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Sensitive mRNA detection using unfixed tissue: combined radioactive and non-radioactive in situ hybridization histochemistry

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Summary. In the present study some experimental parameters for in situ hybridization histochemistry (ISHH) have been analysed using ^{35}S-labelled and alkaline phosphatase-conjugated probes, in order to develop a reproducible double-labelling procedure. We have compared the total exclusion of tissue fixation with tissue sections fixed by immersion in formalin. In addition, the effect of dithiothreitol was assessed both when combining radiolabelled and non-radioactive probes on a single tissue section and when the probes were used separately. Hybridization of unfixed tissue resulted in stronger specific labelling and lower background both for radiolabelled and alkaline phosphatase-conjugated probes. No loss in tissue preservation was seen at the light microscopic level after hybridization of unfixed tissue. High concentrations (200 m*M*) of dithiothreitol strongly suppressed background when using ^{35}S-labelled probes, whereas in the non-radioactive procedure, alkaline phosphatase labelling could only be achieved with very low dithiothreitol concentrations (<1 m*M*). This incompatibility led to a protocol using unfixed tissue sections and a sequential hybridization procedure, with the radiolabelled probe and high concentrations of dithiothreitol in the first step and the alkaline phosphatase-conjugated probe without dithiothreitol in the second step.

Introduction

In situ hybridization histochemistry (ISHH) is based on the identification and localization of specific target nucleotide sequences (e.g. mRNA) in different types of preparations such as tissue sections or slide-mounted cultured cells. The use of labelled nucleotide probes makes it possible to detect the hybrid formed between the complementary strands of the probe and the target sequence. Advantages of ISHH compared to hybridization techniques based on RNA prepared from tissue homogenates, such as Northern blot and solution hybridization, include increased sensitivity together with preservation of the cellular organization. This is of particular importance in studies of complex organs including the brain, where effects induced experimentally may be analysed by studying changes in mRNA levels in individual cells with ISHH.

ISHH has also been used in combination with other histochemical techniques. For example, in combination with immunohistochemistry, ISHH can provide information about the coexistence of multiple compounds in a single cell (Gendelman et al. 1985; Young et al. 1986b; Guitteny et al. 1988; Seroogy et al. 1988; Ronnekleiv et al. 1989; see Uhl 1986; Valentino 1987; Young 1990). In addition, the combined use of probes labelled with different types of ligands (Baldino and Lewis 1989; Ichimiya et al. 1989; Young 1989, 1990; Kiyama et al. 1990c, 1991; Ozden et al. 1990) on a single tissue section has made it possible to study the coexpression of several mRNAs within the same cell. ISHH has also been successfully combined with retrograde tract-tracing techniques (Schalling et al. 1986; Wilcox et al. 1986; Burgunder and Young 1988; Gerfen and Young 1988; Young 1990).

Important questions for ISHH methodology concern the handling and treatment of the tissue in order to achieve a high penetration of probes into the tissue while retaining the target nucleotide sequence at its native site within the section. Factors favouring specific hybrid formation, reducing background and preserving the morphology of the tissue are also critical. Here we describe in detail the use of unfixed tissue and the role of dithiothreitol (DTT) to increase the signal to noise ratio. We have attempted to optimize the method for the combination of ^{35}S-labelled and alkaline phosphatase (AP)-conjugated probes (Ichimiya et al. 1989; Kiyama et al. 1990a, b, c, 1991; cf. Baldino and Lewis 1980; Young 1989; Ozden et al. 1990) in order to study the expression of multiple mRNAs within a single tissue section.

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Table 1. Hybridization solutions

	Solution 1	Solution 2	Solution 3	Solution 4
Formamide[a]	50%	50%	50%	50%
SSC (pH 7.0)[b]	4×	4×	4×	4×
Denhardt's[c]	1×	1×	1×	1×
Sodium phosphate (pH 7.0)	0.02 *M*	none	none	0.02 M
Dextran sulphate	10%	10%	10%	10%
Sarcosyl[d]	1%	none	1%	none
DTT[e]	0–200 m*M*	0–200 m*M*	0–200 m*M*	0–200 m*M*
Salmon sperm DNA[f]	500 µg/ml	500 µg/ml	500 µg/ml	500 µg/ml

[a] Deionized (see text)
[b] 1 × SSC is 0.15 *M* sodium chloride, 0.015 *M* sodium citrate
[c] 1 × Denhardt's is 0.02% polyvinylpyrrolidone (Sigma), 0.02% Ficoll (Sigma), 0.02% bovine serum albumin (USB, Cleveland, OH, USA)
[d] Sarcosyl is *N*-lauroylsarcosine (Sigma)
[e] Dithiotreithol (LKB, Bromma, Sweden), 0–200 m*M* means either 0 m*M*, 1 m*M*, 10 m*M*, 60 m*M* or 200 m*M*, added just prior to the hybridization
[f] Sheared and heat denatured (see text), added just prior to the hybridization
The probe is added just prior to the hybridization at the concentration described in the text

Materials and methods

Male Sprague-Dawley rats (body wt. 150–200 g; ALAB, Solna, Sweden) were sacrificed by decapitation, and the brain and adrenal glands were rapidly removed. To minimize the methodological variation all tissues were, whenever possible, mounted together on a cryostat chuck, frozen with carbon dioxide and sectioned at 14 µm in a cryostat (Microm or Dittes, Heidelberg, FRG). Sections were thaw-mounted onto either ProbeOn slides (ProbeOn Microscopic slides, Fisher Scientific, Pittsburg, Pa., USA) or onto microscope slides (Menzel-Gläser, KEBO-Lab, Stockholm, Sweden) pretreated by dipping in a 0.01% poly(L-lysine) solution (PLL; Sigma, St. Louis, Mo., USA). The PLL-pretreated slides were stored at −20° C. Using ProbeOn slides approximately 150 µl of the hybridization solution (all solutions in Table 1 were tested) was applied per pair of slides. These slides contain small spacers, and by applying the hybridization solution onto one slide and inverting a second slide over it, a reservoir containing the solution is formed between the two slides. When PLL-pretreated slides were used, 100–200 µl of hybridization solution (Table 1) was applied per slide. No cover slips were used on PLL-pretreated slides.

