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The immunogold-silver staining method

A powerful tool in histopathology

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Summary. Immunostaining of routinely fixed, wax embedded tissues may present problems to the pathologist since destruction of antigens can lead to false negative results. In an attempt to overcome this problem, we have compared the results of the standard peroxidase anti-peroxidase (PAP) method with those obtained using the newly developed and very sensitive immunogold-silver staining (IGSS) method. Sections from routine histopathological material as well as from normal tissue specimens were used in the comparison. Antisera to a variety of antigens commonly employed in pathology were used, including regulatory peptides and a range of other markers. In all cases the IGSS method was found to give superior or at least equal results to those obtained with the PAP technique. In some cases staining was obtained with IGSS method when the PAP technique gave no result. The intense black reaction product allowed much easier and more rapid screening of immunostained preparations as well as permitting sections to be counterstained with routine histological stains such as haematoxylin and eosin.

It is therefore suggested that immunogold-silver staining is a valuable technique for the pathologist, particularly when examining overfixed or badly processed tissues.

Key words: Histopathology – Immunocytochemistry – Paraffin sections – Regulatory peptides – Staining techniques

Advances in the field of immunocytochemistry have led to the development of highly sensitive and specific techniques for the detection of various antigens. Although most peptides and other markers in tissue sections can be demonstrated easily by the immunofluorescence (Coons et al. 1941 and 1955), immunoperoxidase (Nakane and Pierce 1966) or peroxidase anti-peroxidase (PAP) technique (Sternberger 1974), special methods of tissue

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Table 1. Summary of tissues used in the study

Diseased tissue	Normal tissue
Colon, Crohn's disease	Foetal liver
Colon, with adenocarcinoma	Pancreas
3 Vipomas	Brain stem
Phaeochromocytoma	Spinal cord
2 Merkel cell tumours	Pituitary
Spinal cord, motoneuron disease	Rat cerebellum
Lung, small cell carcinoma	Rat pituitary
Rhabdomyosarcoma from epididymis	Rat hypothalamus
Leiomyosarcoma from vulva	Pig urinary bladder
Lymphoma	Pig snout skin
Lung, Legionella-infected	

fixation are often required. In tissue fixed in conventional fixatives, such as formalin and Bouin's fluid, and embedded in paraffin wax, it is sometimes difficult to obtain consistent, strong immunostaining with the currently available immunocytochemical methods. This problem has been attributed to a number of factors, including loss or alteration of antigenicity by the fixative used. This variability has sometimes hampered the use of immunocytochemistry in routine histopathology. This is particularly the case in neuropathology as peptide neurotransmitters appear to be very susceptible to fixation damage.

Another problem of a completely different nature occurs in PAP immunocytochemistry, where substances are used which may have carcinogenic potential and thus require careful handling.

In 1983, Holgate and co-workers described a new method for immunostaining: "immunogold-silver staining" (IGSS). In principle, this technique is a combination of immunogold staining (Faulk and Taylor 1971; Gu et al. 1981) and a method for the visualisation of gold particles in histological preparations by use of a silver reaction (Danscher 1981). When we applied this method to the detection of regulatory peptides, it resulted in a very high degree of background staining. We have, therefore, recently modified the technique to allow the demonstration of peptidergic nerves and endocrine cells since it can often provide a number of advantages in comparison with conventional immunocytochemical techniques (Springall et al. 1984). These advantages include greater sensitivity than the PAP technique (Holgate et al. 1983; Springall et al. 1984), a high contrast of specific immunostaining against a low level of unwanted background staining and avoidance of carcinogenic reagents.

In the present study, we have compared the PAP and the IGSS method for the visualisation of antigens in a wide range of tissues from tumour and non-tumour cases, all routinely fixed and embedded in paraffin wax. We have used antibodies to regulatory peptides and other antigens including intermediate filaments, alpha fetoprotein, factor VIII, neuron specific enolase (NSE), carcinoembryonic antigen and S-100.

Table 2. Primary antisera (rabbit antisera and rat or mouse monoclonal antibodies) used in this study. The dilution shown is that which gave the maximal detection of antigen with the lowest level of background staining

