are the most important aspects since they may affect the stability of the hybrid. In general, probes with greater GC content will form more stable hybrids, since GC base pairs are stabilized by three hydrogen bonds in contrast to the two hydrogen bonds that stabilize AT/UT base pairs. However, if the GC content of the oligonucleotide is too high (>65%), non-specific labelling may occur since the thermal stability of the probe itself will be greater. The probe length and GC content, in addition to the formamide

and salt concentrations in the hybridization buffer, will also determine the appropriate conditions for hybridization and post-hybridization treatments.

Good results were obtained using the Oligo Primer Analysis Software version 5.1 from NBI/Genovus (http://www.natbio.com), with the following settings:

- Oligonucleotide length approximately 45 bp.
- Percentage GC content between 55-65.
- Duplex (interprobe complementarity) formation.
- Hairpin (intraprobe complementarity) formation, energies in kcal/mol need to be as near to zero as possible.
- Checked for related sequence homology by BLAST to minimize non-specific hybridization.
- Correct orientation checked prior to ordering.
- Ensuring the probe was synthesized by the highest purification procedure.
- Store stocks kept at 1 □g/ul in depc-treated water, working solutions at 5 ng/ul.

Some of the *in-situ* probes, were designed by Sarah Hunt at the Sanger Centre using the computer programme for choosing PCR and DNA sequencing primers (Hillier and Green), original default parameters assumed.

Figure 62 lists the *In-situ* Probe Sequences chosen from the PCR expression profile patterns for this study.

Figure 62: *In-situ* Probe Sequences

Gene Name	Probe sequence	Symbol	Accession number
Epidermal growth factor	GCAGGTGACTGATTTCTCCCTGAGACAGGCACAACCAGGCAAAGG	*Egf*	J00380
Protease 26S subunit, ATPase	GGCGGCACTGGGTGTGAGGTGTTACCAGTGGCAGTTTGCTGGCAG	*Psmc1*	U39302
Ribosomal protein S29	ATGTTCAGCCCGTATTTGCGGATCAGACCGTGGCGGTTGGAGCAG	*Rps29*	L31609
RAR-related orphan receptor alpha	CTTCTTCGTGACTGAGATACCTCGGCTGGAGCTCGCATAGCTCTG	*Rora*	U53228
Sox2	GCGGAGCTCGAGACGGGCGAAGTGCAATTGGGATGAAAAAACAGG	*Sox2*	U31967
Sox4	CCTAAGTCCCTTTCCTGCAGTGCAAAGCCAAAGCGACTCTGGCTC	*Sox4*	X70298
Sox6	CTCCATCCGCTGTACAGGCAAATGGAGAGGTGGCTTGCTTGGAAG	*Sox6*	U32614
Sox15	TTGGGGGGCTGGTACCCAAAGCCTCTGCTCCCTTGGGTAGTTGTG	*Sox15*	X98369
Sox16	TCCTCCACGAAGGGTCTCTTCTCTTCATCGTCCAGCAGCTTCCAC	*Sox16*	L29084
Sox17	GACCTGAGGCTCGAAAGGCTGGGGCAAGGAAGCGTCTAATGTAAC	*Sox17*	D49473
SoxLZ2	GTGAGGTTTGTTGTGCATTATGGGGTGCAGAGGCAGATGGGAGGC	*SoxLZ*	D61689

3.3.4. Radioactive labelling and purification of oligonucleotide probes.

The commonly used radioisotopes for labelling oligonucleotides are ^{32}P and ^{35}S. These produce labelled probes of high specific activity allowing for fast regional localization of mRNA in tissue sections. The ^{35}S provides probes of high specific activities, with good resolution on X-ray film and with excellent cellular resolution after liquid emulsion autoradiography. It also has a longer half-life (87.4 days) and is less hazardous to use than ^{32}P.

Tailing with terminal deoxynucleotide transferase enzyme catalyses the repetitive transfer of mononucleotide units from a deoxynucleoside triphosphate to the 3'-OH terminus of the synthetic oligonucleotide with the release of inorganic pyrophosphate. The length of the poly (dATP) tail is in the order of 15 - 25 residues and can be checked by PAGE. The number of residues transferred is influenced by enzyme concentration, oligonucleotide/isotope molar ratio, and the duration of the incubation.

3.3.4.1. Radioactive ^{35}S-labelling of oligonucleotide probes.

For each of the eleven oligonucleotide probes (see above Figure 62):

In a sterile 1.5 ml eppendorf tubes on ice the following was prepared:

- 1.25 □l 10 x One-Phor-All Buffer
- 7.25 □l depc-treated water.
- 2.0 □l (10 ng) oligonucleotide
- 1 □l terminal deoxynucleotidyl transferase enzyme
- 1 □l [^{35}S]deoxyadenosine '5 (□-thio)triphosphate

Using label as fresh as possible, ^{35}S label stored at -70 ^{o}C and thawed immediately before use, behind a screen, taking extreme care when removing the foil cover if new, the remainder subaliquoted and stored at -70^{o}C.

- Total volume of the contents of the reaction tube was checked as 12.5 µl, mix gently by pipetting up and down with a gilson, to avoid air bubbles.
- Incubated immediately in a waterbath at 32 ^{o}C for 1 - 1.5 hrs.
- The reaction was stopped with the addition of 40 µl depc-treated water.
- Labelled probe was purified using a sephadex G50 spin column. This separates unincorporated nucleotides from the labelled probe.

3.3.4.2. Purification of Probe

- The 'wings' of a disposable 1 ml syringe were clipped, and place into a sterile 15 ml falcon tube such that it did not fall into the tube and yet the lid can be satisfactorily applied.
- The syringe plunger was removed and used to plug the bottom of the syringe with autoclaved siliconized glass wool.
- Using a 5 ml Gilson, pre-swollen G50 Sephadex in TENS buffer, was quickly and gently poured into the 1 ml syringe at an angle, any bubbles were then removed by sharply flicking/shaking the syringe.
- The TENS buffer elutes into the Falcon tube as the syringe was filled with with the Sephadex G50 slurry. Removing the air bubbles by gently tapping the syringe.
- Excess TENS buffer drained into the Falcon tube and discarded.
- The whole assembly (Falcon tube and syringe) was covered with the Falcon tube cap and spun at 2000 rpm for precisely 2 mins in a low speed centrifuge.

Discarding the eluate. The packed G50 column reaching around the 0.9-1 ml mark on the syringe.

- The sephadex-filled 1ml syringe, was removed, to allow the sterile decapped eppendorf tube (appropriately labelled according to the probe used) to be placed in the Falcon tube and the Sephadex G50 syringe column replaced on top.
- 2 µl 1M DTT was added to the sterile decapped eppendorf tube, at the base of the Falcon tube.

3.3.4.3. Removal of unincorporated label

- 52.5 µl of probe solution was then pippeted onto the centre of the top of the Sephadex column and spun precisely as before (2000 rpm for 2 mins). The purified labelled probe in about 50 µl volume was collected in the decapped eppendorf tube, containing the DTT.
- Mixed by vortexing and kept on ice.
- 2 µl of probe solution was transferred to a hot dot (sample range 0.5 - 2µl, SL 8329 # B-QC-7001, 48 cont. HCT.dot.) for counting in the Scotlab Easicounter 4000. Counts per minute recorded below: usually in the range of 5,000-20,000 cpm; calculate dpm [cpm x efficiency of the counter, the Scotlab Easicounter 4000 is assumed to have an efficiency rate of 7%; thus cpm / 7 x 100 = dpm] and diluted in hybridization buffer such that 100 µl will give in the region of 80 – 400,000 counts and preferably in excess of 100,000 dpm/slide.
- Figure 64 is a table showing ^{35}S incorporation into probes, with the relevant volumes to give 100,000-200,000 dpm/slide.

Figure 63: Table of label incorporated into Probes

Probe	cpm	dpm $x10^3$	vol for 2 slides	Dpm x 103 per slide
Egf	2016	14	28□l	196
Psmc1	12435	88	4□l	176
Rps29	9201	65	6□l	195
Rora	2679	19	21□l	199.5
Sox2	6162	44	9□l	198
Sox4	704	-		
Sox6	9183	61	6□l	183
Sox15	3482	25	16□l	200
Sox16	4223	29	14□l	203
Sox17	5956	42	9□l	189
SoxLZ2	3849	27	14□l	189

- Quality of the labelling was checked prior to removing the sections from storage in alcohol and proceeding with the hybridization step.

3.3.4.4. Incubation of sections with labelled probe

- Slides with the appropriate sections were removed from the alcohol, placed in a slide rack and air-dry thoroughly.
- Labelled probe diluted in hybridization buffer to give 100 – 200,000 dpm/100 □l hybridization buffer/slide – see above.
- Slides placed in a sterile Nunc petri dish, (holds approximately 20 slides).

- 100 µl aliquots of hybridization buffer applied to each slide, as 4-5 droplets evenly across the section ensuring uniformly distributed over the section.
- Strips of parafilm were used to cover the sections (the inner surface of the Parafilm is next to the sections); avoiding air bubbles. Dispersing 4-5 droplets of the hybridization buffer over the section and stand for a few seconds before applying the Parafilm.
- The dish was humidified with a piece of Kleenex tissue soaked in 50% formamide/4 x SSC (in-situ wetting solution).
- The petri dish was wrapped with saran wrap.
- Incubated at 42^{o}C overnight.

3.3.5. Post-hybridization treatments

Labelled sections were washed to remove non-hybridized probe under stringency conditions that will reduce background without losing the signal to verify the specificity of the probe for the specific mRNA. RNase contamination is no longer a concern and aseptic techniques were not observed after this point.

3.3.5.1. Slide washing

- 2 L wash solution, placed in three sandwich boxes, maintaining one at 55^{o}C.
- Slides were lifted up from the bottom of the petri dish, using a razor blade, and one at a time, in order, placed under 1 x SSC at room temperature to remove the parafilm coverslip. The parafilm was gently lifted off, without smearing the section, and the slide washed by agitation to remove excess hybridization buffer and unhybridized probe. Slides were then placed in order, in the rack and kept under the 1 x SSC, until the full complement of slides was collected.

- The rack of slides was transferred to a box of wash solution at 55^{o}C for 30 mins.
- The rack of slides was then transferred to a fresh box of wash solution at 55^{o}C for 30 mins.
- The rack of slides was then transferred at room temperature sequentially through the following boxes:
    - 1 X SSC for 5 seconds.
    - 0.1 X SSC for 5 seconds.
    - 18 ohm water for 5 seconds.
    - 70% Ethanol for 2 seconds.
    - 95% Ethanol for 2 seconds.
- The sections were then thoroughly air-dried at room temperature before exposure to dry X-ray film.

3.3.6. Autoradiography

All autoradiographic procedures must be carried out in a light-tight dark room with suitable safelight illumination with appropriate filters and a 15 W bulb (see section 3.1)

3.3.6.1. Exposure to X-ray film

- Card was placed in a cassette, without an intensifying screen and, using double sided sticky tape, rows were created to place the dried slides, in order, side by side, in a perfectly straight line. C^{14} standards at one edge.
- In the dark room, a sheet of X-ray film was placed over the arranged slides, the cassette close securely and seams of the cassette taped with masking tape.