Preparation of probes

Oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems, Foster City, Calif., USA) leaving the protecting dimethoxytrityl group at the 5′-hydroxyl group intact. They were subsequently purified on OPC-columns (Oligonucleotide Purification Cartridge, Applied Biosystems). The oligonucleotide probes were complementary to nucleotides 595–642 of rat phenylethanolamine *N*-methyltransferase (PNMT) mRNA (Weisberg et al. 1989), nucleotides 1671–1714 of rat neuropeptide Y (NPY) mRNA (Larhammar et al. 1987) and nucleotides 1441–1488 of rat tyrosine hydroxylase (TH) mRNA (Grima et al. 1985). The AP-conjugated probes were complementary to nucleotides 1223–1252 of rat TH mRNA (TH-AP; Grima et al. 1985) and nucleotides 397–429 of rat vasoactive intestinal polypeptide (VIP) precursor mRNA (VIP-AP; Nishizawa et al. 1985). The TH-AP probe was purchased from NEN (Boston, Mass., USA), and the VIP-AP probe was supplied by Dr. P. Emson, MRC Group, Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, UK. The alkaline phosphatase conjugation of oligonucleotide probes has been previously described (Ruth et al. 1985; Agrawal et al. 1986; Jablonsky et al. 1986; Haralambidis et al. 1987).

PNMT, TH and NPY probes were radiolabelled with deoxyadenosine 5′-[α-thio]triphosphate [^{35}S] (NEN) at the 3′-end using terminal deoxynucleotidyl transferase (TdT; Amersham, Amersham, UK). Eighty nanograms (5 pmol) oligonucleotide in 2 µl autoclaved and deionized water were mixed with 2.5 µl × cobalt reaction buffer (1.4 *M* cacodylate pH 7.2, 300 m*M* *Tris* base, 10 m*M* $CoCl_2$, 1 m*M* DTT), 6 µl deionized and autoclaved water, 12 µl ^{35}S tracer (0.15 mCi) and 3 µl TdT (37.5 U) to a total volume of 25.5 µl. The labelling mixture was incubated at 37° C for 2.5 h and subsequently purified using a Nensorb 20 column (NEN). The specific activity of the radiolabelled probes ranged between 2 and 4×10^9 cpm/µg, and they were stored in 20% ethanol containing 10 m*M* DTT at 4° C until used. DTT was added to avoid possible formation of disulphide bonds.

In situ hybridization

To provide a good basis for the comparison of the various parameters a limited number of probes in well defined systems were used including: TH-containing dopamine neurons in the rat ventral mesencephalon (Hökfelt et al. 1976), NPY and VIP neurons in rat cerebral cortex (Allen et al. 1983; Chronwall et al. 1985; De Quidt and Emson 1986; Gehlert et al. 1987; Fuxe et al. 1977; Lorén et al. 1979), and chromaffin cells in the rat adrenal gland which contain TH, PNMT and NPY (Schalling et al. 1988; Dagerlind et al. 1990a, b).

In the following paragraphs the term fixation means fixation by immersion in 4% paraformaldehyde in 0.1 *M* Sörensens phosphate buffer, pH 7.5 (prepared according to Pease 1962) before the hybridization procedure. The term postfixation means that the fixation (as above) has been done after the hybridization and washing. A scheme of the procedures tested in this study is presented in Fig. 1.

A. ^{35}S-labelled probes. (1) Sections were thawed at room temperature prior to use.
(2) Without any pretreatment, some sections were hybridized using either of the hybridization solutions (Table 1), for 16–24 h at 42° C with 10^7 cpm of the ^{35}S-labelled probes/ml hybridization solution. The remaining sections were fixed for 5 or 30 min, washed with phosphate-buffered saline (PBS) for 2 × 5 min, dehydrated with ethanol (60% for 1 min, 80% for 1 min, 95% for 2 min, 100% for 1 min), delipidated with chloroform (5 min), transferred through 99% and 95% ethanol (1 min each), air-dried and hybrid-

Table 2. Buffers and substrates used for detection of the alkaline phosphatase (AP)-conjugated probes

Buffer A	100 m*M* *Tris*-HCl pH 7.5, 150 m*M* NaCl
Buffer B	100 m*M* *Tris*-HCl pH 9.5, 100 m*M* NaCl, 50 m*M* $MgCl_2$
Buffer C	10 m*M* *Tris*-HCl pH 7.5, 150 m*M* NaCl, 10 m*M* EDTA
AP substrates	75 mg of 4-nitroblue tetrazolium chloride (NBT; Boehringer Mannheim, Mannheim, FRG) was dissolved in 1 ml 70% dimethylformamide (Sigma) and 50 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Boehringer) was dissolved in 1 ml 100% dimethylformamide (see De Jong et al. 1985; Van Noorden and Jonges 1987). The stock solutions were diluted to final concentrations of 340 µg/ml (NBT) and 170 µg/ml (BCIP) with buffer B

ized using either of the hybridization solutions (Table 1) for 16–24 h at 42° C.
(3) Sections were washed with 1 × SSC (Table 1) at 55° C for 60 min with four changes of SSC followed by a final wash in 1 × SSC starting at 55° C and slowly cooled to room temperature (approximately 30 min).
(4) Some tissue sections, both unfixed and fixed, were briefly dipped in deionized water, dehydrated (transferred through 60%, 80% and 100% ethanol for 30 s each) and air-dried. The remaining tissue sections (unfixed and fixed) were postfixed for 5 or 30 min, washed with PBS for 2 × 5 min, dehydrated with ethanol (60% for 1 min, 80% for 1 min, 95% for 2 min, 100% for 1 min), delipidated with chloroform (5 min), transferred through 99% and 95% ethanol (1 min each) and air-dried.
(5) Tissue sections were apposed to Hyperfilm βmax autoradiography film (Amersham) for 1–70 days, which was developed in LX 24 developer (Kodak) for 2 min and fixed in AL4 fixer (Kodak) for 15 min.
(6) Tissue sections were then dipped in NTB2 Nuclear track emulsion (Kodak, Rochester, N.Y., USA), diluted 1:1 with distilled water, and exposed for 14–21 days. Sections were developed for 3 min (D19 developer, Kodak), fixed for 5 min (Unifix, Kodak) or 6 min (Kodak 3000 A and B fixer) and mounted in glycerol. All sections were analysed in a Nikon Microphot-FX microscope using brightfield and darkfield illumination. In some cases an IGS ('immuno gold staining') epipolarization filter set (Nikon) was used in combination with darkfield illumination.

