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Review

Just cool it! Cryoprotectant anti-freeze in immunocytochemistry and in situ hybridization☆

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Abstract

Immunohistochemical techniques offer specificity as well as flexibility for visualizing antigens. Their use with freely floating sections provides a high signal-to-noise ratio and has become a gold standard for brain and a number of other tissues. Yet this approach initially suffered from inability to keep the antigenicity in tissue sections and required immediate processing of all cut sections. Use of sucrose solutions enabled storage at refrigerator temperatures for a few days but longer-term storage was risky and either bacterial/fungal growth or evaporation of the storage solution compromised the integrity of the tissue. Our discovery 25 years ago that tissue sections can be stored for many years at −20 °C in an anti-freeze cryoprotectant solution with no loss of antigenicity solved this problem and has become widely used. More recently the utility of tissue stored for many years in anti-freeze cryoprotectant was pushed to new levels by testing new non-radioactive in situ hybridization (ISH) techniques that are based on modern immunocytochemistry. This review touches upon these advances in immunocytochemical technology using examples from neuroscience applications.

Keywords: Cryoprotectant anti-freeze; Immunocytochemistry; In situ hybridization

1. Where we started: cryoprotective anti-freeze for immunocytochemical studies

Identification of naturally occurring compounds (transmitters and their transporters, cell-specific markers, receptors, enzymes, transcription factors, cytoskeletal elements, second messengers, etc.) with immunocytochemistry comprises a common approach in neuroscience research. In the early 1980s as basic immunocytochemical techniques improved, scientists recognized that brain and spinal cord sections that were freely floating and processed for antigen localization had more even staining and lower background [7,13] than tissue which was either frozen or paraffin-embedded and processed on slides using the same antibodies. For neurons, this approach provided the ability to discern the three dimensional features of the dendrites and axons. As described in our 1986 Peptides study [21], using freely floating sections initially suffered from an inability to maintain antigenicity in tissue sections stored in buffers for more than a few days; with time, the tissue became more fragile as well. Thus, rapid processing of all cut sections was required. Should all the sections not be used for the initial staining, the remaining tissue had to be discarded. As our understanding of brain chemistry became more sophisticated and more antibodies became available, too often one wished that the discarded tissue could have been saved for use with the new reagents. Our discovery that tissue sections stored for many years (now over 20 years) at −20 °C in an anti-freeze cryoprotectant solution had no loss of antigenicity, solved this problem [21]. The time and dollars saved by enabling this long-term storage are enormous and in many laboratories cryoprotectant anti-freeze is routinely employed.

One simple example of how useful this approach is stems from the fact that purchased antibodies can vary with the lot either in total quality when polyclonal antibodies are used or in titer for monoclonal antibodies. Thus, it becomes imperative that new batches of antibodies be validated to ensure comparable quality across experiments. For many years, my laboratory has studied pathway activation using localization of the immediate early gene product Fos. Since animal subjects can also vary in their responsiveness, a change in the pattern of staining when testing antibodies leaves open the question of whether the difference is due

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Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology

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Abstract

Use of an ethylene glycol based cryoprotectant solution has been found to be effective for the long-term storage of brain tissue either in block form or as freely floating sections prior to immunocytochemical processing. Storage of tissue in the solution at 20 °C or 4 °C for up to 3 months produced no adverse effects upon tissue morphology, nor was LHRH immunoreactivity diminished or accompanied by elevated non-specific staining. Furthermore, ultrastructural analysis of cryoprotected tissue revealed excellent preservation of cellular morphology. It is anticipated that this method can find use when it is necessary or desirable for the investigator to retain tissue for later immunocytochemical or electron microscopic processing.