Antiserum to	Donor	Type	PAP dilution overnight, 4° C	IGSS dilution 90 minutes, room temp.
Neuropeptide Y (NPY)	rabbit	polyclonal	1/2,000	1/2,000
Vasoactive intestinal polypeptide (VIP)	rabbit	polyclonal	1/2,000	1/5,000
Pancreatic glucagon	rabbit	polyclonal	1/5,000	1/20,000
Calcitonin gene-related peptide (CGRP)	rabbit	polyclonal	1/2,000	1/16,000
Neurotensin (NT)	rabbit	polyclonal	1/16,000	1/16,000
Adreno-corticotrophic hormone (ACTH)	rabbit	polyclonal	1/2,000	1/4,000
Luteinising hormone (LH)	rabbit	polyclonal	1/800	1/2,000
Prolactin	rabbit	polyclonal	1/1,800	1/4,000
Follicle stimulating hormone (FSH)	rabbit	polyclonal	1/600	1/2,000
Thyroid stimulating hormone (TSH)	rabbit	polyclonal	1/900	1/2,000
Serotonin (5-hydroxy-tryptamine, 5HT)	rabbit	polyclonal	1/10,000	1/10,000
Met-enkephalin (M-ENK)	rabbit	polyclonal	1/4,000	1/4,000
Human chorionic gonadotropin (HCG)	rabbit	polyclonal	1/10,000	1/20,000
Neuron specific enolase (NSE)	rabbit	polyclonal	1/2,000	1/4,000
Neurofilament protein (NF) (150 kd)	rabbit	polyclonal	1/2,000	1/4,000
Neurofilament protein (NF – MCA) (150 and 200 kd)	rat	monoclonal (super-natant)	1/2	1/50
Carcinoembryonic antigen (CEA)	rabbit	polyclonal	1/1,000	1/8,000
Factor VIII	rabbit	polyclonal	1/1,000	1/2,000
Alpha fetoprotein (α FP)	rabbit	polyclonal	1/1,000	1/2,000
Glial fibrillary acidic protein (GFAP)	rabbit	polyclonal	1/500	1/2,000
S-100	rabbit	polyclonal	1/4,000	1/4,000
Desmin (Des)	rabbit	polyclonal	1/100	1/5,000
Leucocyte Common Antigen (LCA)	mouse	monoclonal (super-natant)	undiluted	1/10
Legionella	rabbit	polyclonal	1/500	1/4,000
Gastrin	rabbit	polyclonal	1/5,000	1/10,000
Somatostatin-14 (SST-14)	rabbit	polyclonal	1/10,000	1/20,000

Table 3. Comparison of results of results of immunostaining obtained on serial sections by the PAP and IGSS methods

Tissue	Fixative	Staining for	PAP	IGSS
Colon, Crohn's disease	F	Fact. VIII	strong+	strong+
		VIP	medium	strong
		S-100	strong	strong
Colon, adenocarcinoma	F	Fact. VIII	medium+	strong+
		CEA	strong	strong
		S-100	strong	strong
VIPoma 1	B	VIP	weak	strong
	F	VIP	n.d.	strong
VIPoma 2	B	NT	medium	medium
	B	VIP	medium	strong
	F	VIP	weak	strong
VIPoma 3	B	VIP	negative	weayk
		Glucagon	medium	strong
		HCG	medium	strong
		NSE	medium	medium
Phaeochromocytoma	B	NF	negative	strong
		TH	negative	strong
		M-ENK	medium	strong
		NSE	medium	strong
		NPY	medium	strong
Merkel cell tumour 1	B	NSE	weak	strong
Merkel cell tumour 2	F	NSE	medium[a]	medium
Epididymis, rhabdomyosarcoma	FM	Desmin	strong	strong
Vulva, leiomyosarcoma	FM	Desmin	medium	strong
Lymphoma	F	LCA	strong	strong
Lung, legionella infected	FM	Legionella	strong	strong
Spinal cord motoneuron disease post mortem	F	CGRP	medium	strong
		NF	negative	medium
		NF-MCA	n.d.	strong
Spinal cord post mortem	F	CGRP	medium	strong
		NF-MCA	n.d.	strong
Foetal liver	F	αFP	medium	strong
Brain stem (pons) post mortem	F	GFAP	weak+	strong+
Pancreas, post mortem	B	Gluc	strong	strong
Pituitary, post mortem	FM	LH	strong	strong
	FM	Prolactin	strong	strong
	FM	FSH	strong	strong
	FM	ACTH	strong	strong
	FM	TSH	strong	strong

Table 3 (continued)

Tissue	Fixative	Staining for	PAP	IGSS
Rat pituitary	B	NSE	strong	strong
		ACTH	strong	strong
Rat cerebellum	B	NF	negative	strong
		NF-MCA	n.d.	strong
		NSE	medium	strong
Rat hypothalamus	B	NF	negative	strong
		NSE	medium	strong
Pig urinary bladder	B or F	NF	weak	strong
		NF-MCA	n.d.	strong
		NSE	weak	strong
		S-100	medium	strong
Pig snout skin	B	NF	weak	strong
		NF-MCA	n.d.	strong
		NSE	medium	strong
		S-100	medium	strong
Pig antrum	B	Gastrin	strong	strong
		SST-14	strong	strong
Pig duodenum	B	SST-14	strong	strong

Abbreviations: F = formalin; FM = formol mercury; B = Bouin's fluid; + = trypsinisation necessary; n.d. = not done