B. Alkaline phosphatase-conjugated probes. Steps (1–3) were identical to those described in section A except for the hybridization temperature which was either 37° or 42° C. Two pmol of the TH-AP probe or 6 pmol of the VIP-AP probe were added per ml of hybridization solution (either solution described in Table 1).
(4) Some tissue sections, including both unfixed and fixed, were postfixed for either 5 or 30 min, washed with PBS for 2 × 5 min, dehydrated with ethanol (60% for 1 min, 80% for 1 min, 95% for 2 min, 100% for 1 min), delipidated with chloroform (5 min), transferred through 99% and 95% ethanol (1 min each), air-dried and then transferred to step 5. The remaining tissue sections (unfixed and fixed) were briefly dipped in deionized and autoclaved water (30 s) and then directly transferred to step 5.
(5) Sections were incubated in buffer A (Table 2) for 30 min at room temperature.
(6) Sections were incubated in buffer B (Table 2) for 10 min at room temperature.
(7) Sections were incubated in an AP-substrate solution (Table 2) at room temperature for 24–48 h in the dark to visualize the AP reaction product.
(8) Sections were washed at room temperature with either a stop-buffer (buffer C, Table 2) or with 1 × SSC (Table 1) for 1–48 h with several changes.
(9) Some sections from both the unfixed and fixed groups not postfixed (step 4) were now postfixed.
(10) Sections were mounted in glycerol and analysed in brightfield to localize the sites of the AP-reaction product.

C. Combination of ^{35}S-labelled and alkaline phosphatase-conjugated probes. Parallel experiments were carried out using a 'simultaneous' (one step) or a 'sequential' (two step) hybridization procedure for the detection of both ^{35}S-labelled and AP-conjugated probes within a single tissue section. The 'simultaneous' procedure was performed according to steps (1–10) in section B at either 37° C or 42° C, and the hybridization solution (all solutions in Table 1 were tested) contained both types of probes at the concentrations described. After visualization and analysis of the AP reaction product in brightfield, coverslips were removed and sections were rinsed in PBS (5 min), dipped in deionized and autoclaved water (30 s), briefly dehydrated in ethanol (65% and 95% for 30 s each) and then processed for autoradiographic localization of the radiolabelled probe by dipping in liquid emulsion (section A, step 6).

The 'sequential' procedure involved a primary hybridization at 42° C with the radiolabelled probe (section A, steps 1–3) using either of the hybridization solutions (Table 1). Sections were then dipped in deionized and autoclaved water (30 s) to remove SSC, air-dried and thereafter hybridized a second time at either 37° C or 42° C with the AP-conjugated probe (section B, steps 1–10) using either of the hybridization solutions (Table 1). Tissue sections were analysed in brightfield to localize the sites of the AP reaction product. Coverslips were removed and tissue sections were processed for autoradiographic localization of the radiolabelled probe (section A, step 6). The simultaneous microscopic visualization of both probes was done either with brightfield illumination alone or in combination with an IGS filter set.

High radioactive and non-radioactive background was observed after dipping in liquid emulsion. This increase appeared to result from the buffers and substrates used in the non-radioactive procedure (see the 'Results and discussion'). Thus a new set of hybridization experiments was carried out following the two-step procedure, where sections, prior to dipping in liquid emulsion, were coated twice by dipping for 15 s in Collodion (0.3% in chloroform; Bio-Rad, Microscience Division, Watford, UK), air-dried and dipped once again (Young 1989), while other slides were left uncoated. In addition, sections previously hybridized only with a ^{35}S-labelled probe (section A) were either incubated only with the buffers (30 min with buffer A followed by either 10 min or 24 h with buffer B) or incubated with the buffers and substrates in combination prior to dipping in liquid emulsion.

D. Hybridization solutions. The four different hybridization solutions used in sections A–C are described in Table 1. The salmon sperm DNA (Sigma) was dissolved in deionized and autoclaved water to a concentration of 10 mg/ml, sheared using a MSE Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Sussex, UK) and stored at −20° C until used. The salmon sperm DNA was denatured by boiling for 5 min before being added to the hybridization solution. Formamide (Baker Chemicals, Deventer, The Netherlands) was deionized with analytical grade mixed bed resin (AG 501-X8; Bio-Rad Laboratories, Richmond, Calif., USA) until the pH was below 5.6 (Sambrook et al. 1989).

The hybridization solutions (Table 1) were stirred overnight in a light-tight, covered Erlenmeyer flask to dissolve the dextran