Keywords: Immunocytochemistry; Peptides; Morphology; Electron microscopy; Cryoprotectant

to the antibody or the degree of stimulation achieved. Having sections in anti-freeze cryoprotectant allows use of the same tissue in the antibody screens thus allowing precise comparisons across antibodies even in situations where the original antibody lot has long been depleted. This is illustrated in Fig. 1. The tissue is from an animal treated with estrogen and progesterone to induce Fos in luteinizing hormone releasing hormone (LHRH) neurons. The section on the left was stained in 1989 shortly after tissue acquisition. LHRH neurons (stained brown) show Fos (black) staining in their nuclei. In 2001, 12 years later, we acquired better Fos antibodies and as is seen in the section on the right, in an adjacent section, LHRH neurons still show Fos labeling, although with the newer antibody, the staining is more intense, and the background is lower.

2. The next horizon: cryoprotection of freely floating sections for RNA detection

2.1. Freely floating tissue in in situ hybridization (ISH)

This idea was first introduced in studies of gene expression in whole embryos employing non-radioactive ISH methods [20]. It is a highly successful strategy that obviates the problem of how to handle fragile, embryonic tissue and is still widely used. For ISH on tissue sections, freely floating tissue has only recently been tested. The utility of this approach was less obvious for radioactive ISH since it was not known whether having thicker sections would make detection of the radiolabel more difficult. However, once tested, use of floating sections for radioactive ISH, produced quite satisfactory results [16]. Nonetheless, techniques that employ fresh frozen sections or paraffin-embedded tissue mounted directly onto slides are among the most common ISH strategies [1]. While powerful for detection of low levels of RNA, maintaining low background with mounted tissue when signals are weak is sometimes difficult, and this is a particular problem at the edges of the tissue. This diffuse label is due to either an inability to remove unreacted probe or “stickiness” of the probe to tissue constituents other than appropriate nucleotide sequences [23]. Removal of unreacted

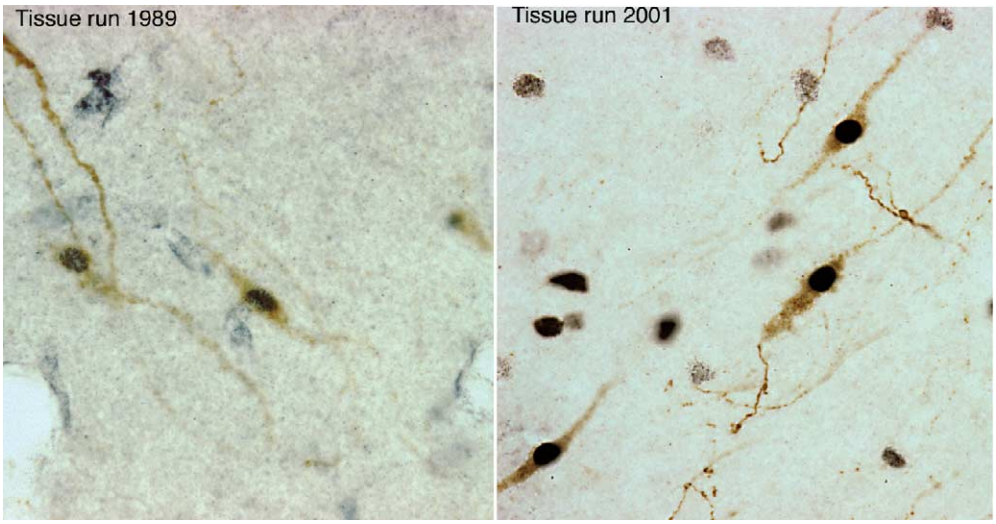

Fig. 1. Demonstration of the utility of anti-freeze cryoprotected tissue for antibody screening. The figure shows two sections from an animal euthanized in 1989 reacted for Fos and LHRH. (a) The section was stained with a sheep anti-Fos from Cambridge Research Laboratories only a short while after the tissue was generated. (b) The adjacent section was stained in 2001 using a rabbit anti-Fos from Oncogene Sciences.

probe is more efficient in freely floating sections, reinforcing the utility of this approach.