[a] In this case, the antiserum to NSE had to be used at a higher concentration than shown in Table 1 (1/800) to obtain a positive in PAP reaction

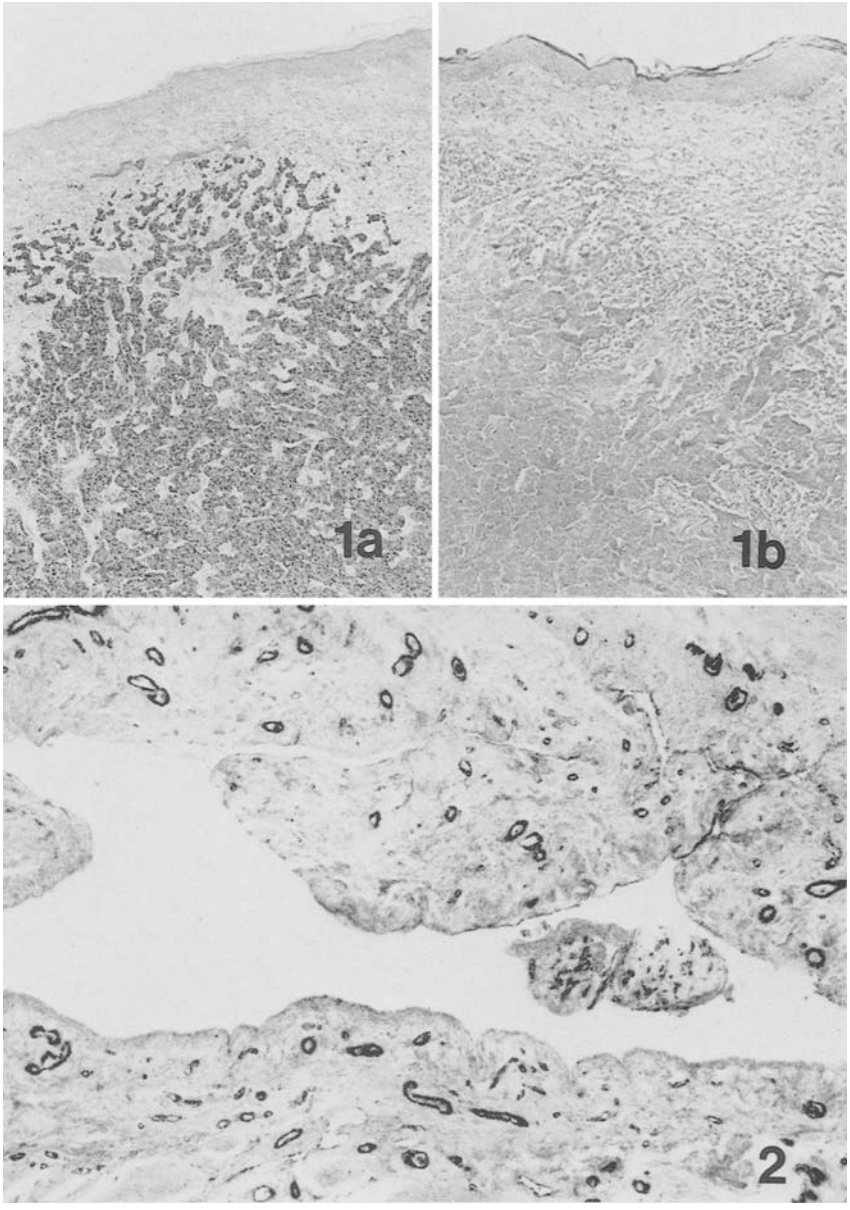
Materials and methods

A. Tissues. A brief summary of all the tissues used in this study is given in Table 1. All pathological cases had been diagnosed previously on the basis of histological and clinical data.

Tissue specimens were collected from surgery, fixed overnight in phosphate-buffered 10% formalin or formol-mercury, or for 4 h in Bouin's fluid, dehydrated and cleared in a series of graded alcohols and xylene, and vacuum embedded in paraffin wax at 60° C. In addition to these freshly obtained and optimally processed specimens, a range of cases was also selected from the routine histopathology files. To compare the effects of different fixatives on immunostaining and for testing second layer antibodies, tissues from rat and pig were used. Serial sections (2 to 12 μm thick) were mounted on poly-L-lysine coated slides to increase section adherence (Huang et al. 1983). Alternate consecutive sections were stained with the PAP technique and the IGSS method.

B. Trypsin pre-treatment. In some cases where the PAP technique has given weak or equivocal immunostaining, trypsin pre-treatment may increase the reactivity by revealing the antigenic sites (Huang et al. 1976). After rehydration, some formalin-fixed sections (Table 3) were therefore pre-treated for 20 or 30 min at 37° C with an aqueous solution of 0.1% trypsin and 0.1% calcium chloride, adjusted to a pH of 7.8 with sodium hydroxide. After trypsinisation, these sections were well rinsed in deionized water and immunostained.