- The cassette was placed in a cool dark place (cupboard or drawer) away from any other source of radiation.
- The length of exposure of the labelled sections to film depends upon several factors including the specific activity of the labelled probe, and the mRNA and its abundance in the relevant sections under investigation, and varies from 3 days to 3 weeks.

3.3.6.2. Development of the X-ray film

Developed in an automatic developer at appropriate settings or as follows:

- Kodak D19 or Ilford D19 developer at 21-23^{o}C for 5 mins.
- Running water for 30 secs.
- Rapid fix (Kodak) for 5 mins.
- Wash in running cool water for 30 mins.
- Air dry for 30 - 60 mins.

Label the film carefully and store in an X-ray envelope.

3.3.7. Emulsion autoradiography

For the cellular localization of mRNA transcripts, dip the sections in liquid photographic emulsion in a dark room under a safe-light.

X-rays were examined to judge, which slides were worth exploring further. It is important to dip sections as soon as possible after autoradiography.

The following was brought together in the darkroom:

- A small water bath with water, preset to run at 43^{o}C.
- Ilford K5, which had been stored at 4^{o}C for no less than 3 months.

- A 50 ml falcon tube containing 40-50 ml of freshly made 600 mM ammonium acetate with glycerol at 0.5% prewarmed to 43^{o}C.
- A glass rod, a dipping chamber, prewarmed in the water bath.
- A flat metal plate placed on an ice tray.
- Staining racks.
- A light-proof container, tape and scissors.

Procedure:

- Approximately 20 mls worth of the liquid photographic emulsion shreds (K5) by volume were placed into a clean 50 ml falcon tube and 'melted' in the 43^{o}C water bath, using the glass rod to ensure no bubbles/lumps occur whilst adding an equal volume of ammonium acetate solution.
- The K5/Ammonium Acetate solution was placed in the dipping chamber held in the water bath, at 43^{o}C.
- A couple of the spare slides were dipped to check the quality of the emulsion and to remove air bubbles.
- Taking the slide, with the section upper most, each slide was slowly immerse in the dipping solution twice with a smooth action ensuring an even coating.
- The underside of the slide was scrapped on the chamber and the back of the slide mopped with a paper towel to remove excess emulsion sticking to the cooled metal plate. The slide was then placed on the cooled metal plate, for approx 10 mins, with emulsion uppermost, to set.
- The dipping chamber reservoir was topped up as necessary to ensure sufficient emulsion was available for the slides to be dipped.

- Once the emulsion was set, the slides were placed in a staining rack and positioned upright in a light proof box containing silica gel for 1-2 hrs at room temperature, to dry.
- Finally, the slides were placed in a slide box with silica gel, sealing the edges, and wrapping in foil. Left at room temperature for a further 2-3 hrs prior to placing at 4^{o}C marked with the expected development date (it pays to have a few at an advanced date, in order to develop early). Development could be anything from 1-12 weeks depending on the strength of hybridization signal.

3.3.7.1. Development of emulsion-coated sections

Emulsion coated sections were removed from 4^{o}C 30 - 60 mins prior to processing, to allow slides to reach room temperature.

Meanwhile, the following was prepared in staining troughs:

- Developer. The temperature of the developer solution should be 20^{o}C $\pm$ 1^{o}C as temperature affects the speed of development (an increase of 1^{o}C adds approx. 15 secs to the developing time). This solution has a short life, and after two or three racks of slides, should be remade.
- Freshly made stop solution (see 3.2.23).
- Fix solution (see 3,2,24). (An alternatively solution is Rapid fixer HYPAM)
- Wash: single distilled/deionised water.

In the darkroom - test slides were transferred to a rack and processed as follows:

Developer:	6 mins
(longer time will give bigger grains, but also more background)	
Stop:	2 mins
Fix:	6 mins
Water:	5 mins
(keep slides moist until required)	

Only the development time is critical, the others are recommended minimum times. At this point it is prudent to check the test slides under the microscope before proceeding with the remainder.

Slides were washed in a sink with slowly running water for 30 mins - 2 hrs.

3.3.8. Counterstaining

A number of different stains may be used to counterstain sections following in-situ hybridisation. Methylene blue is quick and easy, but Hematoxylin and Eosin (H&E) is used here, resulting in the nucleus blue/purple (*hematoxylin*) and cytoplasm pink (*eosin*).

- Slide racks were removed from sink one at a time, remembering to keep slides moist at all times, rinse in double distilled water.
- Immersed in hematoxylin for 10 mins.
- Rinsed in tap water until blue (approx. 5-10 mins).
- Excess hematoxylin was removed with 1% acid ethanol until background clears and nuclei remain blue, should take only a few seconds. If nuclei remain too dark you may subsequently have problems seeing the grains against a dark

background.

- Returned to tap water until blue (approx. 5-10 mins).
- Immersed in 1% eosin for 2 mins.
- Returned to tap water until cytoplasm is pale pink and emulsion relatively clear (approx. 5-10 mins).
- Dehydrated in the following:
- 70% Ethanol for 5 mins (may be left longer if eosin stain is too strong).
- 95% Ethanol for 5 mins
- 100% Ethanol for 5 mins x 3
- Xylene for 5 mins (slides can be left here at this stage).
- Slides removed one at a time from the final xylene.
- A couple of drops of DPX mountant was applied to each section, covered with a tissue polished coverslip and any air bubbles pushed out with tweezers (if dehydration and clearing has not been completely thorough, droplets will appear under the cover slip obscuring the view of the section).

Slides were then ready for observation under the microscope.

3.3.9. Protocol for capturing images

The analysis of the in-situ hybridisation autoradiographs and dipped slides was performed at Pfizer Cambridge with the assistance of Alistair Dixon, Peter Wooding, and Janet Small, and later at the Anatomy department with the assistance of Marie Watkins and Professor Martin Johnson.

• CCD relay camera was connected to the computer with a lens. Either the MTI CCD 72 connected to a Nikon PN-11 52.5 plus / NIKKOR 105mm 1:2,8 # 244228 when

viewing from the illuminator light box, or the Leica DC 200 connected to a Leica DN RB microscope when viewing microscopically.

- M4 icon starts the image software.
- Initially the **graphic window** opens
- Using the scan area toggle ensure the following sensitivity fields are set:

Target defined in: current channel.		Target size: off
Target scan mode: normal.		Filled: off
Measures: group.		Single pixel: count
Keep only last scan shapes		
Segmentation range		highlighting
Width: levels 91	density ROD	red
Centre: 0.755	*high 0.4540*	none
	Low 2.4082	

- Using the options toggle check the following fields:

Targets defined in: channel 1(ch1) or channel 2 (ch2)

Target scan mode:

Normal

Measures:

Group

Targets

Mean size 1

Keep only last scan shapes

- In the **transform window** the following fields were used:

	Target accent	
Channel: source: 1		2: destination

Toggle the following icons

Modify:

kernal size

7 X 7

3 X 3 63 X 63

N channels:

Channel selection: all channels

Filters: target accent

Point operators: none

• In the **calibration window** and viewing a slide, the following settings applied:

Toggle the establish icon to set the distance as a X 40 lens

Calibrated

Distance in μm		cal. directions
Horizontal:	84 pixels 40μm	horizontal and vertical
Vertical:	84 pixels 40μm	

• Ensuring the microscope is visible to the camera at the slide at the top left hand side of the microscope, near the camera was positioned to 50:50 and the computer image adjusted with the fine focus of the microscope.

• The middle mouse button moves the curser from the computer screen to the image screen. From the toolbox icon a shape was selected for the area of interest. With the Ctrl key held down the mouse will increase or decrease the size of the area for scanning. With the Alt key depressed the area for scanning can be rotated.

• Ctrl plus Page Up or Page Down keys switched the image between channels.

• With the above settings, the silver grain representation was in channel 2 and the full image was in channel 1. Through selecting a defined area and confirming with the

right hand mouse button, counts were established as relative optical density (ROD). Fields ranging from ROD, scan area, total target area to count density are chosen from the options toggle in the graphic window and data stemming from this exercise was stored in the graph window for copying and pasting into excel for subsequent analysis.

- Images were copied and saved through either copying the whole image or pasting a selected area of interest onto a black background in another channel and saved.
- Subsequent manipulation of the images was carried out in Adobe photoshop and transferred to power point for printing.

3.3.10. Brief summary of *In-situ* Protocol

Tissues from embryo day stages 8.5d, 9.5d, 10.5d, 13.5d, 15.5d and 17.5dpc were prepared as described, and sections were cut, fixed and stored under ethanol. Probes were prepared as described above. *Sox4* was the only probe that failed to provide sufficient counts to proceed with hybridization, and was not pursued further. Each of these labelled probes was then incubated with sections. Slides were processed as described, put down to film for 6 days, re-exposed for a further 21 days, then dipped and left for a further 21 days, developed and counterstained.

Legends for Figures 64 and 65:

Figure 64: Sections of the autoradiographs captured with a digital camera.

Figure 65: A selection of slides, at x 6 magnification, captured on film.

Figure 64: Autoradiograph composition of slide images

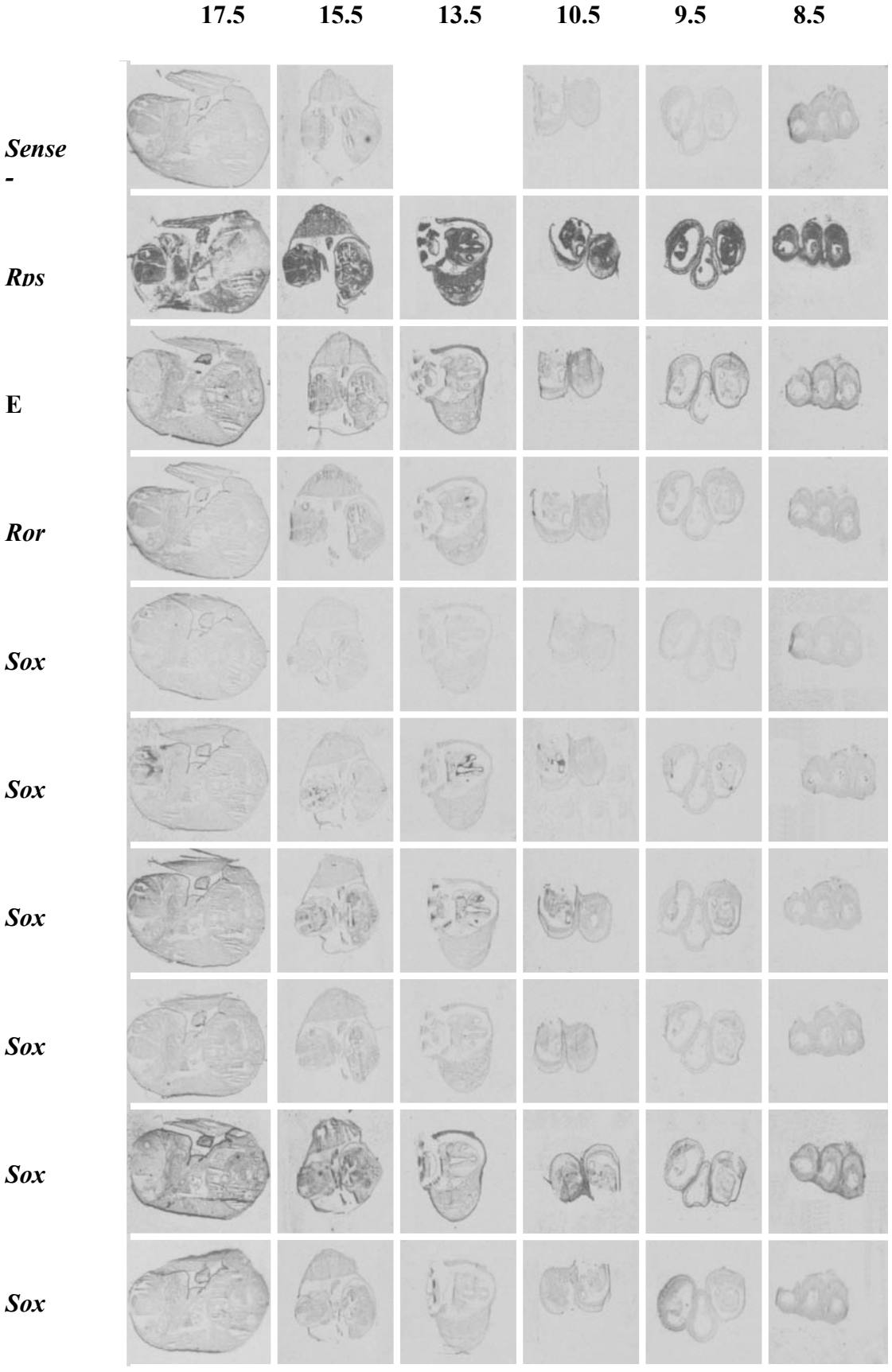
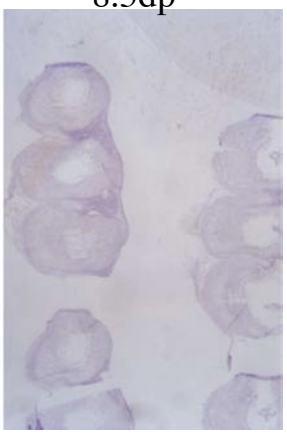