It was an obvious next step to test whether tissue stored in cryoprotectant anti-freeze for many years would still enable detection of RNA. One of the greatest concerns in storing tissue for ISH is staving off RNase activity. The initial studies that used anti-freeze cryoprotection were those of de Olmos et al. [10] who described this solution for preserving horseradish peroxidase activity in tract-tracing experiments that used paraformaldehyde fixed sections. So from the onset, it was clear that the storage solution per se does not block all enzyme activity. Clearly this observation applies to endogenous enzyme activity as well, since RBC peroxidase activity is also well maintained in the stored sections (sometimes necessitating its blockade when localizing nearby antigens) and the same is true for the nitric oxide synthase visualized with the diaphorase reaction. A study by Lu and Haber [16] surveyed tissue from rats, primates and humans fixed in 4% paraformaldehyde and stored in cryoprotectant anti-freeze for up to 3 years. The results made clear that mRNA was well preserved even after long term storage. A follow-up on that study now determined that even much longer storage (>10 years) did not compromise detectable mRNA levels (Haber, personal communication). Yet despite these advantages, one disadvantage that accompanies freely floating sections for isotopic ISH is the added costs of large quantities of probes. Non-radioactive ISH on the other hand is far less expensive.

Our laboratory has now tested extensively non-radioactive ISH procedures (using biotinylated riboprobes) on sections that were fixed and stored in cryoprotectant anti-freeze for many years (12 years), and like Lu and Haber, we have observed excellent preservation of the RNA signals that are indistinguishable from those in tissue prepared only a short time prior to hybridization (Fig. 2). Yet the ability to use cryoprotected tissue for non-radioactive ISH procedures extended beyond the ability to maintain RNA. Namely, the preservation of RNA and protein antigenicity in freely floating sections made easy the application of double labeling strategies combining ISH and immunocytochemistry (immunofluorescence and immunoperoxidase) for detection of protein signals in the same cells [5]. Examples of this are shown in Fig. 3 and show how powerful the ability of tissue to retain both RNA and protein antigenicity can be in floating tissue that was cryoprotected.