C. PAP method. In order to compare the IGSS method with a standardized, well established and highly sensitive immunocytochemical method, the PAP technique of Sternberger (1979) was chosen. In combination with rabbit primary antisera, goat anti-rabbit IgG (Dako, Copenhagen, Denmark) was used as bridge molecule in a dilution of 1/200. Rabbit PAP complex



Figs. 1 a and b. Immunostaining for neuron specific enolase in a surgical specimen of a Merkel cell tumour, fixed in Bouin's fluid. 5 μm thick wax sections. Intense staining is obtained by use of the immunogold-silver method **1 a**), whereas PAP staining gives a rather weak reaction **1 b**). Both counterstained with haematoxylin. (× 80)

Fig. 2. Immunogold-silver staining using antibodies to factor VIII in muscle wall of human colon, fixed in phosphate-buffered formalin and wax-embedded. Intensely stained endothelial lining of blood vessels can be seen even at very low magnification. 5 μm wax section, counterstained with eosin. (× 80)

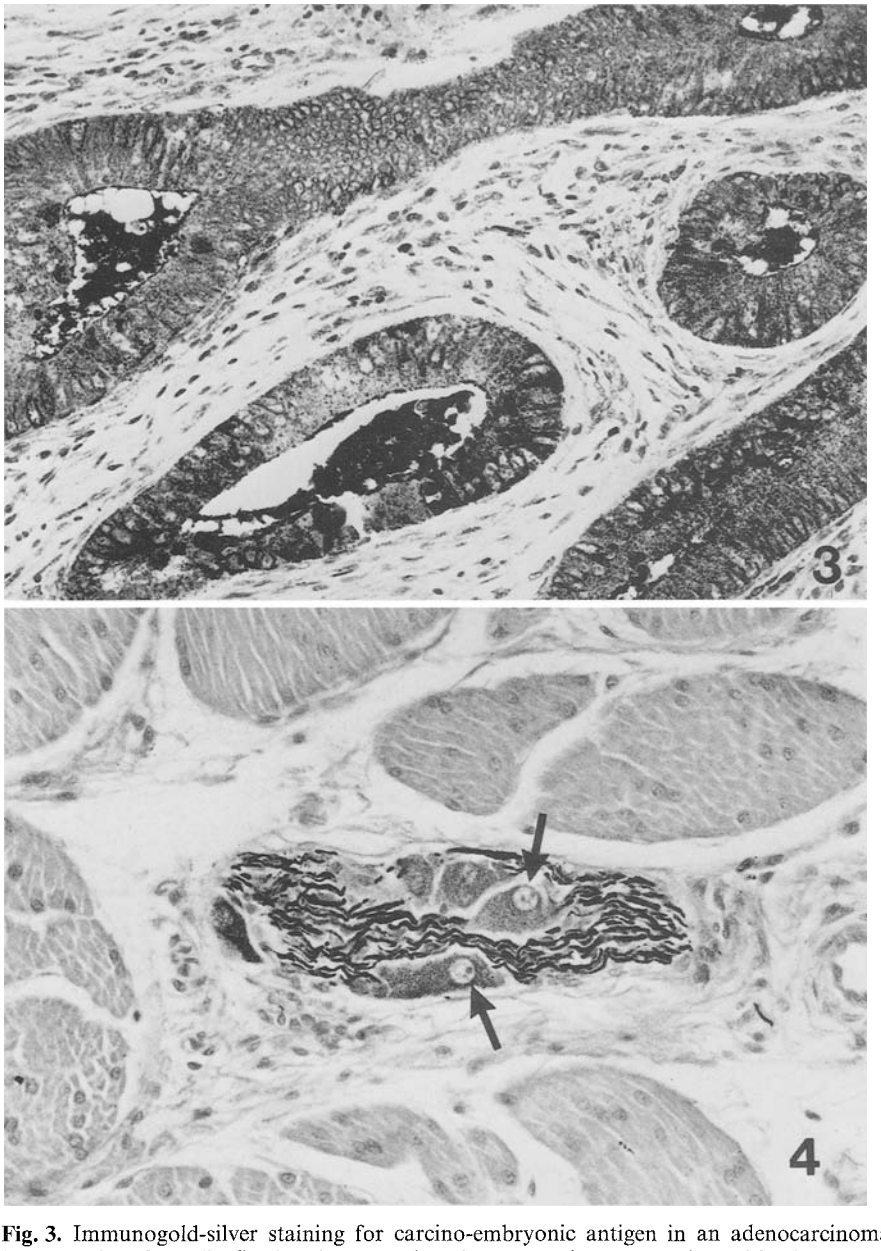


Fig. 3. Immunogold-silver staining for carcino-embryonic antigen in an adenocarcinoma of human colon, formalin-fixed and wax-sectioned at 5 μm, shows strongly positive immunoreaction. Counterstained with haematoxylin and eosin. (× 400)

Fig. 4. Small intramural ganglion in formalin-fixed urinary bladder of pig, stained by the immunogold-silver method with a rabbit antiserum to chicken neurofilament proteins. Nerve fibres are intensely stained, whereas perikarya (*arrows*) show a coarse granular and lighter reaction. 5 μm wax section, counterstained with eosin. (× 400)

(Dako) was applied in a dilution of 1/300. The chromogen used for the visualisation of peroxidase was 3,3′-diaminobenzidine-tetrahydrochloride. PAP-stained sections were counterstained lightly with haematoxylin.