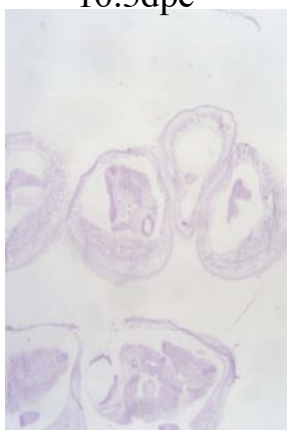
Figure 65: Images of H&E stained tissue sections,

8.5dp

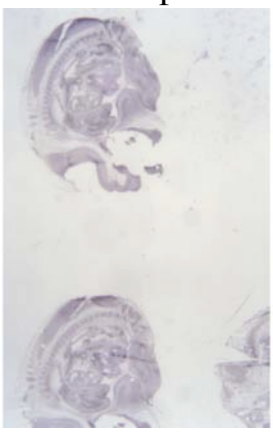


8.5dp

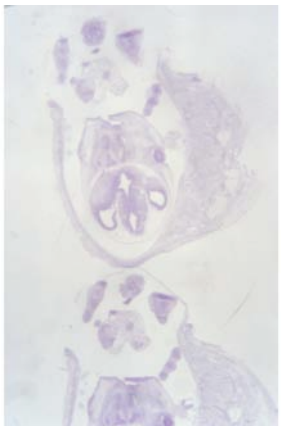
10.5dpc



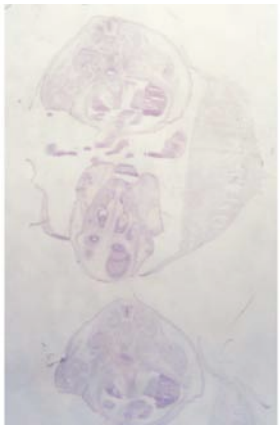
12.5dp



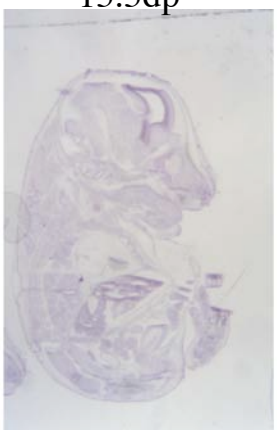
13.5dp



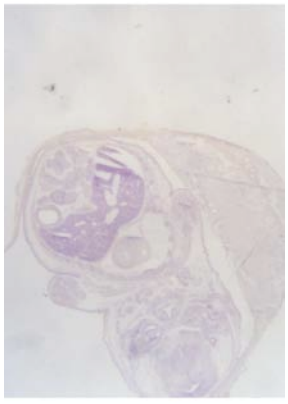
13.5dp



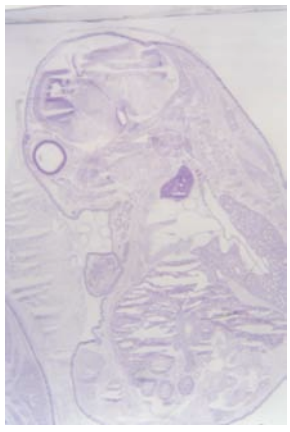
15.5dp



15.5dp



17.5dpc



3.4. Results

3.4.1. Visual Analysis of Slides

A composite of the autoradiographs of slide images is illustrated in Figure 64, showing the 10 probes used in this study on each of 6 staged foetuses. This figure gives an over view of the various probes and their broad localization. It also illustrates the importance of tissue orientation at the time of cryostat sectioning and the low resolution obtained through autoradiography. Figure 65 illustrates the different orientations of the staged embryo sections that were taken. This range of orientations was an unfortunate result of cryostat sectioning the fetuses in their embryonic sacs. Thus, some sections were sagittal (17.5d), some transverse (12.5d, 15.5d), and others of indeterminate orientation (15.5d, 13.5d, 12.5d, 10.5d, and 8.5d), the precise orientation being revealed only at the point of microsopic examination. Appendix 2 highlights the main characteristics used to determine the foetal staging.

The top row of Figure 64 shows hybridisation of the negative control sense probe (*Psmc1)*, which shows no signs of specific hybridisation, other than at the edge of the sections. The positive control probe for the housekeeping gene *Rps 29* is shown in the second row in Figure 64 and hybridises throughout all tissues of all sections, as appropriate for a housekeeping gene. Of the remaining probes in this figure *Egf*, and *Sox16* show hybridisation at the edge of the tissue sections, that appear distinct from non-specific edge effects found with the negative control. *Rora, SoxLZ, Sox15* and *Sox17* showed very little evidence of specific hybridisation, *Sox2* and *Sox6* show the most interesting hybridisation signals to internal structures.

Through dipping the slides, a closer examination of each section was made possible. The process of dipping slides in a photographic emulsion enables a more precise localisation of the radioactive hybridisation position. Microscopic

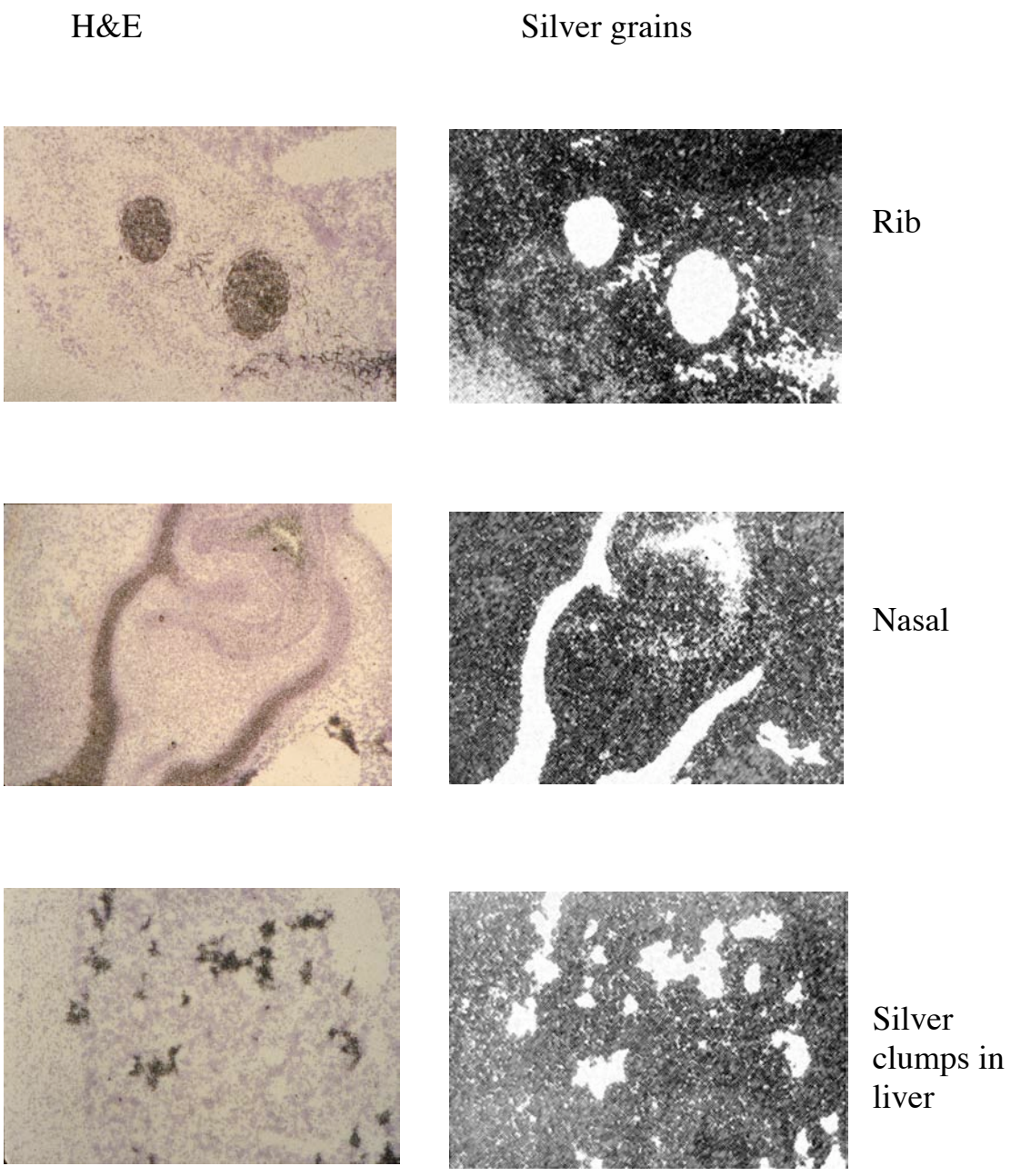
identification of silver grains under dark-field, when compared to the H&E staining assists the process of locating regions of hybridisation. The slides are coated in a gelatinous solution containing silver salts; beta particles from the S^{35}, which radiate out from the section, reduce silver in the emulsion to metallic silver (halide). On developing the sections the sensitized silver ions show as black specks under a light microscope and reflect light in the dark-field. It is this dark-field image that is most informative for data analysis. All resulting figures of *in-situ* counterstaining and silver grains are at x10 magnification with the exception of Figure 75, which is at x40 magnification.