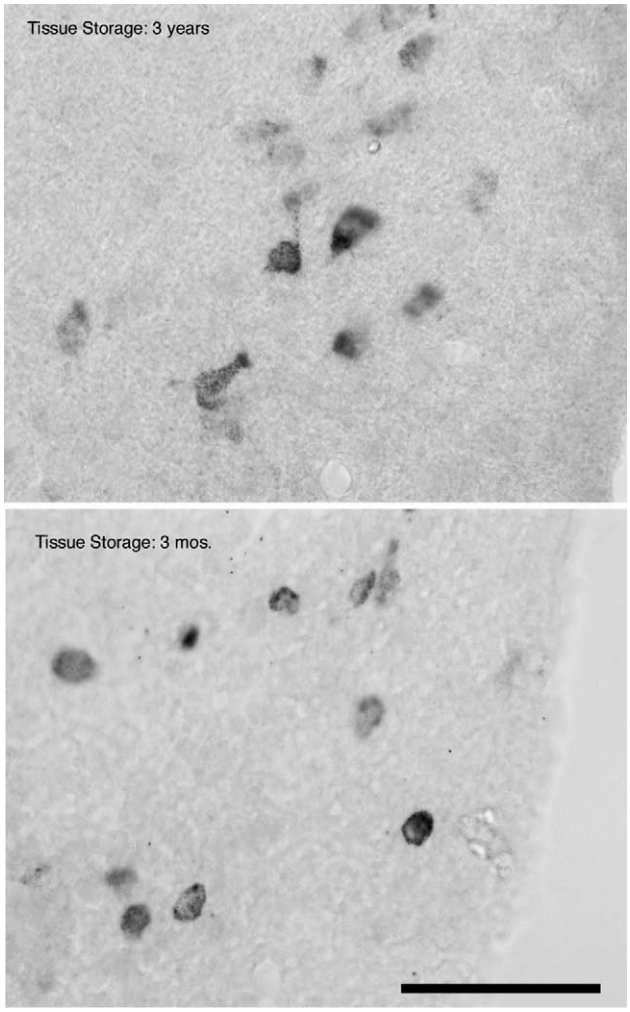

Fig. 2. Preservation of tyrosine hydroxyalse (TH) mRNA in tissue that is stored in cryoprotectant anti-freeze and later processed for ISH using biotinylated riboprobes. Note the signal intensity and its clarity are well preserved whether the tissue was stored for 3 years or only 3 months. We have observed similar preservation of RNA for up to 12 years (data not shown). Bar = 50 μm.

2.2. Quantification of non-radioactive ISH

The last frontier to be tamed in non-radioactive ISH in cryoprotected tissue is the ability to have sensitive detection that permits quantification of the levels of RNA detected. While scientists have worked diligently in understanding the foundations of quantifying ISH with radiolabeled probes, doing so with non-radioactive signals was less common and thus the data on such approaches was minimal. Quantifying an RNA signal with a non-radioactive method must use a method of detection that is graded (so that there are clear distinctions between weakly and strongly labeled cells). The lower the tissue background, the more easily weakly labeled cells are discerned. The choice of approach, therefore, can influence the ability of the data to be quantified.

Basically, non-radioactive methods for ISH were introduced in 1983 [2] and represent a class of approaches based on the use of probes labeled with molecules that either fluoresce directly [22], can easily bind to fluorescent molecules (for example biotin reacted with streptavidin-labeled fluorophores) or are visualized after immunocytochemical reaction directed against the tag. The list of potential tags is continuously expanding but most commonly includes acetyl aminofluorene, biotin, digoxigenin or fluoroscein. Non-radioactive in situ hybridization procedures solve the problem of signal spread since the visualized product of the hybridization reaction is deposited upon the nucleotides