D. IGSS method. Immunogold-silver staining was carried out as previously described by Springall and co-workers (1984). In brief, incubation with highly diluted primary antiserum specific for the antigen to be detected was followed by treatment with gold-adsorbed secondary antisera raised against immunoglobulin of the species in which the primary antiserum was produced. The colloidal gold was then visualised by use of a physical silver development solution.

Sections stained with the IGSS method were counterstained with conventional haematoxylin and eosin, eosin or haematoxylin alone or toluidine-blue.

E. Specificity tests. Controls used for the PAP and IGSS methods included replacement of primary antisera with diluent alone or normal rabbit, rat or mouse serum, as appropriate. In addition, in the IGSS technique the gold-labelled second antibody was replaced by diluent in order to ensure that the silver enhancement solution did not itself react with the tissue.

F. Primary antisera. All the primary antisera used have been described previously. Rabbit antisera, as well as rat and mouse monoclonal antibodies were used. Antisera characteristics are shown in Table 2.

G. Gold-adsorbed antibody for IGSS. For rabbit primary antisera, goat anti-rabbit IgG adsorbed to gold particles of 5 nm diameter, 'GAR G5', was used as second layer, at a dilution of 1/250 in 0.05 M tris buffer, pH 8.2, containing 0.8% bovine serum albumin. When rat or mouse monoclonal antibodies were applied as first layers, goat anti-rat IgG 'GARa G5' (1/100) or goat anti-mouse IgG 'GAM G5' (1/100) affinity-purified antisera in the same diluent were used. These optimal dilutions were established by testing concentrations of 1/10 to 1/500. All gold-adsorbed antisera were obtained from Janssen Life Sciences Products, Beerse, Belgium.

Results

In many cases where the PAP method provided staining, the IGSS method gave a much greater contrast with a higher dilution of the primary antibody and allowed easier reading of the preparations at a lower magnification. Sometimes, staining was obtained with the IGSS method where only a weak or a negative PAP reaction was seen.

Strongly positive cells showed a dense black homogeneous staining, whereas weakly or intermediately positive cells appeared to have coarse granular cytoplasmatic staining. The pattern of staining, especially of that obtained in tumour tissues, was in most cases similar to that demonstrated by the PAP method, but in some cases IGSS revealed more positively stained structures than the PAP method. The results are summarized in Table 3.

The fixatives used allowed positive results with IGSS in all cases tested. Using this method, no significant difference in results was found between Bouin's- or formalin-fixed rat or pig tissues. After trypsinisation for 20–30 min, the sensitivity of both staining methods was found to be strongly increased for some antigens (Table 3).

None of the control sections showed any immunostaining. In most cases, the degree of background staining in the IGSS sections was very low, provided that the shortest possible silver development times were used. In general, many primary antibodies could be used at a considerably higher dilution for the IGSS method than that necessary for the PAP method (Table 2).