Results from dipped sections for *Egf, Rora, SoxLZ, Sox15, Sox16* and *Sox17* showed few regions suggesting specific *in-situ* signals and those regions were faint in comparison to *Rps29, Sox2* and *Sox6.* Because the object of this exercise was to compare *in-situ* results with those from RTPCR, it was decided to focus on the *Sox2* and *Sox6* data from this series of sections. The results were also compared with those from published papers on gene expression, which were more extensive for these two genes. There was very little literature for the *Sox* genes LZ, 15, 16 & 17, all of which gave weak *in-situ* signals .

The *Egf* probe hybridised to the outer embryonic sac of the early stages (8.5-12.5dpc), placental tissues of 15.5dpc, yolk sac of 17.5dpc, with binding to few other identifiable tissues (see eye below), and gave a clumping effect in the liver (data not shown). This latter effect was regarded as an artefact, since the sense probe, *Psmc1,* also gave this sort of signal. This negative control probe highlighted a number of other potential artefacts, illustrated in Figures 66 and 67, notably an edge effect to some sections, and autofluorescence from cartilage and retina pigment layer. These

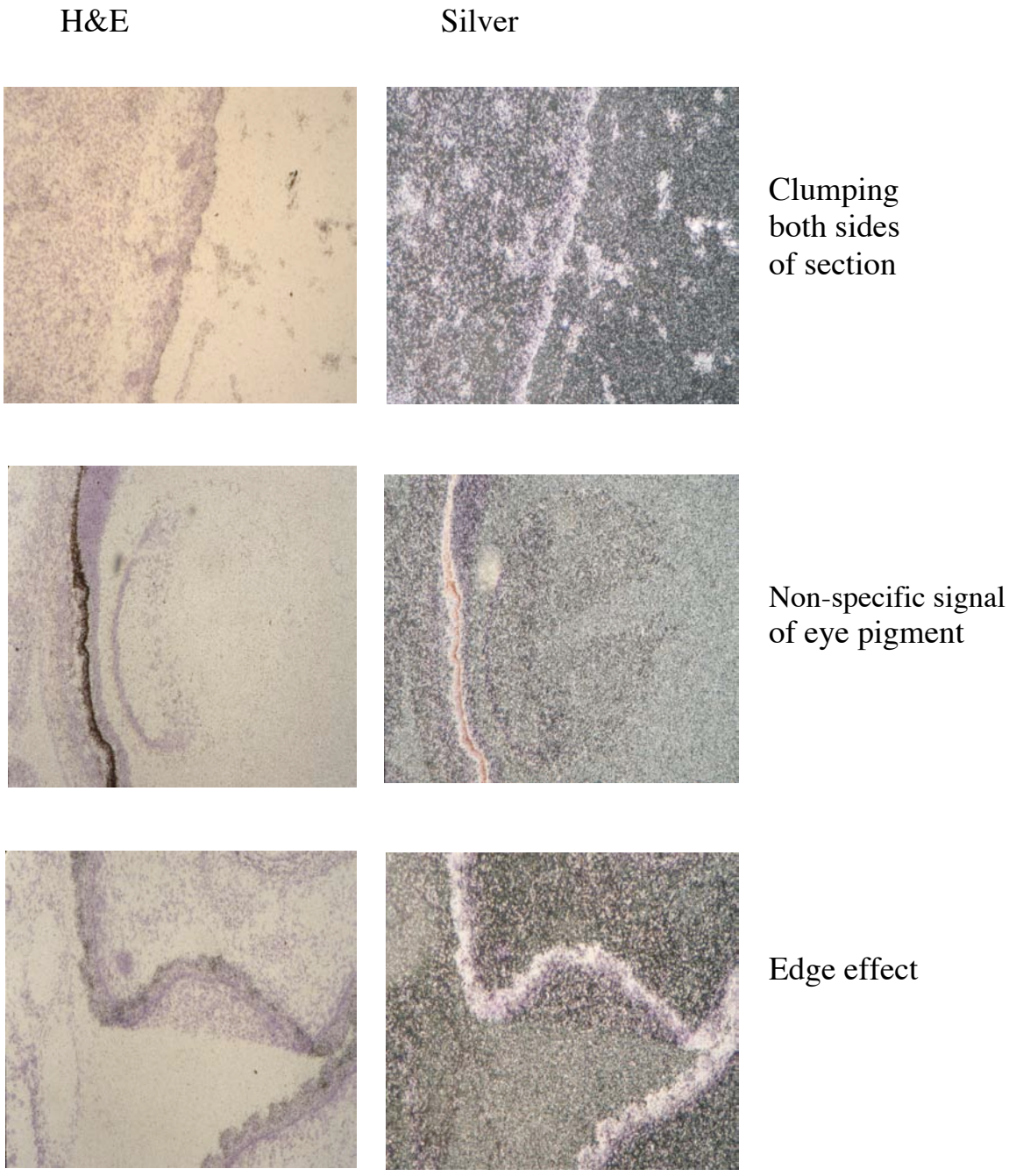
artefactual effects were ignored as non-specific signals when found during the charting of expression profiles using the two more successful probes *Sox2* and *Sox6*. Specific hybridisation for these two probes are set out as tables in Figures 68 and 69.

Figure 66: Artifacts of Silver Staining.



Microscope images at x 10 magnification, from H&E staining and silver dipping, captured on film, illustrating non-specific signal.

Figure 67: Silver Staining Artifacts



Microscope images at x 10 magnification, from H&E staining and silver dipping, captured on film, illustrating non-specific signal.

Figure 68: Chart of *Sox2 In-situ* hybridisation

Tissue/day	Forebrain/Head region	Midbrain	Hindbrain	Spinal cord	Heart	Lung	Liver	Intestine
17.5	Telencephalon Sensory layer of retina	Dorsal thalamus	Mescencephalon	Ependymal layer of spinal canal	ND	Lining cells of oropharynx	faint	ND
15.5	Telencephalon Cells lining the nasal passage Sensory layer of retina Optic chiasma	Dorsal thalamus Olfactory epithelium Recess of 3rd ventricle	Mescencephalon	Ependymal layer of spinal canal	NA	Lining of mouth, Olfactory epithelium. (Rathke's pocket) Bronchi	ND	ND
13.5	Semilunar ganglion Cochlea	Dorsal thalamus	Mescencephalon	Ependymal layer of spinal canal	NA	NA	ND	NA
12.5	Telencephalon	Present	Myelencephalon	Spinal canal	NA	NA	ND	ND
10.5	Ventricle edge of Telencephalon	ND	Myelencephalon	NA	NA	NA	NA	NA
8.5	Forebrain Stomodaeum region	NA	NA	Neural fold	NA	NA	NA	NA
Tissue/day	**Oesophagus/ stomach**	**Urogenital**	**Kidney**	**Gonads**	**Bladder**	**Forelimb/Rest of body**	**Placenta**	**Yolk sac**
17.5	Duodenum	ND	Kidney	NA	ND	Epidermis Cartilage	ND	NA
15.5	Mucosal lining of stomach	Lining of urogenital sinus	ND	NA	NA	Epidermis	NA	NA
13.5	Oesophagus Mucosal lining of stomach	ND	ND	NA	NA	Epidermis	ND	ND
12.5	Stomach	ND	ND	NA	NA	Epidermis	ND	ND
10.5	NA	NA	NA	NA	NA	NA	ND	ND
8.5	NA	NA	NA	NA	NA	NA	ND	ND

ND – signal Not Detected
NA - tissue Not Available for informative diagnosis

Figure 69: Chart of *Sox6 In-situ* hybridisation

Tissue/day	Forebrain/Head region	Midbrain	Hindbrain	Spinal cord	Heart	Lung	Liver	Intestine
17.5	Sensory layer of retina Ganglion	ND	ND	ND	ND	ND	Present	ND
15.5	Nasal capsule. Cortex Meckel's cartilage (jaw)	Present	ND	Ependymal layer of spinal canal Spinal ganglion.	NA	Trachea	Present	ND
13.5	Telencephalon Nasal Cartilage Meckel's cartilage	ND	ND	Ependymal layer of spinal canal	NA	Bronchi	Present	NA
12.5	Neopallial cortex Nasal Cartilage Meckel's cartilage	Midbrain Striatum	Medulla	ND	NA	NA	ND	ND
10.5	Telencephalon Neural fold	Myelencephalon	ND	NA	NA	NA	NA	NA
8.5	Squamous endoderm	NA	NA	Neural fold	NA	NA	NA	NA
Tissue/day	**Oesophagus/ stomach**	**Urogenital**	**Kidney**	**Gonads**	**Bladder**	**Forelimb/Rest of body**	**Placenta**	**Yolk sac**
17.5	ND	ND	ND	NA	ND	Cartilage	ND	NA
15.5	Duodenum	ND	ND	NA	NA	Cartilage	NA	NA
13.5	ND	ND	ND	NA	NA	ND	ND	ND
12.5	ND	ND	ND	NA	NA	NA	ND	ND
10.5	NA	NA	NA	NA	NA	NA	Present	ND
8.5	NA	NA	NA	NA	NA	NA	ND	ND

ND – signal Not Detected
NA - tissue Not Available for informative diagnosis

Of the 90 cDNAs represented in the mouse foetal panel, those not represented in the *in-situ* analysis are the 5 controls – two glycogen blanks, genomic mouse, rat and human DNA, 11 adult tissues, a single 9.5dpc and two 11.5dpc samples. Of the remaining 71 tissue samples, only 50 tissues were presented for analysis, those missing were not identifiable in sections available (as shown by "NA" in the charts). For all those tissues that were identified in sections and so available for analysis, the concordance between RTPCR and *in-situ* analysis was expressed in percentage terms for each of the two *Sox* oligo probes analysed here.

For *Sox2*, there were 31 cases in which a signal was detected in both the *in-situ* and the RTPCR tissue samples, and 4 cases in which no signal (ND) coincided with a negative value for RTPCR. Collectively, this gave a 70% score where the RTPCR signal was confirmed by the *in-situ* signal.

The remaining 30% (15) of cases were all where there was no signal (ND) detected *in-situ,* but where a positive value had been found by RTPCR. There were no cases of tissues showing a signal from *in-situ* analysis, with a negative signal in the RTPCR. Reported expression of *Sox2* primarily concerns the preimplantation stages [1], developing CNS [2], and the lens [3] [4]. Precise sub-localisation of *Sox2,* expression was found here for the following areas: the ganglion, amacrine, synaptic and plex layers of the eye (17.5dpc), cells lining the oropharynx (17.5dpc), Rathke's pocket (15.5dpc), lining of the urogenital sinus (15.5dpc), the vibrissae (15.5dpc), the bronchi of the lung (15.5dpc), the urethra (13.5dpc), and the ependymal layer of the spinal cord (13.5 and 15.5dpc), and much of the developing brain and spinal cord from 8.5dpc through to 17.5dpc, confirming it's importance as a lens and CNS developmentally important gene.