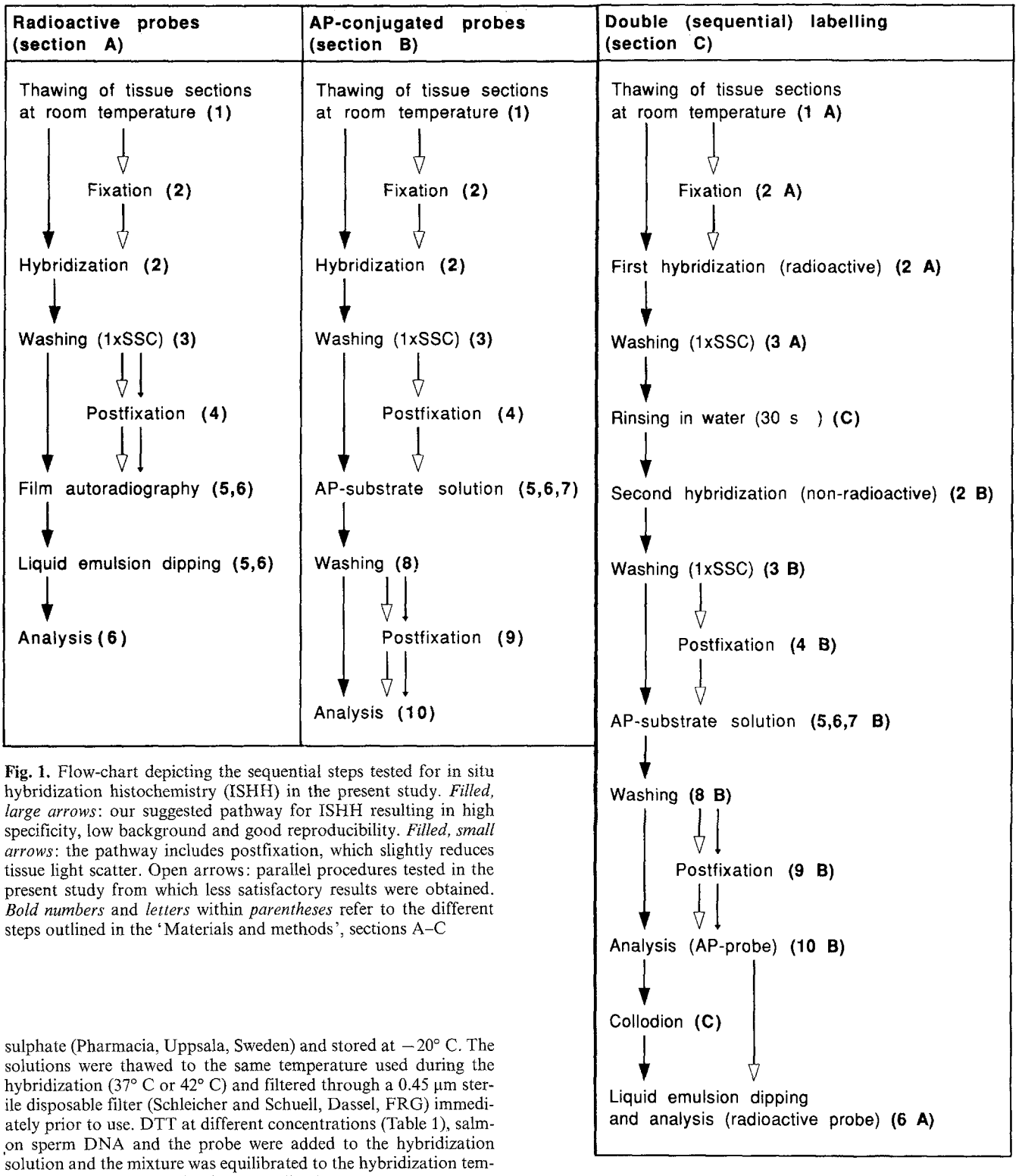

Fig. 1. Flow-chart depicting the sequential steps tested for in situ hybridization histochemistry (ISHH) in the present study. *Filled, large arrows*: our suggested pathway for ISHH resulting in high specificity, low background and good reproducibility. *Filled, small arrows*: the pathway includes postfixation, which slightly reduces tissue light scatter. Open arrows: parallel procedures tested in the present study from which less satisfactory results were obtained. *Bold numbers* and *letters* within *parentheses* refer to the different steps outlined in the 'Materials and methods', sections A–C

sulphate (Pharmacia, Uppsala, Sweden) and stored at −20° C. The solutions were thawed to the same temperature used during the hybridization (37° C or 42° C) and filtered through a 0.45 μm sterile disposable filter (Schleicher and Schuell, Dassel, FRG) immediately prior to use. DTT at different concentrations (Table 1), salmon sperm DNA and the probe were added to the hybridization solution and the mixture was equilibrated to the hybridization temperature (37° C or 42° C) prior to application.

Results

Comparison between unfixed and formalin-fixed tissues resulted in some general observations, which appeared to be valid after both film autoradiography and liquid emulsion dipping. Our tested and recommended procedures are presented in Fig. 1.

^{35}S-labelled probes

1. The specific hybridization signal was consistently strong and the background reduced, using unfixed tissue (cf. Fig. 2a, d). In addition to the general background observed in fixed tissue there was, especially after long autoradiographic exposure, an increased incidence of artefactual labelling (cf. Fig. 2b, e).