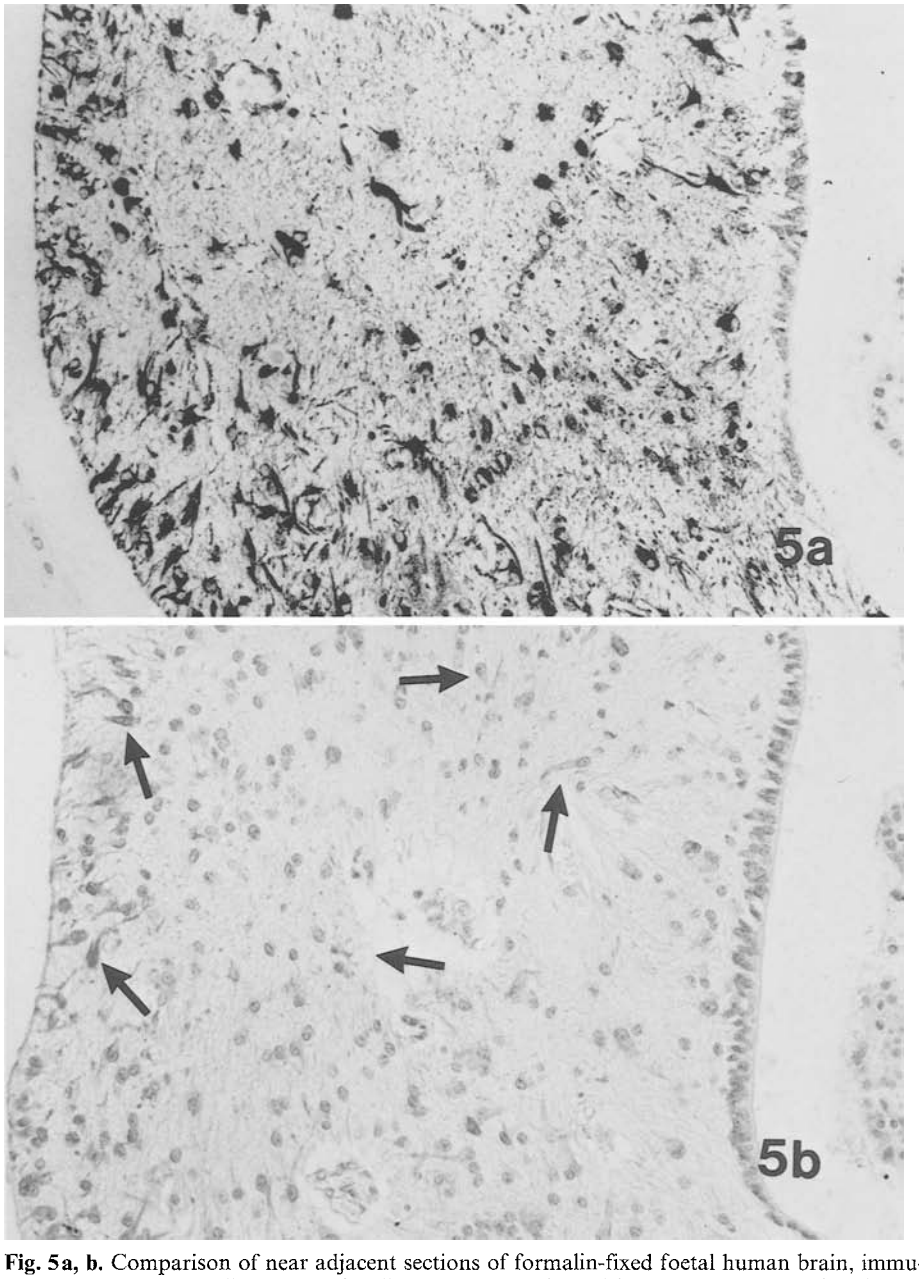
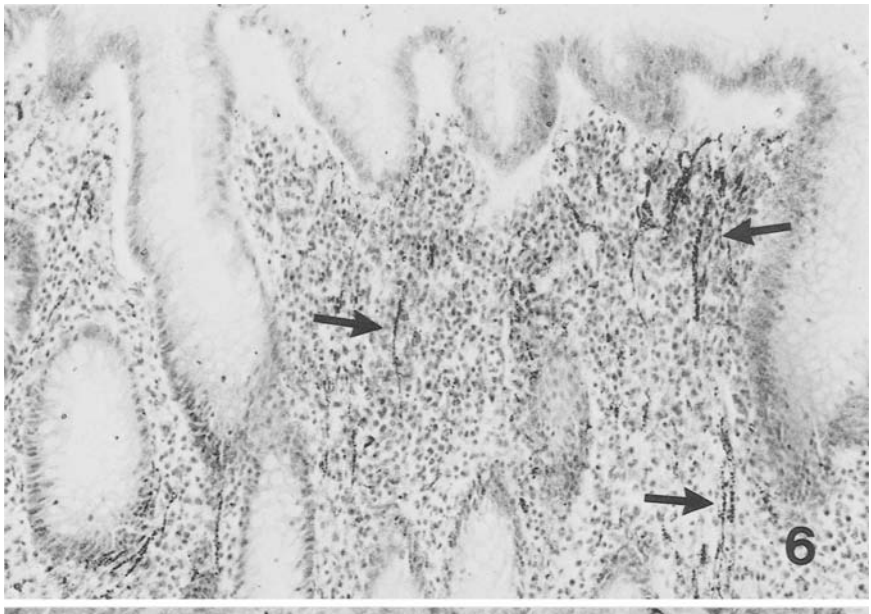


Fig. 5a, b. Comparison of near adjacent sections of formalin-fixed foetal human brain, immunostained with antibodies to glial fibrillary acidic protein and lightly counterstained with haematoxylin. Fig. **5a** shows an intense black reaction-product in astrocytes and their processes produced by the immunogold-silver staining method whereas the PAP method **5b**) revealed only weakly immunopositive astrocytes (*arrows*). 5a and b were printed under the same conditions. 5 μm thick wax sections. (× 300)