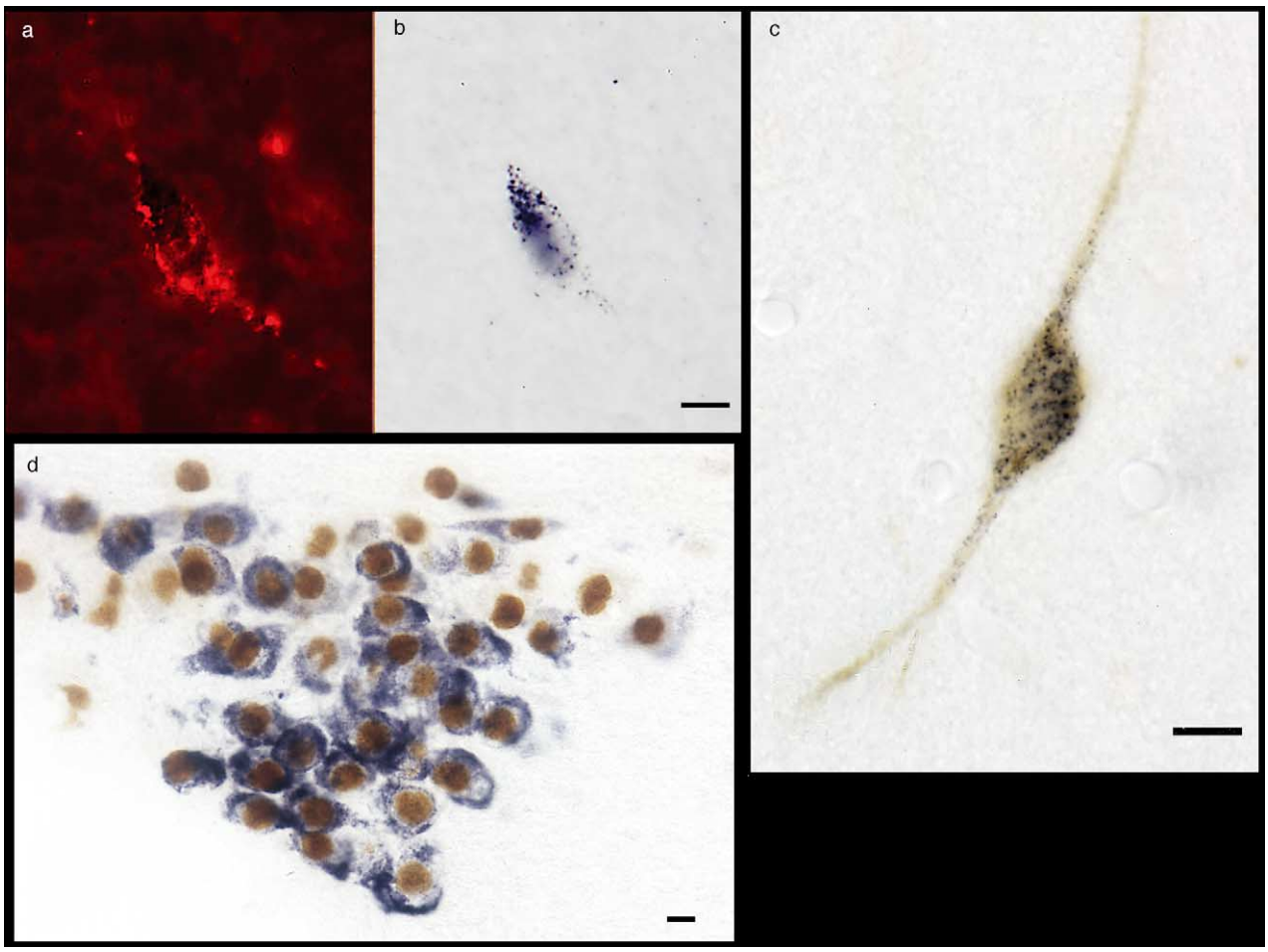

Fig. 3. Examples of double labeling in freely floating tissue that was first reacted for ISH using biotin-labeled probes (blue/black product), then for ICC with immunofluorescence or immunoperoxidase. Sections shown in (a and b), and (c) were reacted for TH mRNA followed by TH protein with ICC. (a) The protein was stained using immunofluorescence, (c) the protein was stained with DAB in an immunoperoxidase reaction, (d) vasopressin mRNA is localized first followed by the immediate early gene product Fos (brown) in an animal which received a hypertonic saline injection to induce Fos. Bars = 10 μm.

within the cells rather than above them, offering superb cellular resolution. This feature is illustrated in Fig. 4 in which a 50:50 mixure of probes was applied, one of which was labeled with S35 and visualized with autoradiography, the other with digoxigenin (visualized following application of an alkaline phosphatase tagged anti-digoxigenin and reaction with a substrate for alkaline phosphatase which yields a blue/purple product). The greatest limitation of non-radioactive methods initially was thought to be their sensitivity. For example, early studies comparing radioactive and non-radioactive ISH approaches indicated that use of biotin/avidin peroxidase procotols had sensitivities far below those of radiolabeled ISH [8]. A subsequent analysis determined that use of digoxigenin-labeled probes with alkaline phosphatase detection, compared well to detection with probes labeled with S35 [12,14]. Overall, when various methods for non-radioactive ISH were compared, most investigators concluded that digoxigenin or biotin-labeled probes with alkaline phosphatase detection was preferable to systems that used peroxidase for visualization of the probe. More recent data indicates that with adjustment of the procedures for detection, both enzyme systems can perform well [9,11]. One can easily find data to support superiority of any one approach over another. Thus, any conclusion of method superiority needs to be interpreted cautiously. Both for fluorescent and non-fluorescent immunocytochemical approaches to ISH, methods for amplification of signals are now available and these greatly improve detection of even low levels of nucleotides [6,15,18,19,24] but if linearity with deposition of the visualized product is lost, quantitation can become less reliable. For achieving quantitative data fluorescence is difficult due to fading; immunocytochemically-based methods (with enzyme detection) as a group are the most successful and it is in this domain that the freely floating cryoprotected tissue emerged as having clear advantages.