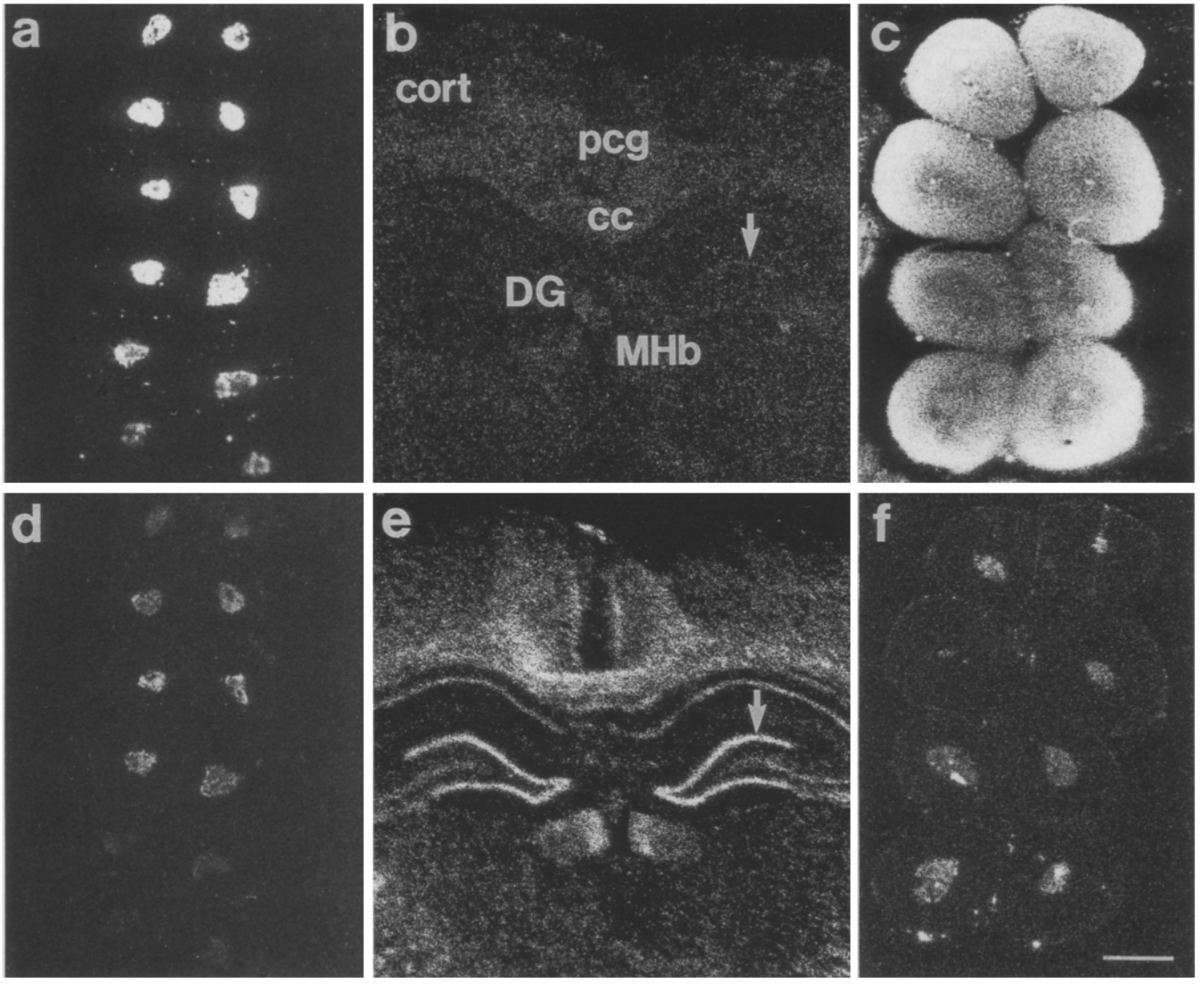

Fig. 2a–f. Film autoradiograms from the radioactive hybridization procedure. **a, d** Unfixed (**a**) and fixed (**d**) adjacent sections of rat adrenal glands hybridized with a probe complementary to phenylethanolamine *N*-methyltransferase (PNMT) mRNA. The two sections were hybridized together and exposed to film for 1 day. Note strong hybridization signal in unfixed tissue. Differences seen between pairs of adrenals show the changes in mRNA levels with time after a single dose of reserpine (see Schalling et al. 1988). **b, e** Unfixed (**b**) and fixed (**e**) adjacent coronal sections of rat brain using the PNMT probe. Artefactual labelling using fixed tissue (**e**) in pyramidal layer of hippocampus (*arrows*), cerebral cortex (*cort*), corpus callosum (*cc*) and medial habenular nucleus (*MHb*) is shown. It is known that these brain areas do not express PNMT. *pcg*, Posterior cingulate cortex; *DG*, dentate gyrus. Sections were exposed to film for 70 days. **c, f** High background is seen in all areas of the adrenal gland when using low concentrations (<1 m*M*) of dithiothreitol (DTT) in the hybridization solution (**c**) compared to the very low background seen using high concentrations (200 m*M*) of DTT. Note specific labelling of the adrenal medulla (**f**). The probe is complementary to neuropeptide (NPY) mRNA and the sections were exposed to film for 2 days. *Bars*: (**a, d**), 0.7 mm; (**b, e**) 1 mm; and (**c, f**) 0.8 mm

2. At the light microscope level, the morphological preservation of unfixed tissue after the hybridization procedure was comparable to that seen in fixed tissue.
3. Liquid emulsion-dipped sections of unfixed tissue analysed in darkfield showed increased tissue light scatter. This was particularly strong in areas with high myelin content (tracts/white matter) as well as over other types of cells with high lipid content (e.g. steroid producing adrenal cortical cells) (cf. Fig. 3a, b). The light scatter was slightly reduced after postfixation (not shown).
4. Kodak 3000 A and B fixer seemed to result in lower tissue light scatter compared to Kodak Unifix after liquid emulsion dipping (not shown; see the Discussion).
5. The use of an IGS epipolarization filter set, in combination with darkfield illumination, strongly reduced tissue light scatter (cf. Fig. 3a, b).
6. No changes in the signal to noise ratio were seen after postfixation.
7. Low concentrations (1–10 m*M*) or absence of DTT resulted in high background (cf. Fig. 2c, f).
8. Hybridization solution 1 (containing both sodium phosphate and Sarcosyl; Table 1) resulted in stronger

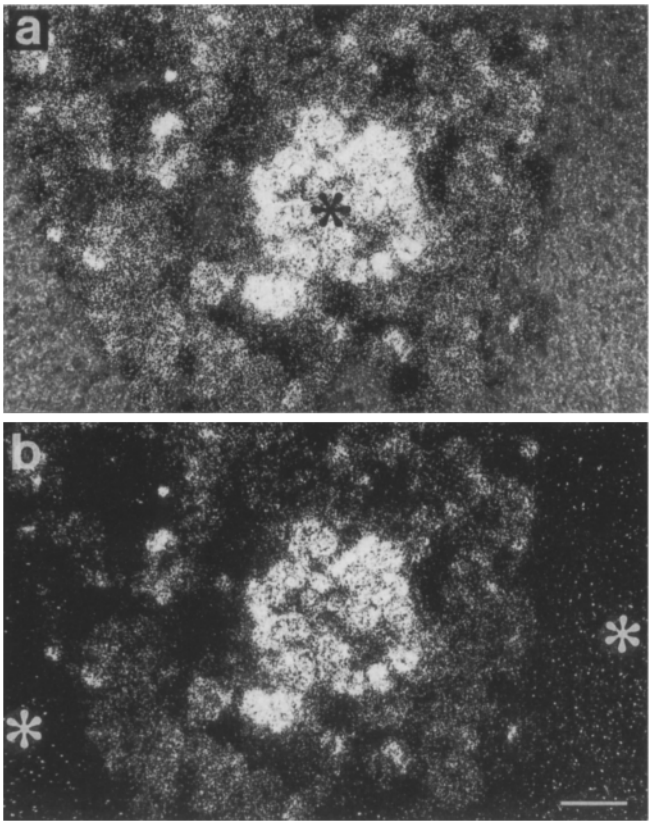

Fig. 3a, b. Section of rat adrenal gland hybridized with a probe complementary to NPY mRNA, and examined under (**a**) darkfield illumination or (**b**) in combination with an immuno gold staining (IGS) epipolarization filter set. Note reduced tissue light scatter, especially in the adrenal cortex (*white asterisks* in **b**), when using the IGS filter set. The section was exposed to liquid emulsion for 2 weeks. Strongly labelled cell island (*black-asterisk* in **a**) represents noradrenergic intrinsic ganglion cells in the adrenal medulla (see Dagerlind et al. 1990a). *Bar*, 0.1 mm

specific labelling and lower background than solutions 2, 3 and 4.