For *Sox6*, there were 20 cases where there was signal for *Sox6* in both the *in-situ* and the RTPCR tissue samples, and 2 incidences where no signal (ND) was coincident with a negative value for RTPCR. Collectively, this gave a 56% score where the RTPCR signal was confirmed by the *in-situ* signal. The remaining 44% (28) of cases were again where no signal (ND) was found in the *in-situ* analysis, but where a positive signal had been found by RTPCR. Again, there were no cases of tissues showing signal from *in-situ* with a negative signal in the RTPCR. The majority of the known literature relating to *Sox6* expression reinforces the view of this gene as an essential component of the developing CNS and cartilage formation [5-9]. Confirmation of this gene's involvement in cartilage formation is shown here through hybridisation to cartilage structures in the forelimb, ribs, Meckel's and nasal cartilage. It was also found in the 10.5 dpc placenta, 15.5 dpc duodenum, the liver from 13.5 – 17.5 dpc, trachea of 15.5 dpc and bronchi of lung at 13.5 dpc indicating that it may have a wider role in development.

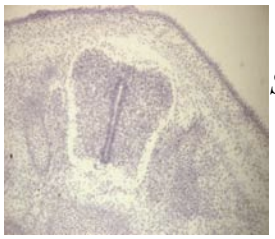
One of the most striking regions of expression was the spinal cord region in 13.5dpc sections (Figure 70 for 10 of the probes demonstrates the benefit of silver dipping). These regions of silver grain staining have been quantified using the method described in 3.3.9 and shown below graphically as Figure 80. A closer image of the *Sox2* probe hybridisation to the spinal cord is shown as Figure 71, which identifies the target area more precisely as the ependymal layer of the spinal cord as shown by the diagram in Figure 72 (p.269, The Mouse It's Reproduction and Development by Roberts Rugh).

The top four panels of Figure 73 illustrate a region of the lung in 15.5dpc mouse. *Sox2* is compared to the control sense probe (*Psmc1*) showing specific labelling of the bronchi by the probe for *Sox2*, illustrating that the *in-situ* technique

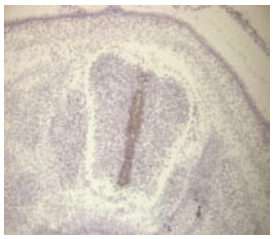
provides useful localisation information undetected by RTPCR alone. The lower four panels of Figure 73 shows the snout region of the 15.5dpc mouse, illustrating specific hybridisation of *Sox2* to the primordial vibrissae follicles (whiskers growth site), again compared to the sense probe.

Figure 74 is of the olfactory region of the 15.5dpc mouse, showing *Sox2, SoxLZ, Sox6 and Sox16* probe hybridisations, together with the H&E counterstaining to identify the region of interest. This figure shows a strong signal for the *Sox2* probe, *SoxLZ* shows no specific signal, *Sox6* and *Sox16* are showing signal at the outer perimeter of the olfactory region, most likely cartilage. In comparison, the cartilage artefactual signal illustrated in Figure 66 when viewed under the microscope is a far brighter and intense signal.

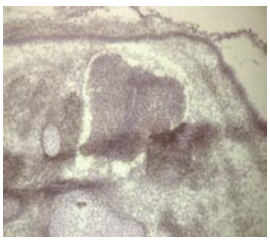
Figure 70: 13.5d region of spinal cord hybridisation results



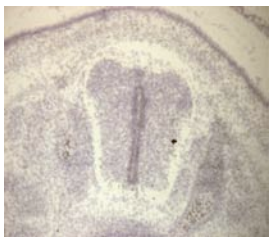
Sense - Psmc1



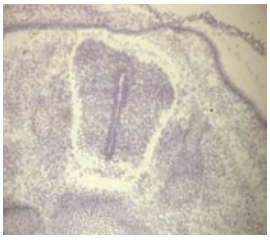
Sox 2



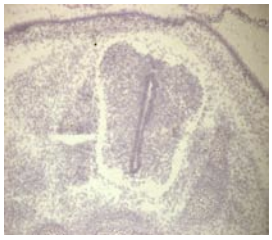
Rps2



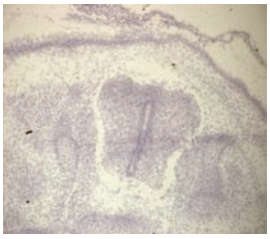
Sox 6



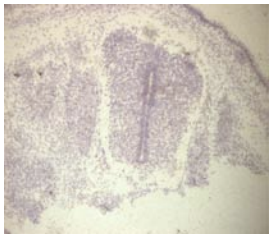
Egf



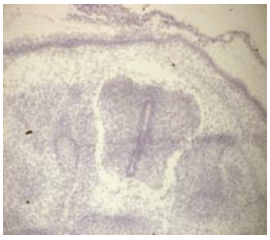
Sox 15



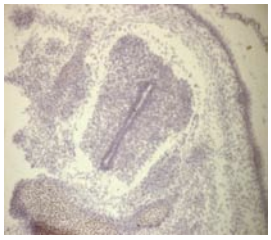
Rora



Sox 16



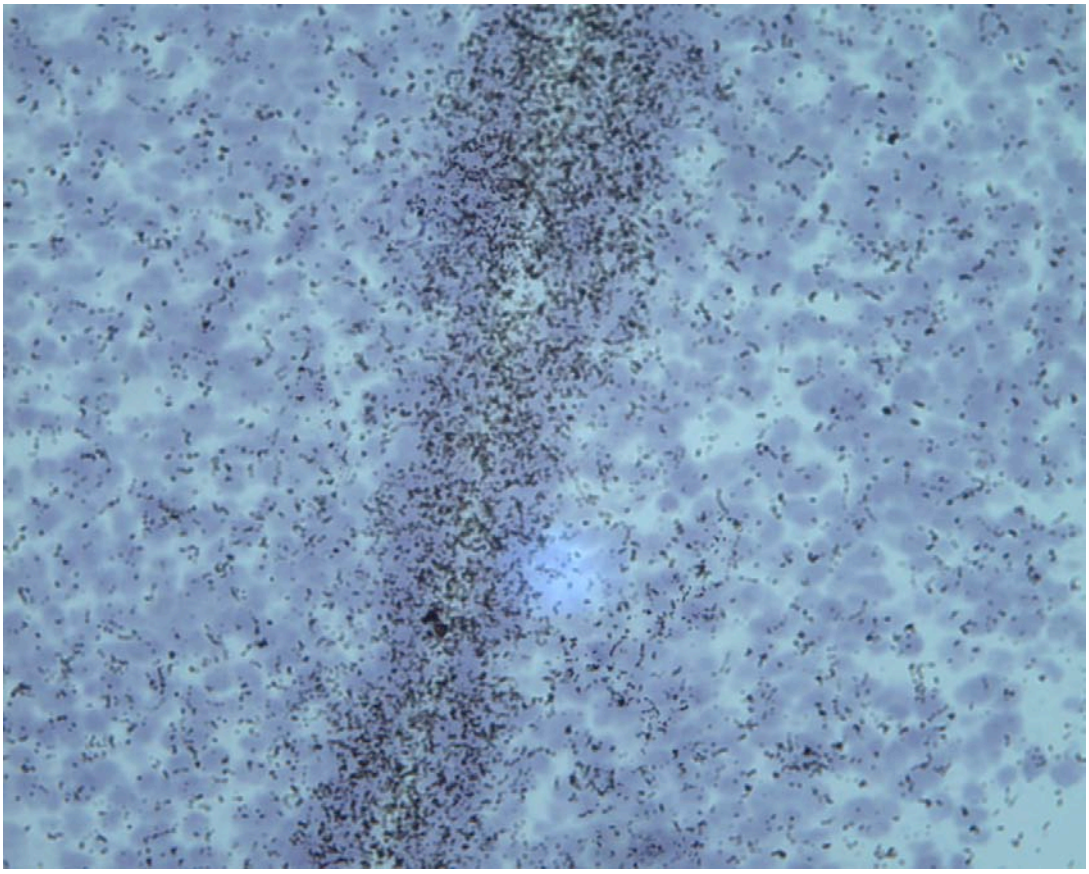
SoxLZ



Sox 17

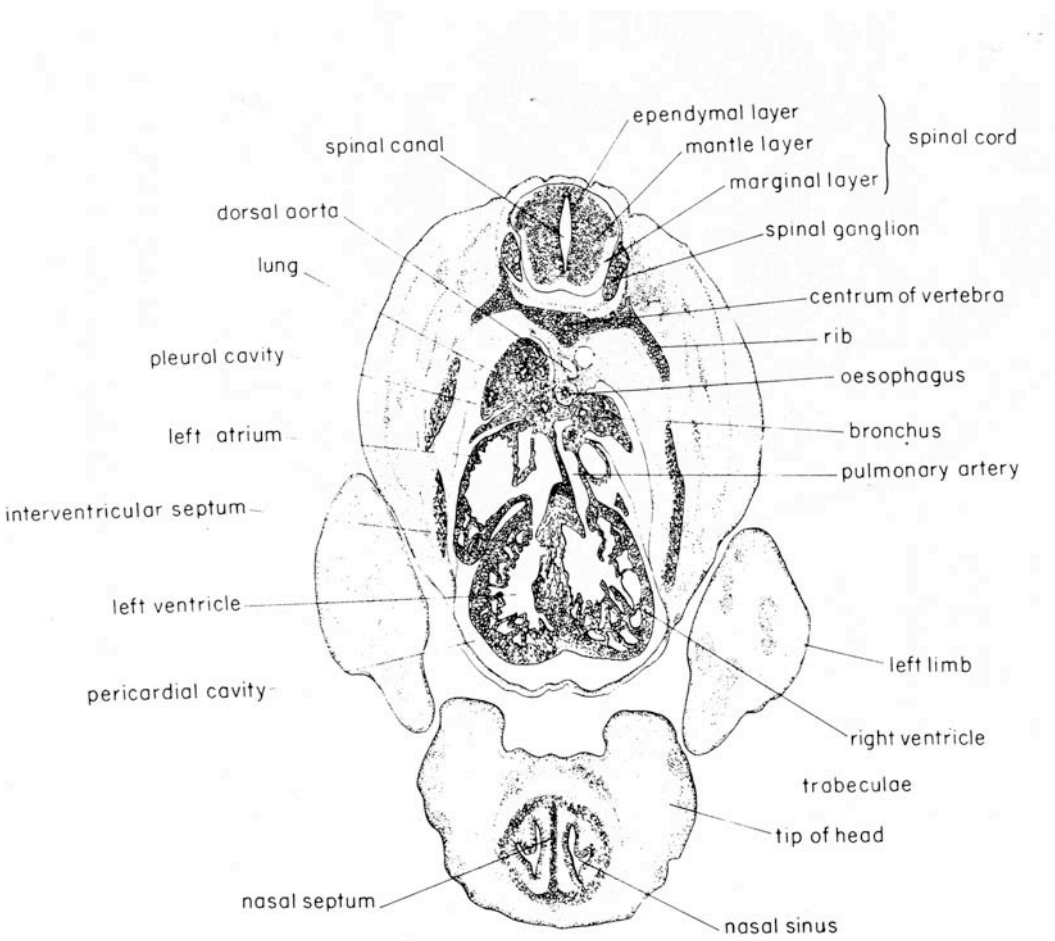
Spinal cord view of 13.5d sections, silver dipped, x 10 magnification. Illustrating the ependymal layer with all 10 probes.