When we began use of non-radioactive ISH, most investigators suggested that digoxigenin/alkaline phosphatase

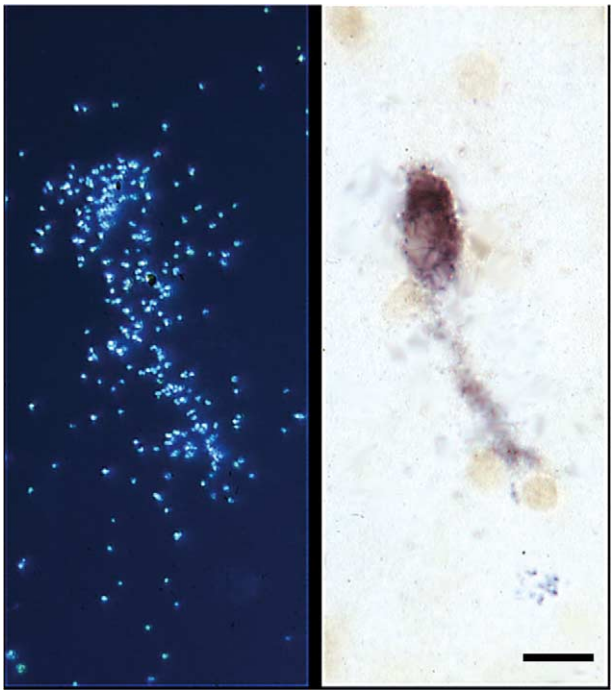

Fig. 4. Example comparing the localization of mRNA for LHRH using a mixture of an S35-labeled riboprobe: (a) grains in darkfield following autoradiography and a digoxigenin-labeled proble, (b) blue/purple reaction product viewed under brightfield optics (Hoffman and Smith, unpublished). Note the difficulty in discerning the cell borders when the grains are viewed compared with the same cell viewed as the alkaline phosphatase product. Bar = 10 μm.

methods were better than peroxidase-based methods due to the high background seen with use of biotin/peroxidase methods. During a study of biotin-amplification in immunocytochemistry, we had systematically varied all the components of ABC peroxidase methods. Our experience indicated that if anti-biotin antibodies were used to detect the incorporated biotin and the antibodies were maximally diluted, much of the background was eliminated [4]. Further reductions in background were then achieved through adjustment of the concentration of the secondary antibody, dilution of the AB reagents, and dilution of DAB/peroxidase mixtures. Without experience in how changing concentrations of reagents in the immunocytochemical portion of the method affected the staining outcome, we too would have concluded that biotinylated probes detected immunocytochemically with anti-biotin were inferior to digoxigenin/alkaline phosphatase probe-detection designs. We now conclude that the two methods are equally sensitive, but that the biotin/immunoperoxidase approach offers better resolution of the RNA. A direct comparison of the two approaches could not be made until each of the approaches was adjusted so as to provide the best signal and low background. An example of the effect of simply reducing the concentration of the anti-biotin in the reaction is seen in Fig. 5.

In considering the ability to quantify the amount of nucleotides within a cell, a number of different measurement strategies have been used with non-radioactive ISH. A large number of these studies counted the numbers of cells detected as their measure of expression. In addition, for digoxigenin-labeled probes detected with NBT blue, the optical density of the reaction product served as a reliable measure of the amount of RNA present in the cells [17]. Either of those methods could be applied to either mounted or freely floating sections. With freely floating sections and immunocytochemical detection of either digoxigenin or biotin-labeled probes, we found that the latter visualized with immunoperoxidase staining with nickel