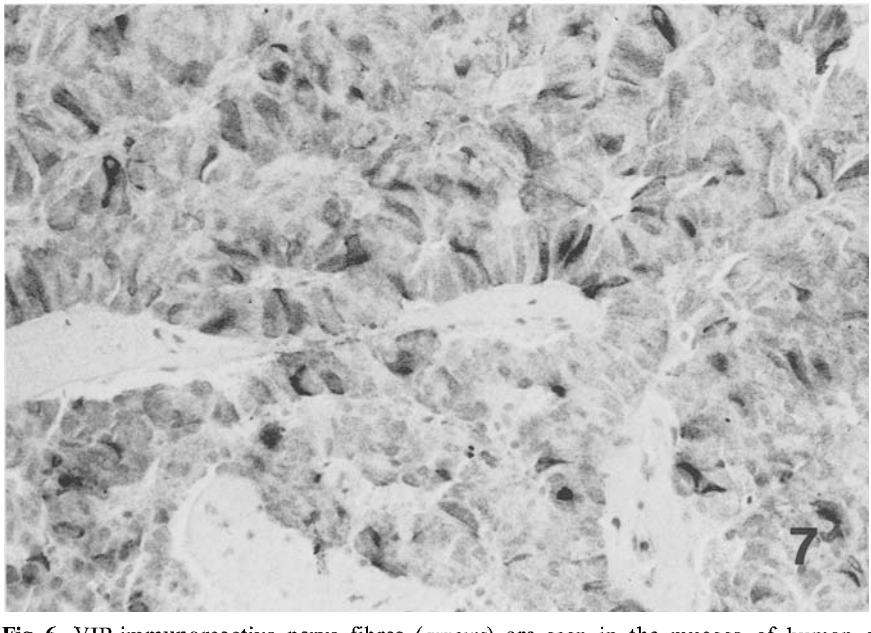


Fig. 6. VIP-immunoreactive nerve fibres (*arrows*) are seen in the mucosa of human colon from a case of Crohn's disease stained by the immunogold-silver method. Formalin-fixed specimen cut at 4 μm and counterstained with conventional haematoxylin and eosin. (× 220)

Fig. 7. VIP-immunoreactive cells in a formalin-fixed VIPoma are stained intensely by the immunogold-silver method allowing rapid screening of sections at low magnification. Adjacent sections stained by the PAP method gave very weak staining. 7 μm thick wax sections, counterstained with haematoxylin and eosin. (× 220)