Alkaline phosphatase-conjugated probes

1. The AP reaction product was consistently strong using unfixed tissue whereas no clear difference between unfixed and fixed tissue was seen with regard to background labelling (cf. Fig. 4a, b).
2. At the light microscope level, the morphological preservation of unfixed tissue after the hybridization procedure was comparable to that seen in fixed tissue.
3. A strong AP reaction product overlying the nucleus was observed in fixed tissue, whereas in unfixed tissue the same cell population exhibited cytoplasmic labelling (cf. Fig. 4a, b).
4. Postfixation of both unfixed and fixed tissue directly after the washing procedures, but before the enzyme-substrate reaction, resulted in a strong AP reaction product overlaying the nucleus (not shown, cf. Fig. 4b).
5. Postfixation of unfixed tissue after the enzyme-substrate reaction resulted in strong AP reaction product that could be compared to that seen with unfixed tissue without postfixation (not shown, cf. Fig. 4a, d–f).
6. DTT strongly affected the AP reaction product; even very low concentrations (1 m*M*) almost completely abolished the signal (Fig. 4c).
7. Hybridization solution 2 (without sodium phosphate and Sarcosyl; Table 1) resulted in stronger specific labelling and lower background compared to solutions 1, 3 or 4 (Fig. 4a, d–f).
8. Hybridization at 37° C slightly increased the levels of the AP reaction product compared to hybridization at 42° C (not shown).

Combination of ^{35}S-labelled and alkaline phosphatase-conjugated probes

The attempt to combine ^{35}S-labelled and AP-conjugated probes met with several problems. One was the fact that high concentrations of DTT were needed to suppress background using ^{35}S-labelled probes (cf. Fig. 2c, f). On the other hand, even low concentrations (1 m*M*) of DTT resulted in very weak or total lack of AP reaction product (Fig. 4c). As a consequence of the two procedures tested, 'simultaneous' and 'sequential', only the latter method could be successfully applied, and the results described below are thus only from this procedure.
1. Double-labelled tissue sections dipped in liquid emulsion resulted in high background of both silver grains and nitroblue tetrazolium (NBT) precipitate. Reapposing these sections to film resulted in a strong specific signal and low background (not shown).
2. Sections that had only been processed for radioactive ISHH but then incubated (24 h) in buffers A, B or C (Table 2) showed a total loss of the specific radioactive signal. The very same sections had previously resulted in a strong signal after exposure to autoradiography film. When these dipped sections were reapposed to film, a strong specific signal and low background were again observed, comparable to that seen after the first exposure to film. However, when decreasing the incubation time in buffer B to 10 min, a strong specific signal with low background was seen after dipping in liquid emulsion. The combination of the buffers and substrates resulted in high background of both NBT precipitate and silver grains after liquid emulsion (not shown).
3. Coating the slides with Collodion before dipping in liquid emulsion markedly reduced the background for both types of probes (Fig. 4g, h). However, Collodion coating reduced the specific radioactive signal, although upon increasing the exposure time a high signal to noise ratio could be obtained. Collodion coating also prevented the loss of radioactive signal produced by buffer B.
4. The two-step (sequential) procedure could only be successfully applied when a primary hybridization using a ^{35}S-labelled probe was followed by a second hybridization with an AP-conjugated probe (Fig. 4g, h). When the radioactive hybridization was carried out after the non-radioactive hybridization, high radioactive background was observed (not shown).