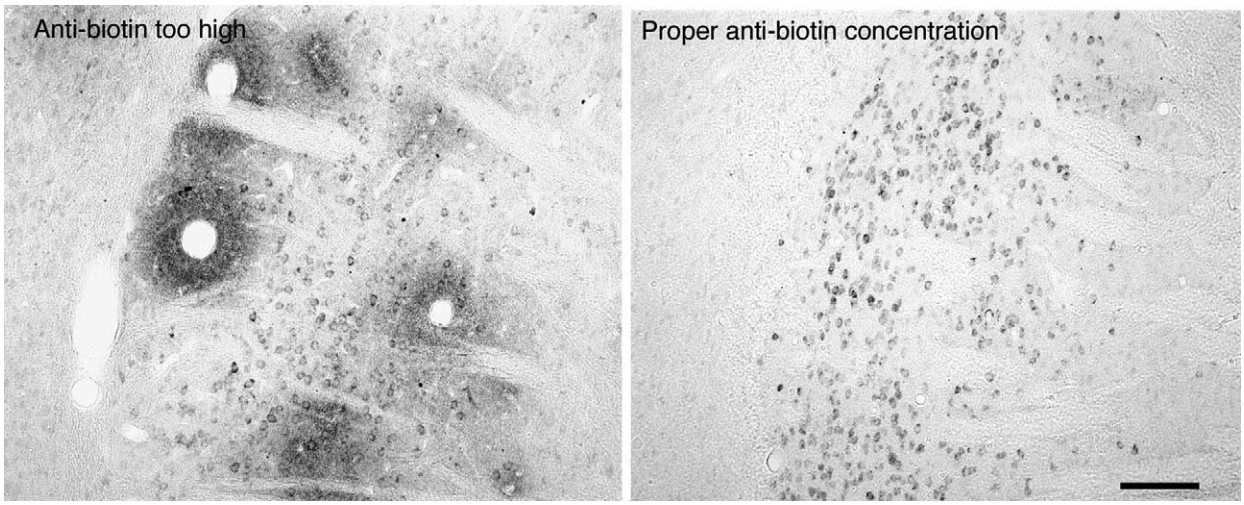

Fig. 5. Changing the concentration of the anti-biotin affects the ability to detect a clear ISH signal. (a) ISH reaction of a section through the striatum of a rat in which the antibiotin was employed at a concentration that was too high to provide a clean signal. (b) Dilution of the antibiotin in the reaction eliminates the background staining and yields a more intense reaction product in the positive cells (staining employed an ABC reaction with NiDAB as the chromogen). Bar = 100 μm.

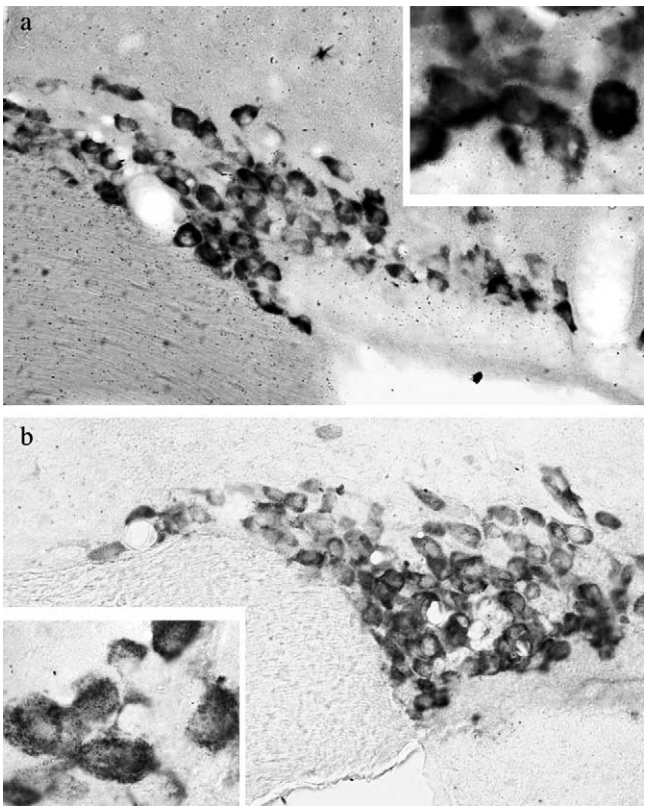

Fig. 6. Comparison of the localization of vasopressin mRNA in the supraoptic nucleus of the hypothalamus with ISH using (a) a digoxigenin-labeled riboprobe and NTB blue alkaline phosphatase visualization and (b) biotin-labeled riboprobes with NiDAB ABC immunoperoxidase detection. The inserts show a few cells at higher magnification.