Figure 71: Ependymal Layer of 13.5dpc spinal cord, hybridised to *Sox2* (x40)



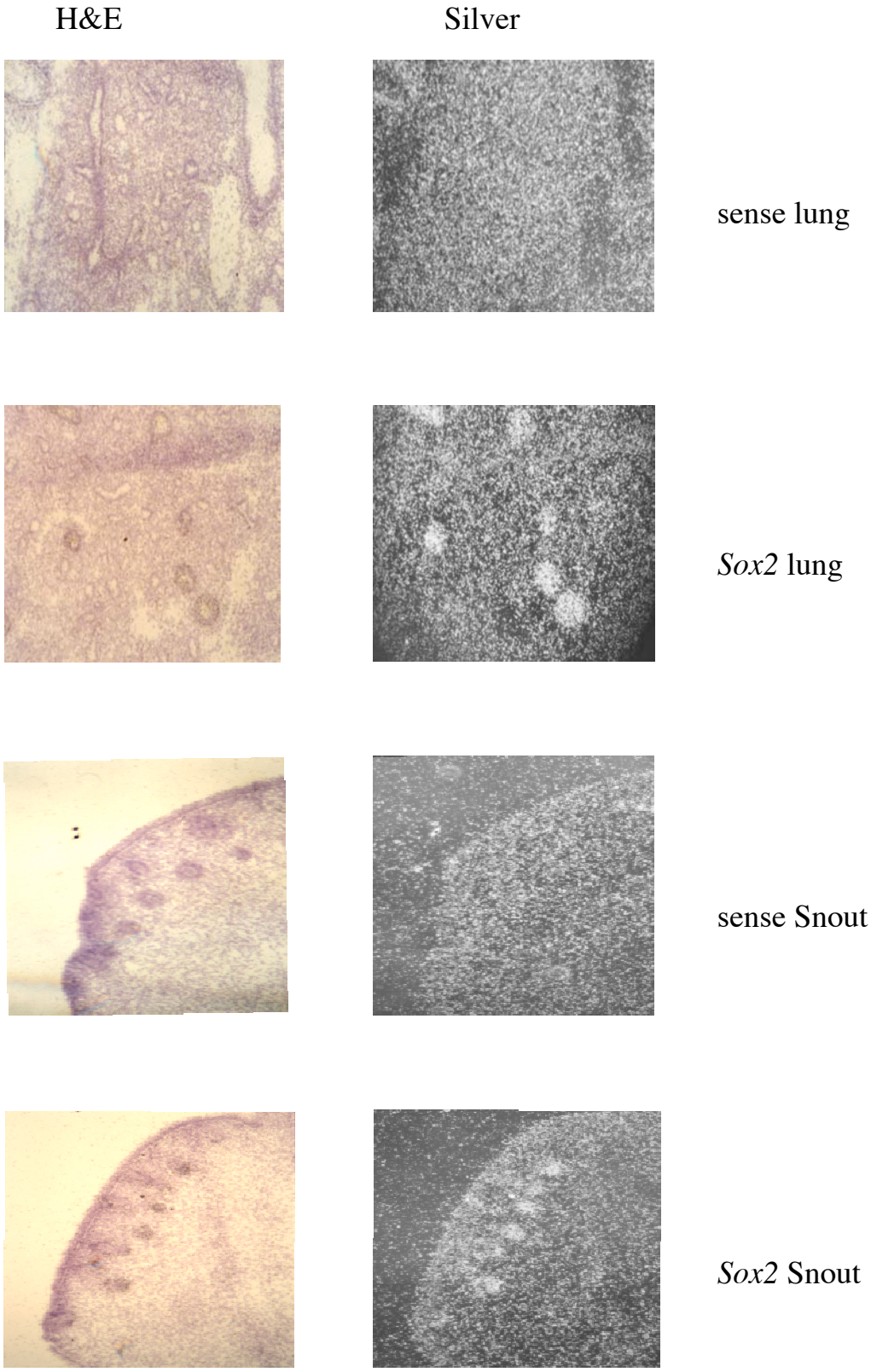
Spinal cord view of 13.5d sections, silver dipped, x 40 magnification. Illustrating the ependymal layer for the *Sox2* probe.

Figure 72: Illustration of a transverse section of a mouse embryo at 14 days gestation



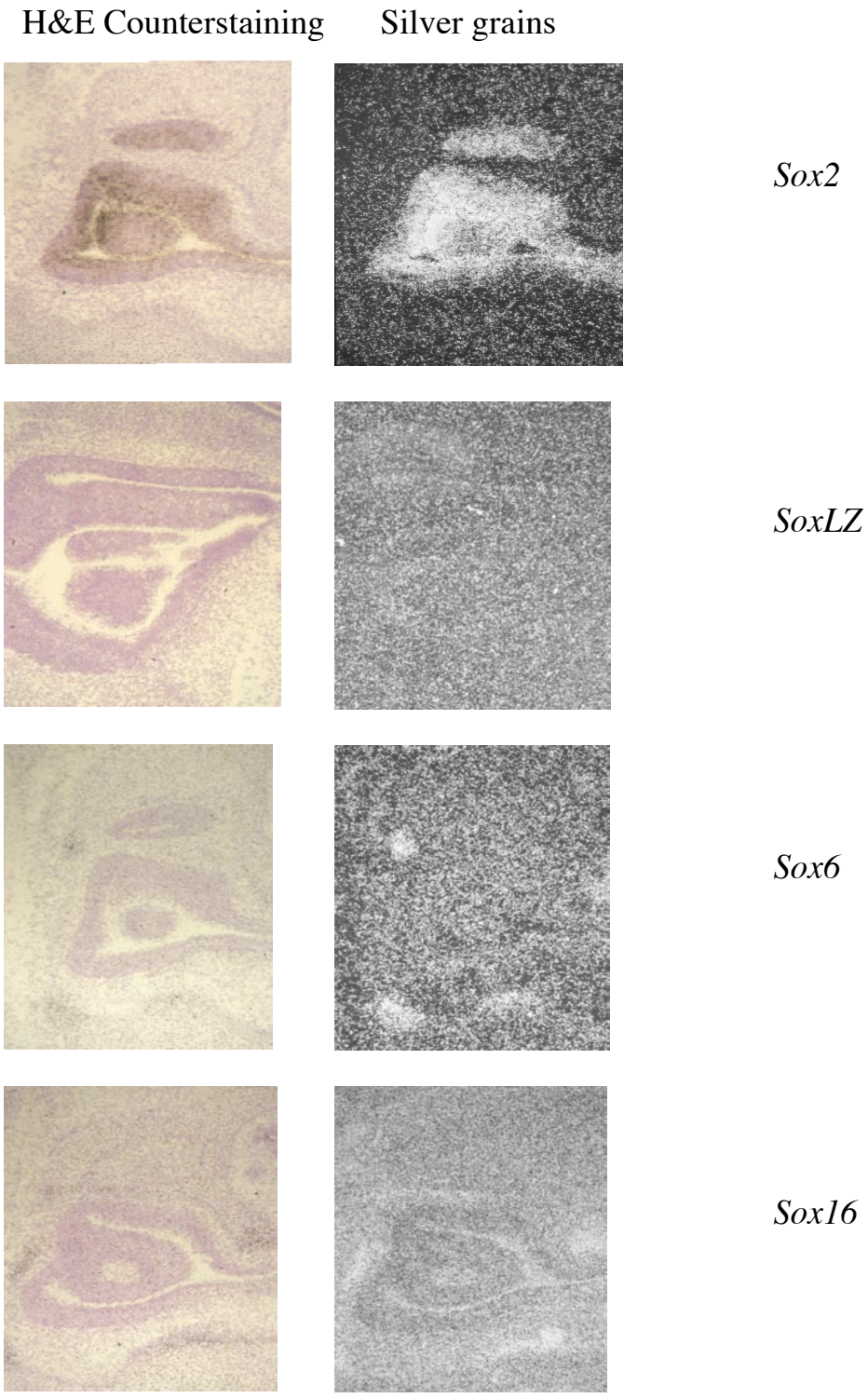
Reference: The Mouse It's Reproduction and Development. Roberts Rugh

Figure 73: Lung and Snout regions



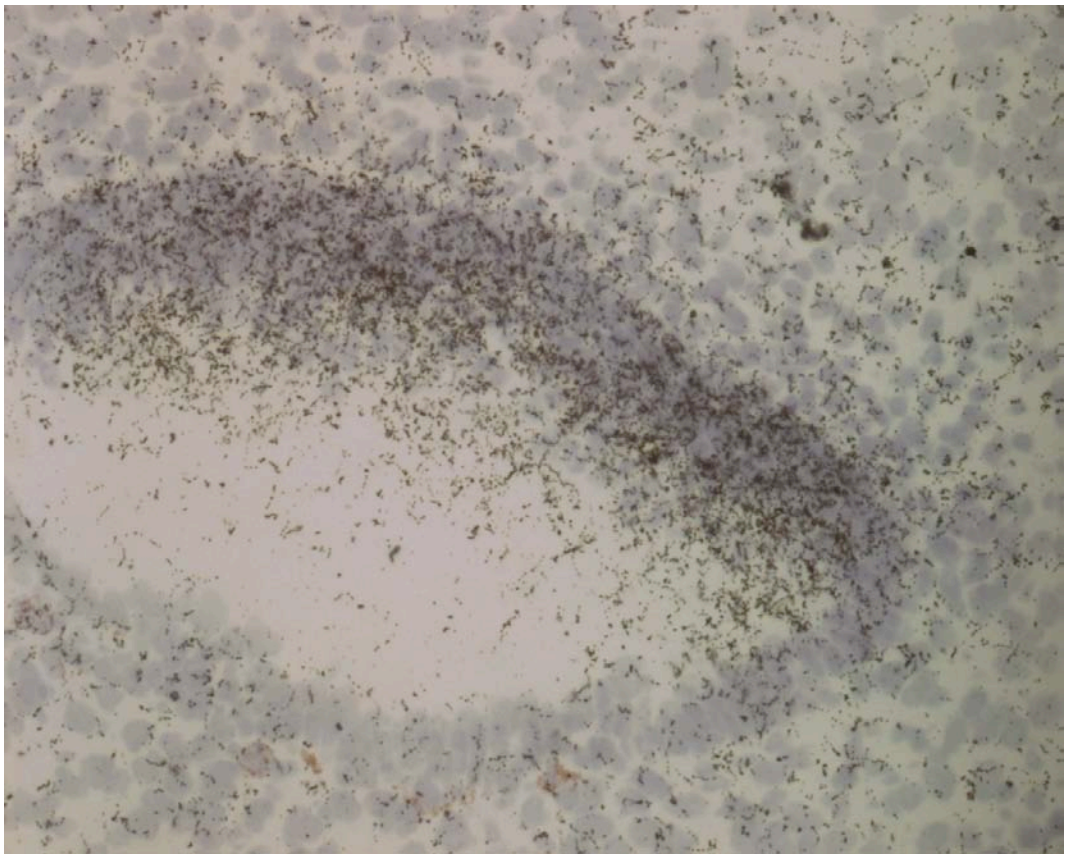
Microscope images at x 10 magnification, from H&E staining and silver dipping, captured on film, illustrating signal due to specific hybridisation.