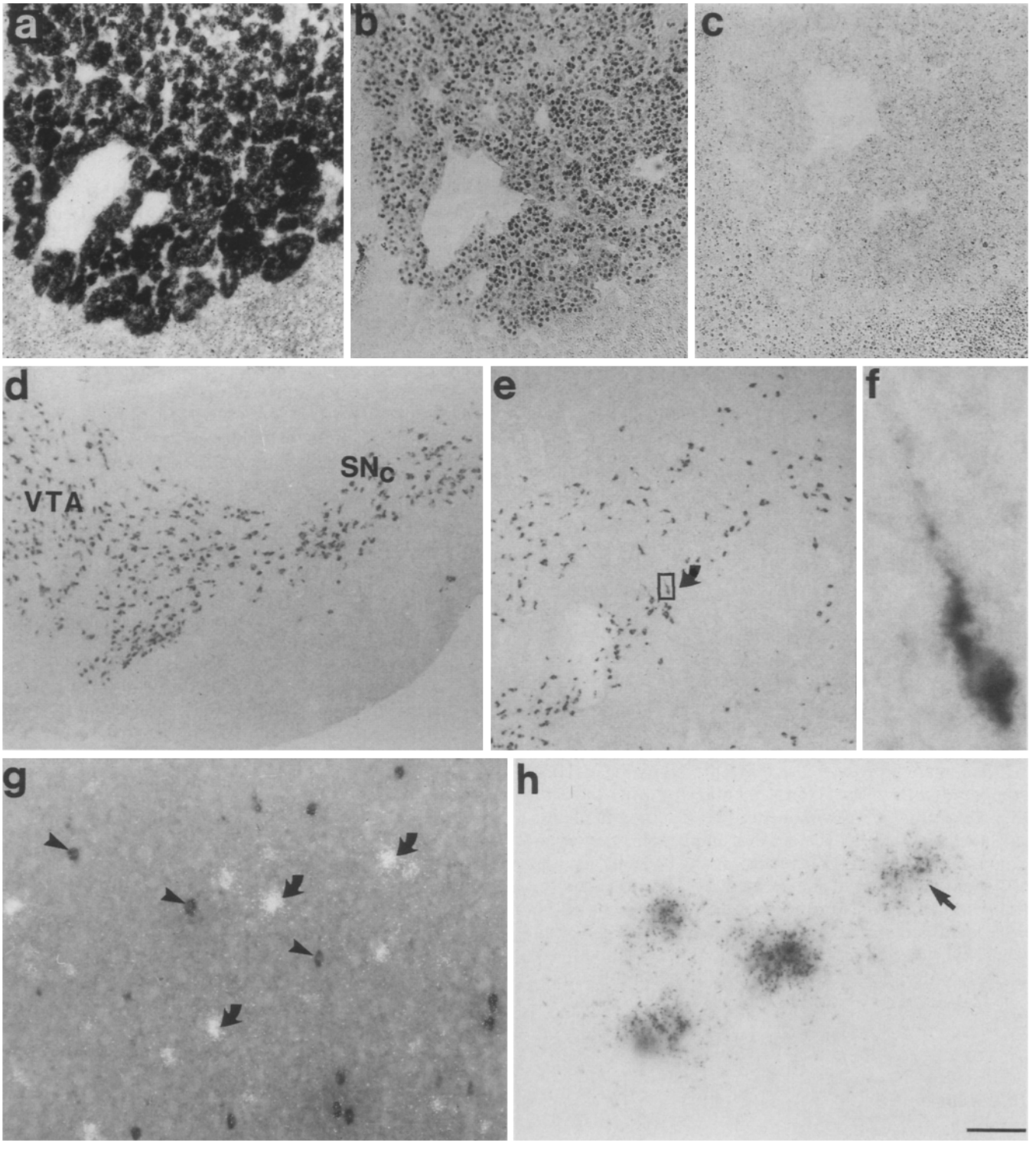

Fig. 4a–h. Brightfield photomicrographs showing the **a–c** rat adrenal gland, **d–f**, **h**, ventral mesencephalon and **g** cortex hybridized with probes complementary to **a–f**, **h** tyrosine hydroxylase (TH) mRNA and probes complementary to **g** NPY and vasoactive intestinal polypeptide (VIP) mRNA, respectively. **a–c** The alkaline phosphatase (AP)-conjugated probe complementary to TH mRNA shows strong cytoplasmic labelling in all chromaffin cells using unfixed tissue (**a**), whereas fixed tissue shows reduced cytoplasmic labelling and strong nuclear labelling (**b**). **c** Unfixed tissue hybridized with the AP-conjugated TH probe and using a hybridization solution containing 1 m*M* DTT. Note the absence of labelling when including DTT in the hybridization solution. **d** The AP-conjugated TH probe shows strong labelling in substantia nigra pars compacta (*SN_c*) and the ventral tegmental area (*VTA*). **e, f** Ventral mesencephalic dopamine cells labelled with the AP-conjugated TH probe shown in overview (**e**) and (*box*) at high power (**f**). **g** Labelled cells in rat cerebral cortex after the two-step (sequential) hybridization procedure using a radioactive probe complementary to NPY mRNA (*arrows*) and an AP-conjugated probe complementary to VIP precursor mRNA (*arrowheads*). Brightfield illumination was used in combination with an IGS epipolarization set. **h** High magnification of TH mRNA containing cells in the ventral mesencephalon after the two-step (sequential) hybridization procedure using the radioactive TH probe and the AP-conjugated TH probe. The probes are complementary to different sequences of the TH mRNA; thus no competition at the hybridization sites will take place. The micrograph shows cells labelled with both AP reaction product as well as with silver grains. The cell indicated by the *arrow* seems to be covered only by grains, but AP reaction product can be visualized by changing focus. The AP enzyme-substrate reaction (**a–h**) was carried out for 2 days. Sections (**g, h**) were coated with Collodion before being exposed to liquid emulsion for 21 days. *Bars*: (**a–c**) 70 μm; (**d, e, g**) 100 μm; (**f**) 5 μm; and (**h**) 30 μm

5. 1 × SSC seemed to be more effective in removing excess NBT from the tissue sections than the stop-buffer (buffer C, Table 2), resulting in reduced background after dipping in liquid emulsion.

ProbeOn slides versus PLL pretreated slides

The background was consistently lower using ProbeOn slides compared to PLL pretreated slides. In addition, ProbeOn slides were easy to handle and allowed the use of smaller amounts of hybridization solution per tissue section.

Specificity of hybridization

The probes used in the present study have previously been tested for their specificity using several approaches. The radioactive TH, NPY and PNMT probes have been used in Northern blot experiments and resulted in single bands corresponding to the estimated size of the respective mRNAs (Schalling 1990a; Dagerlind, unpublished observations). All probes have shown labelling of the same cell populations observed using immunohistochemistry. In addition, the specificity of the radiolabelled TH probe has been verified in a double-labelling experiment combining immunohistochemistry and ISHH on the same tissue section (Seroogy et al. 1988) as well as with the ISHH double-labelling method presented in this paper (see Fig. 4h). Furthermore, all probes (except the AP-conjugated probes) used in this study have been compared with probes complementary to other parts of their respective mRNA sequence and resulting in identical labelling patterns (Dagerlind, unpublished observations). Moreover, several probes with the same length, GC (guanine and cytosine) content and specific activity, but complementary to other mRNAs not present in the cells identified in this study did not result in labelling when hybridized on adjacent sections (not shown). Finally RNAase pretreatment of the tissue totally abolished labelling for all probes (not shown).

Discussion

The main result obtained in the present investigation is the finding that unfixed tissue is superior to formalin-fixed tissue for ISHH, resulting in increased specific labelling and reduced background. This procedure has previously been used in our group for radioactive ISHH studies (see e.g. Dagerlind et al. 1990a, b; Schalling 1990; Schalling et al. 1990), but a systematic evaluation has not been carried out. Here we also present evidence that unfixed tissue is a good alternative when combining radiolabelled and AP-conjugated probes on a single tissue section. The use of unfixed tissue is similar to protocols for receptor ligand-binding autoradiography (see Kuhar 1981), but is in contrast to most immunohistochemical procedures where fixation is needed to retain the antigen at its native site (see Hökfelt et al. 1973).

Role of fixation

Although a number of fixatives are potentially available, formalin has been most commonly used in published ISHH procedures (see Uhl 1986; Valentino 1987; Young 1990). Formalin fixation has also been successfully used in combination with ethanol and chloroform treatment (Young et al. 1986a, b; Young 1990), and this combination was used in our initial studies (see e.g. Schalling et al. 1988). However, when working with less abundant mRNA at the single cell level, the signal to noise ratio was sometimes low. Early attempts to understand the role of the fixative were therefore initiated to test various fixation procedures (Schalling et al. 1986; Schalling 1990). As shown here, omission of fixation results in both stronger specific labelling and in reduced background. In fact after formalin fixation, the nonspecific background can appear as artefactual labelling confined to certain cellular compartments of the tissue. Thus, fixatives such as formalin may actually increase non-specific binding of the probe. We have observed this phenomenon particularly in areas with high cell density, such as in the pyramidal cell layer of hippocampus and the granule layer of cerebellum and also in white matter. Additionally, when AP-conjugated probes were used on fixed tissue the labelling pattern was somewhat different from that seen with unfixed tissue. Fixed tissue resulted in a strong AP-reaction product overlying the nucleus rather than the expected cytoplasmic labelling (cf. Kiyama et al. 1990a, b, c, 1991); the reason for this is unclear.