diaminobenzidine (DAB) as the chromogen, provided the ability to resolve the clusters of RNA within the cell's cytoplasm or nucleus [5] that alkaline phosphatase detection of digoxigenin did not (Fig. 6). The RNA clusters could easily be counted with image analysis.

In addition, the resolution of RNA clusters can enable detailed analyses of not only how much RNA is expressed but where [3]. Fig. 7 illustrates the progression of changes in the level of tyrosine hydroxylase RNA in hypothalamic neurons of the hypothalamus that are undergoing up-regulation prompted by termination of the suckling stimulus. The probe we used was generated against a sequence in a single exon of the gene and thus was capable of revealing both heteronuclear (immature) RNA as well as the mature messenger RNA. Note that as expression is stimulated, the first change observed is an increase in nuclear RNA seen as one or two dense sites of hybridization signal. Later, as transcription is further stimulated, cytoplasmic RNA (likely mature mRNA) accumulates. As the level of expression stabilizes, the nuclear signal declines, and the RNA spreads into the dendrites of some of the cells. For this type of detection, a reaction product which cannot spread from the site of production is needed and in my laboratory's experience, biotinylated probes detected with immunocytochemical reaction of the biotin followed by the avidin/biotin complex (ABC) method with NiDAB as the chromogen provides the best means of achieving that level of resolution.

Taken together, the continuous discoveries of new genes and new intracellular signaling molecules coupled with the need to understand how these molecules are regulated and interact to govern cellular function makes advances in both immunocytochemical detection of cell consitutents and understanding of their synthesis with in situ hybridization strategies at a cellular level critical. The use of stored tissue that preserves both protein and RNA equally well makes these assessments feasible, and thus extends the repertoire of techniques that can be applied to tissue from a single animal. At the same time, as we experience concerns over reduction in the level of available funds for such research, we must find cost effective means of saving precious tissue and yet retaining flexibility in how it can be used for analysis. The capacity for storing tissue for long periods without losing antigenicity or nucleotide content, coupled with having available sensitive and inexpensive methods for detection of both RNA and proteins makes this task easier.

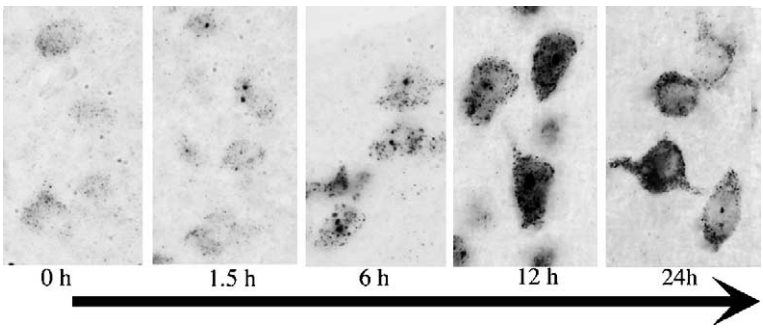

Fig. 7. TH RNA in neurons of the arcuate nucleus during a stimulus (pup removal in a lactating dam) which prompts up-regulation of the TH mRNA. Note that by 1.5 h after pup removal, a strong heteronuclear RNA signal is detected. Six hour after initiation of up-regulation, cytoplasmic RNA levels are increased and by 24 h, the signal extends beyond the soma into the cell's dendrites.

Acknowledgments

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