Figure 74: Olfactory region of stage 15.5d



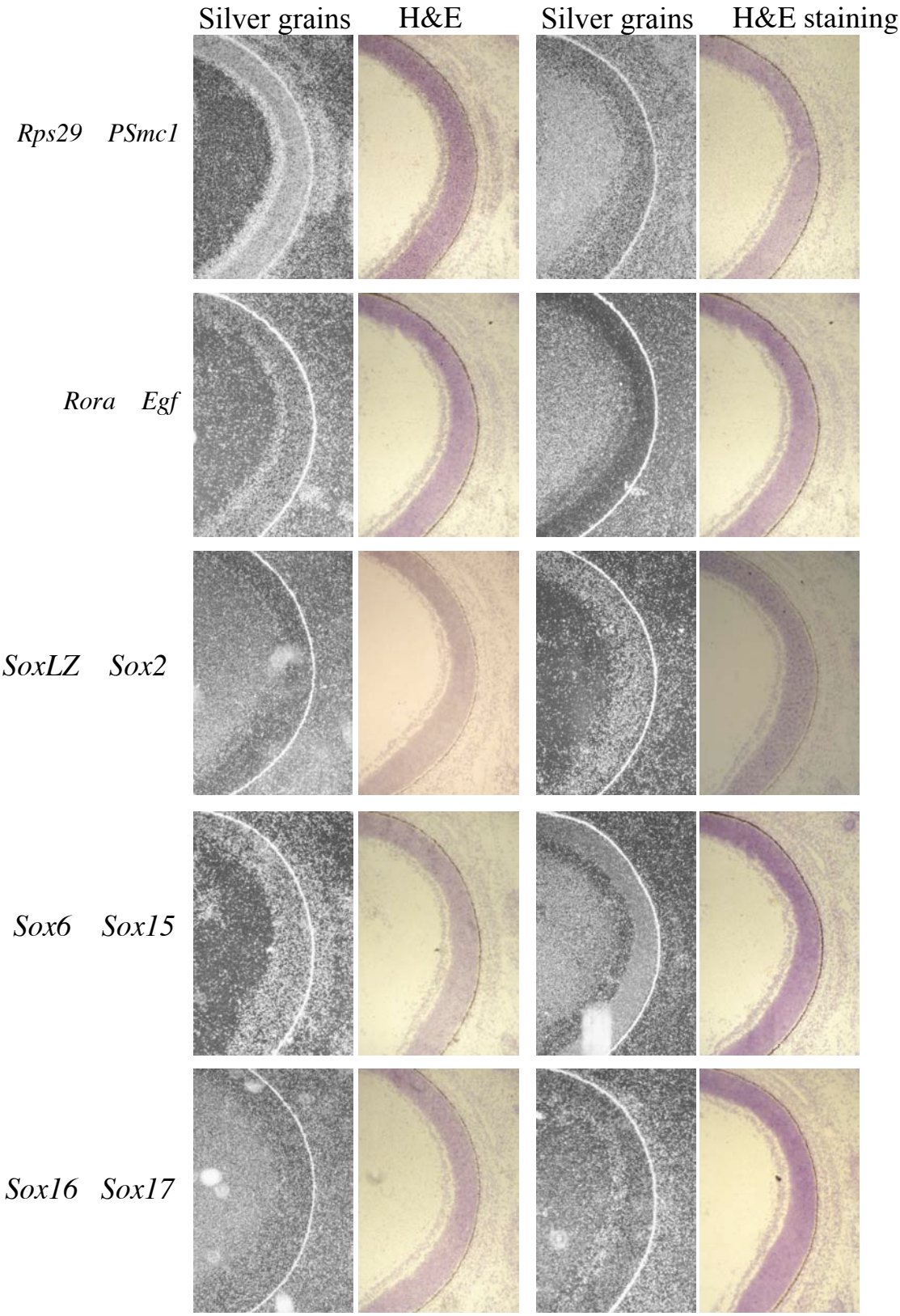
Microscope images at x 10 magnification, from H&E staining and silver dipping, captured on film, illustrating signal due to specific hybridisation in the olfactory region.

Figure 75: 15.5d stage cochlea, hybridised to *Sox2*



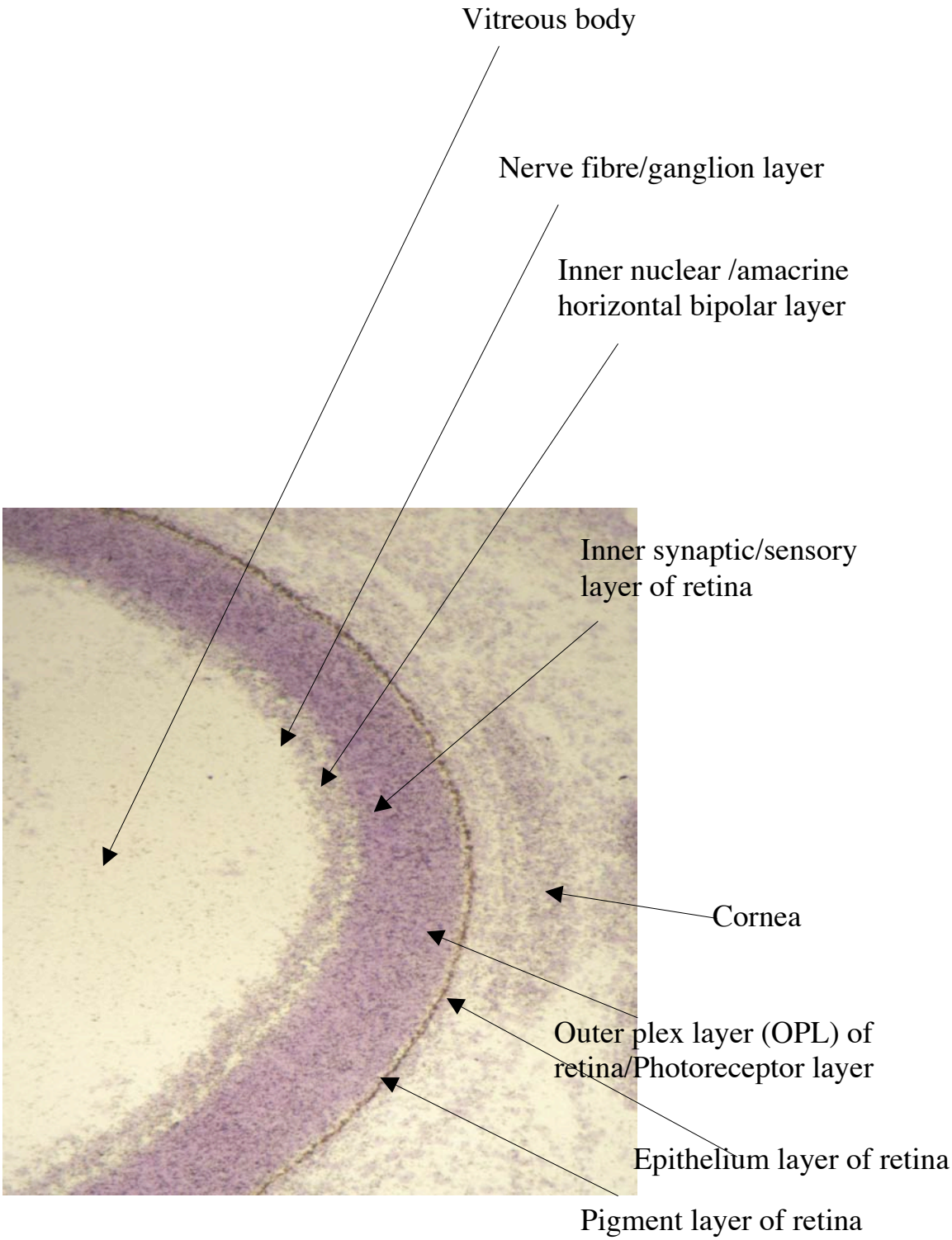
Microscope images at x 10 magnification, from H&E staining and silver dipping, captured on film, illustrating signal due to specific hybridisation in the cochlea.

Figure 76: 17.5d Eye hybridization results



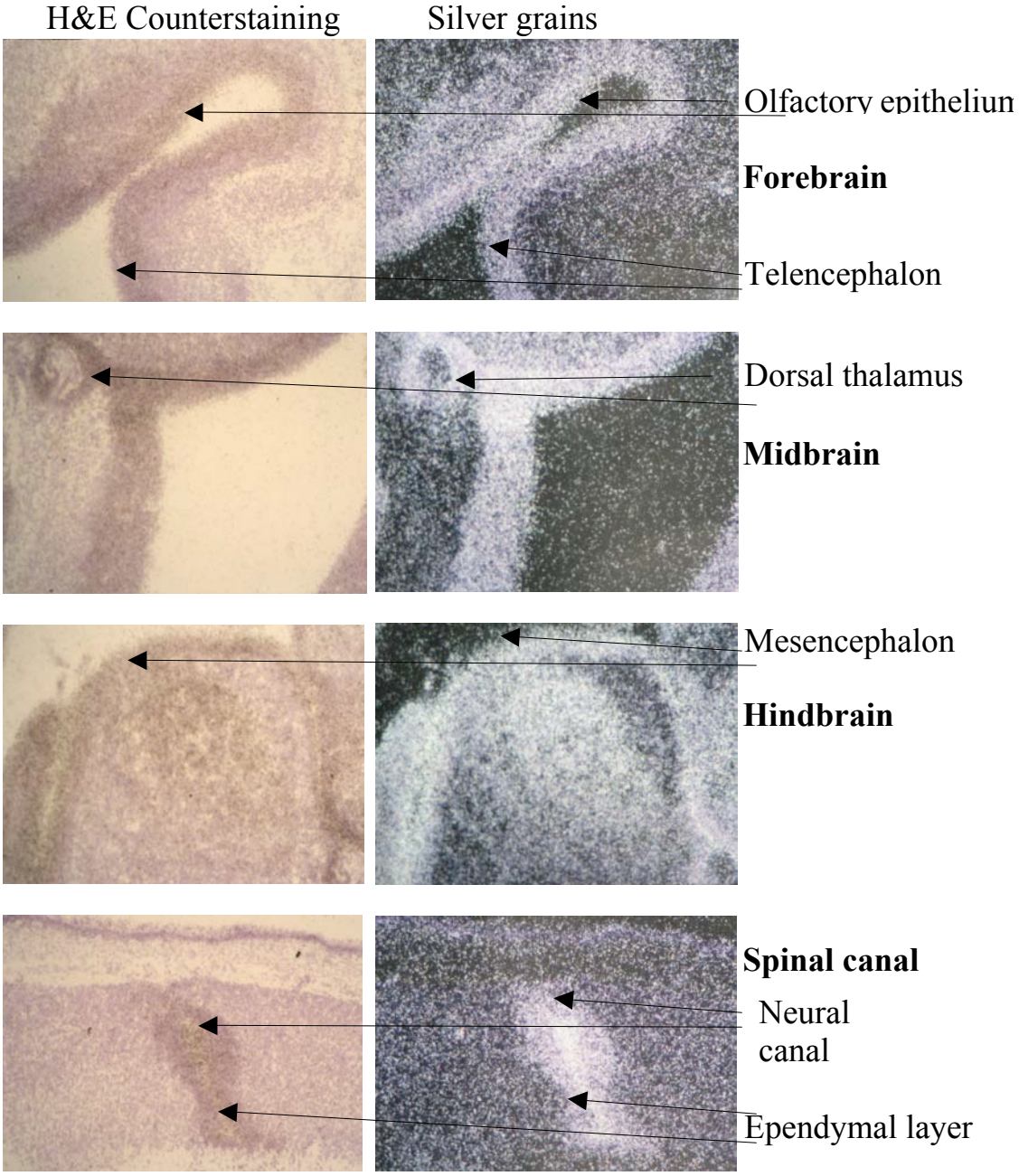
Microscope images x 10 magnification, from H&E staining and silver dipping, captured on film, illustrating signal due to specific hybridisation in the 17.5d eye. The signal in pigmented epithelium is a non-specific artefact.