A further advantage of unfixed tissue is in quantitative studies, where a high degree of reproducibility is required. Fixation of thick slices (approximately 1–4 mm) by immersion before cutting may lead to a gradient of fixation within the tissue block, which in turn may lead to variable hybridization conditions among sections cut from this block. Variations in perfusion fixation between animals may also result in reduced reproducibility. These pitfalls are avoided using sections of unfixed tissue. It should be emphasized that there may be situations where fixation could be advantageous such as when combining ISHH with immunohistochemistry. Also, fixation may inactivate mRNA degrading enzymes and in this way improve ISHH sensitivity.

High tissue light scatter was observed with darkfield illumination using unfixed tissue. The light scatter is most probably due to high lipid/myelin/white matter content of the tissue. However, this light scatter could be reduced with an IGS epipolarization filter set and/or using Kodak 3000 fixer. The reduction seen using Kodak 3000 may be due to its strong fixation capacity resulting in delipidation. Attempts were made to reduce tissue light scatter with a postfixation procedure, which resulted in a slight reduction. However, the introduction of postfixation affected the non-radioactive procedure: an attenuation of the AP-reaction product was observed when the postfixation was done before the enzyme-sub-

strate reaction. Postfixation was therefore always carried out after completion of the enzyme-substrate reaction.

DTT: effect on double labelling

DTT is used to prevent oxidation, thus avoiding the possible formation of disulphide bonds. A high concentration of DTT (200 m*M*) was found to reduce the background using ^{35}S-labelled probes, but higher concentrations did not result in any further decrease in background. In contrast, DTT even at very low concentrations (1 m*M*) strongly reduced the AP reaction product. These results were important in our attempts to devise a double-labelling method, and this incompatibility led to a two-step hybridization procedure. In the first hybridization the radiolabelled probe was used with high concentrations of DTT (200 m*M*), whereas in the second hybridization, using the AP-conjugated probe, DTT was deleted. The two-step procedure, although less rapid than the simultaneous procedure, resulted in high specific labelling and in low background for both probes.

Collodion coating: role for double labelling

In our attempts to combine radiolabelled and AP-conjugated probes, we observed that double-labelled sections dipped in liquid emulsion possessed high background levels of both NBT precipitate and silver grains, with the silver grain background typically overlying areas with intense NBT precipitate. Whether or not the buffers A and B or the substrates used in the non-radioactive procedure could be responsible for the increased silver grain background was evaluated. Thus, tissue sections only hybridized with a radiolabelled probe, but thereafter incubated with the buffers alone or in combination with the substrates were analysed. Here a chemical interaction seemed to occur between buffer B and the substrates used in the non-radioactive procedure and the silver bromide and/or the gelatin components in the emulsion, which resulted in a total loss of the radioactive signal. High background levels of NBT precipitate were found to be due to the developer used in the liquid emulsion dipping procedure. Since autoradiographic developers contain very strong reducing agents, even small amounts of soluble NBT remaining in the tissue sections will be reduced by the developer, resulting in high NBT background. To reduce NBT background a long washing procedure prior to emulsion dipping is needed in order to remove excess, soluble NBT remaining in the tissue.

Moreover, NBT precipitate also increased non-specific levels of silver grains. Collodion coating (Young 1989) before dipping in liquid emulsion markedly reduced both NBT precipitate and silver grain background, probably by creating a barrier between the tissue section and the emulsion. This coating, however, decreased the specific radioactive signal, but by increasing the autoradiographic exposure time high signal to noise ratio was obtained. When studying rare mRNAs in single cells, this coating may make it difficult to obtain a sufficiently strong signal. A recent report by Young and Hsu (1991) indicates that the type of emulsion utilized may influence the signal to noise ratio when combining radioactive and non-radioactive ISHH.

Hybridization temperature

The hybridization temperature for ISHH is usually reduced to approximately 25° C below the theoretical T_m value (melting temperature equilibrium at which half of the potential hybrids are dissociated; Uhl 1986; Valentino 1987; Sambrook et al. 1989; Young 1990). These values may, however, not be optimal for hybridization with the AP-conjugated probes. Jablonsky and coworkers (1986) have shown, using membrane filter hybridization techniques, that oligonucleotide probes carrying a large enzyme have a melting temperature approximately 10° C lower than the unmodified equivalent probe. Since we have chosen a two-step hybridization procedure, it is possible to optimize the hybridization temperature for each step.

Radioactive versus non-radioactive methodology

The radioactive approach is today a well-established procedure and, in addition to localization of a specific mRNA, also allows quantification of the hybridization signal. However, there are reports describing quantitative measurements of mRNA levels also with non-radioactive probes (Kiyama et al. 1990c; see also Van Noorden and Jonges 1987). An advantage with the non-radioactive method is that it can be more rapid than the radioactive procedure for cellular localization of mRNAs, since dipping in liquid emulsion is not needed. Furthermore, the use of hazardous radioactivity is avoided. Enzyme-conjugated probes are also stable much longer than radiolabelled probes, where radioactivity will decay with time. Thus, non-radioactive probes are in many cases good alternatives to radiolabelled probes and of particular importance in combination with radiolabelled probes studying multiple mRNAs in a single tissue section.

Conclusion

The present results suggest some improvements in tissue handling, whereby unfixed tissue increases the signal to noise ratio compared to fixed tissue. We emphasize the role of DTT in reducing the high background often seen with ^{35}S-labelled probes. Furthermore, a double-labelling method combining radiolabelled and non-radioactive (AP-conjugated) probes has been developed. Since we have found that high concentrations of DTT interfere with the detection of the non-radioactive probe, a two-step hybridization procedure was chosen. Thus, the first step includes the radiolabelled probe and high DTT con-

centrations, whereas no DTT is added in the second hybridization with the non-radioactive probe.

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