Figure 77: 17.5d Eye - identification of layers



Microscope image x 10 magnification, from H&E staining, captured on film, illustrating layers of the 17.5d eye.

Figure 79: Neural tissues of 15.5d hybridised to *Sox2*



Microscope images x 10 magnification, from H&E staining and silver dipping, captured on film, illustrating signal due to specific *Sox2* hybridisation in the 15.5d brain.

Figure 75 shows the *Sox2* probe hybridising to the 15.5dpc cochlea.

Figure 76, is a composite of H&E and DF (dark-field) images from the 17.5dpc mouse eye for all probes, and illustrates the variety of tissue layers as the embryo progresses to full term and the probe hybridisation at this stage. Figure 77 illustrates the 17.5dpc eye with the various cell types identified. These data are tabulated as Figure 80.

Figure 78: Table of specific silver staining in the eye

Probe /tissue	Vitreous body	Nervefibre /ganglion layer	Inner Amarcrine /nuclear layer	Synaptic /sensory layer	Outer plex. /nuclear layer
Rps29	-	+	+	+	+
Psmc1	+	-	+	-	-
Rora	-	+	+	-	-
Egf	+	-	+	-	-
SoxLZ	+	-	+	-	-
Sox2	-	+	+	+	+
Sox6	-	+	+	+	+
Sox15	+	-	-	+	+
Sox16	+	-	-	-	-
Sox17	-	+	+	-	-

From the tabulated results it can be seen that *Egf* and *SoxLZ* have similar expression patterns, as do *Sox2* and *Sox6*. It is known from studies in chick that *Sox2* is an important gene in eye development [10], but the co-expression of *Sox6* for this stage has not to date been recorded. The genes *Rps29, Psmc1, Rora, Egf, Sox2, Sox6, and Sox15* (but not *Sox16, Sox17 or SoxLZ)* have all been reported previously as being found in mouse adult eye [11], though the precise location had not been identified.

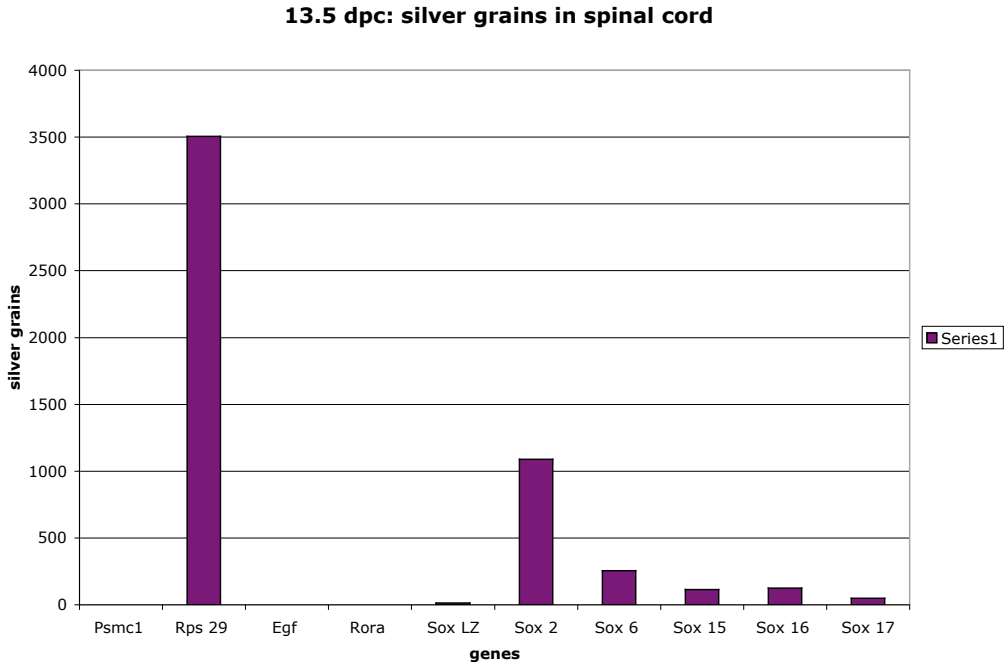
Figure 79 is a composite of various brain regions showing *Sox2* localisation at 15.5dpc. This study confirms the presence of *Sox2* expression in brain and spinal cord [12] for the stages 13.5dpc through to 17.5dpc and begins to provide some anatomical sub-localisation.

3.4.2. Digital Analysis of Slides

The ependymal layer of the spinal cord for 13.5d sections was captured digitally from the microscope and analysed using an MCID image M4 analyser software. This software measures the intensity of silver grains in a user-defined region. The output file contains the scanned area illustrating the total region analysed and total target area as a measure of silver grains present in the scanned area.

Figure 80: Data and Graph of 13.5d ependymal layer silver grain counts

Gene	Scanned area	Total target area
sense	44457	0
Rps 29	12042	3507
Egf	20148	0
Nuc. hor.rec	20148	0
Sox LZ	43765	13
Sox 2	48082	1088
Sox 6	38006	254
Sox 15	23165	115
Sox 16	31722	124
Sox 17	44259	50



The graph illustrates the silver grain concentrations as a measure of probe intensity for the ependymal layer of the spinal cord in Figure 70. The *Sox*2 probe hybridised significantly more to this region than any other probe with the exception of *Rps29,* confirming the visual analysis. The antisense control gene *Psmc1*, showed no recordable signal. *Egf*, which had shown a trace signal at 35 cycles in the adult spinal cord [13], gave no measurable signal in the ependymal layer for the 13.5 dpc spinal cord in this study. *Rora*, had shown a weak to moderate signal at RTPCR in the adult mouse panel [13], but similarly gave no measurable signal in the ependymal layer for the 13.5 dpc spinal cord in this study. As these genes were not included at the RTPCR step for fetal stages, the lack of an *in-situ* signal may be due to these genes not expressing in foetal spinal cord or merely not being present in the ependymal layer. A more thorough study would include these genes at the RTPCR stage for confirmation.

However, for the Sox gene family, RTPCR was undertaken using the fetal panel, and so the results from each approach can be compared. The strong RTPCR signals for *Rps29* and *Sox2* found in the 13.5 dpc spinal cord is localised here to the ependymal layer. In contrast, the strong RTPCR signal for *SoxLZ* is not mirrored in the *in-situ* hybridisation for the ependymal layer, nor is there a signal in tissues surrounding this layer, which may reflect the poor overall signal from this probe at the level of *in-situ* or may be because the region of the spinal cord expressing *SoxLZ* is not represented in this analysis. Both *Sox2* and *Sox6,* gave a strong RTPCR signal at all spinal cord stages; in this analysis *Sox6* is expressed at one quarter of the *Sox2* level in the region of the ependymal layer shown, illustrating a level of quantitation between genes, which can not be deduced from the RTPCR profiles alone. The probes for *Sox15, Sox16* and *Sox17* show 10.5%, 11.5% and 4.5% respectively of the signal represented by the *Sox2* probe, for the ependymal layer of the spinal cord at 13.5 dpc,. The RTPCR profiling for these genes gave trace amounts for the whole spinal cord from *Sox15* and *Sox16* and weak to moderate amounts of signal for *Sox17.* The levels of expression found by RTPCR relate to expression levels within the whole panel for a given gene but for the absolute levels to be in relationship to other genes, the method of *in-situ* hybridisation provides a clearer picture.

Measurements of this nature can be employed to analyse expression over time in a given region, as the software compensates for area differences and can be used to show differences in silver grain intensities for dissimilar sized areas. This type of analysis can be useful in quantifying expression rates in sections, once slide-dipping techniques have been mastered. Common artefacts associated with this technique include (i) high backgrounds due to poor slide handling, storage and poor darkroom facilities, (ii) edge effects as a result of rapid emulsion drying, (iii) uneven emulsion

layering over sections resulting from unmelted emulsion at time of dipping, (iv) loss of silver grains during staining, due to use of alcohol at too low a pH (pH should be 5.2) and/or immersion for too long, and finally (v) talc from gloves dropped in the emulsion can form bright centres on tissue sections, clouding genuine hybridisation data. From my experience in this study, this technique requires careful attention to detail.

3.4.3. Concluding Remarks

Data from the *in-situ* technique can be analysed in a number of different ways.

1. Visually to get a qualitative picture of expression as long as the specificity of hybridisation is secure. Hence rigorous controls and strict attention to methodology is important.

2. Quantitative information from good quality autoradiographs with ^{14}C microscales included as controls. This image can be scanned and the specific region of expression quantified computationally by measuring a defined region in relationship to the control microscales, using the NIH image software freely available from http://rsb.info.nih.gov/nih-image/, or Scion Image software. The optical density of the captured image is compared to the microscale values, resulting in a measure as nanoCuries per gram of tissue weight. This technique gives a numerical relationship to the image, illustrating the different intensities of hybridisation, between sections on the same autorad, or between different autoradographs containing the same set of microscales.

3. Sub localisation and refined anatomical information can be observed microscopically and silver grains counted (see section 3.4.2.) for a more detailed

numerical analysis. Where a refinement of the data is required, silver slide dipping permits closer examination of the defined region to identify the precise position of hybridisation. When the silver grains have been applied as fine particles and their number counted, resulting data give a clearer indication of where the individual probes have hybridised the most.

Through conducting *in-situ* hybridisation, a more detailed knowledge has been gained for some of the probes with confirmatory evidence of expression previously found at the level of RTPCR.

Discrepancies that exist between RTPCR and the *in-situ* results may reflect the lower sensitivity of the *in-situ* technique, or possibly, that a minority sub-component of the tissue was responsible for the PCR signal but proportionately too small to be detected by *in-situ* analysis. It is also possible that the correctly orientated section was not available for analysis by *in-situ*, and a wider ranged *in-situ* study could shed light on more of possible sub-localisation of the expression found at the level of RTPCR. Where an *in-situ* signal was detected, this did allow the localisation of mRNA within composite complex tissue samples used for RTPCR analysis, as illustrated in the charts for *Sox2* and *Sox6* and figures showing defined target areas. Therefore, the two approaches were complementary and additive. Overall, results shown here for the *Sox2* and *Sox6* probes provide evidence that the panel can produce reliable information.

From the diagram (Figure 3, Introduction) illustrating the structure of the *Sox* genes, the two genes *Sox2* and Sox6 are from two different groups B1 and D. They have very different structures and are thought to have different functions in the cell. The B1 group members are regarded as transcription activators [14], involved in

neurogenesis, where as the group D are involved in chondrogenesis and spermogenosis.

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