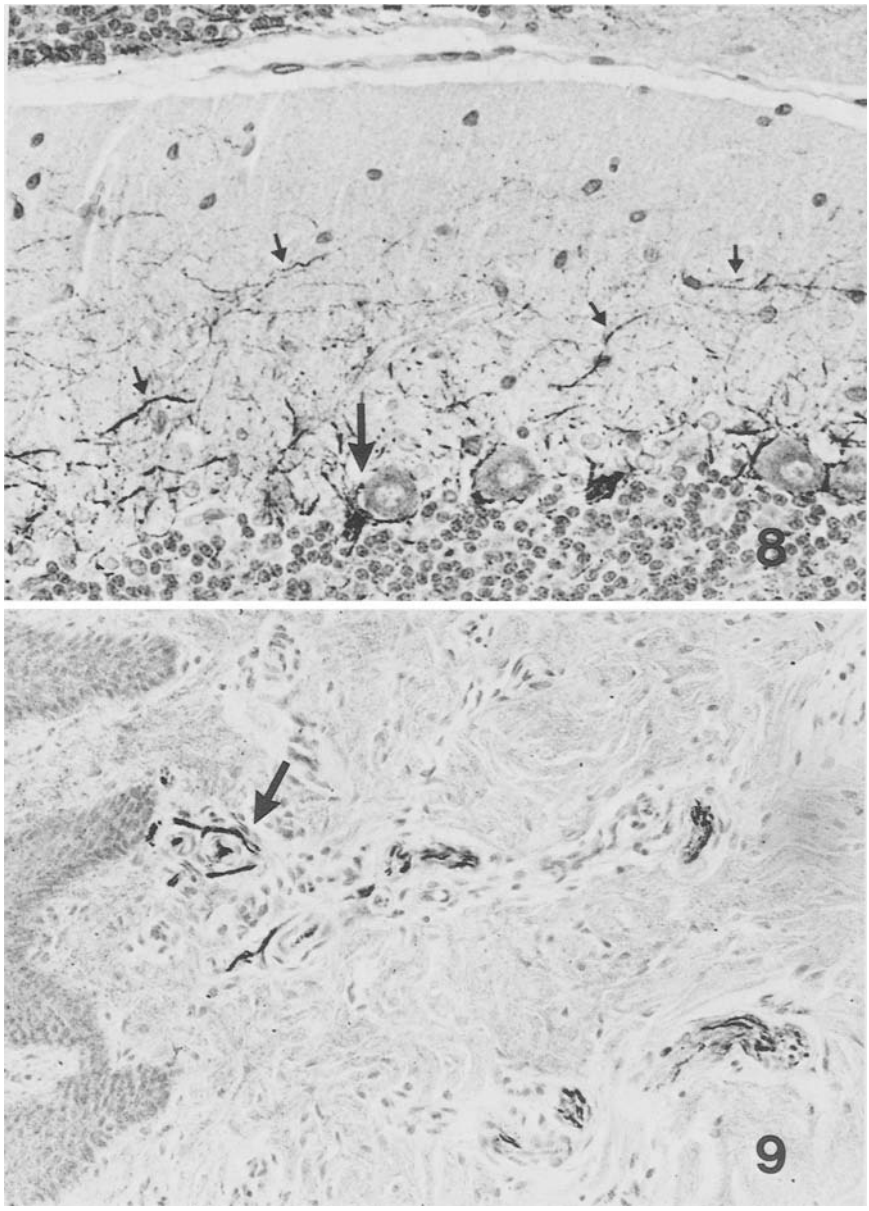


Fig. 8. Rat cerebellum immunostained with monoclonal antibodies to bovine neurofilament proteins and immunogold-silver. Basket cell fibres are revealed in close contact with Purkinje cells (*large arrow*) as well as thin nerve fibres in the molecular layer (*small arrows*). Bouin's-fixed, 5 µm wax section. Counterstained with haematoxylin and eosin to allow assessment of morphology. (× 350)

Fig. 9. Bouin's-fixed and wax-embedded pig snout skin, cut at 5 µm. Neuronal structures in the skin are visualised by use of monoclonal antibodies to bovine neurofilament proteins and the immunogold-silver staining method. The arrow indicates nerve fibres in Meissner's corpuscles. Lightly stained with haematoxylin and eosin. (× 300)

Discussion

The immunogold silver staining method was used for the immunostaining of routinely fixed, paraffin-embedded tissues. This method allowed the visualisation of all antigens investigated, including regulatory peptides and intermediate filaments. Specificity controls showed that the staining was not artefactual. Our findings show that immunostaining could be obtained consistently in optimally processed specimens and usually in those from the routine histopathology files which would have been subject to the less exigent fixation conditions encountered in any routine laboratory. It seems that where fixation has impaired the availability of the antigen, the IGSS method is more sensitive than the PAP method, allowing the detection of the remaining antigenic sites. In some cases staining was obtained with the IGSS method but not with the PAP method.

As with all immunocytochemical staining methods, a number of provisos must be taken into account. It is necessary to titrate the primary antisera, particularly the polyclonal ones, to reduce non-specific reactions and lower the costs. The dilutions for the PAP method shown in Table 2 are those suitable for optimally processed tissue. The dilutions for the IGSS method were optimal for an incubation time of 90 min at room temperature, but it is possible that the primary antisery could be diluted further if overnight incubation were used. Any new batch of gold conjugate must be tested with a known positive control to avoid the problems of possible batch variation. If optimum staining is to be achieved, the development time must be ascertained for each system. It was found that it is extremely important to use very pure distilled water, preferably deionised and double glass-distilled, for all washes before the silver precipitation step and as diluent for the silver solution. It is advisable to produce a range of staining intensities in order to avoid the danger of understaining and missing areas of low antigen content. In cases of overdevelopment some of the silver deposit can be removed by use of a modified photographic cutting reducer (Springall et al. 1984). Negative controls, preferably serial to the test sections, should be included in order to show the level of non-specific background staining.

Although the IGSS method is not necessarily a solution for every problem, it offers some advantages over the PAP method.

Increased sensitivity

Our results demonstrate the higher sensitivity of the IGSS method. However, this increased sensitivity can lead to a higher degree of background staining when low quality primary antisera are used and the method thus requires highly specific primary antisera or monoclonal antibodies.

Ease of screening

The IGSS method gives a very intense reaction product. Therefore, positive reactions can be identified easily using low magnification, thus facilitating fast screening of sections and rapid diagnosis. In addition, the high contrast

obtained with this technique allows the use of conventional counterstaining as an aid to assessment of the morphology.

Efficiency of the staining procedure

The IGSS method takes less time to carry out than the PAP method; it can be completed within four hours. The PAP method can also be carried out using short incubation times, but this requires a higher concentration of the primary antibody which may lead to an increase in non-specific staining as well as incurring greater expense.

Cost

The cost of the IGSS method is less than or equal to that of the PAP method (depending on the price of the primary antibody). A kit for IGSS is commercially available (Janssen Life Sciences Products, Beerse, Belgium).

In conclusion, the IGSS method may become a useful additional diagnostic test for many histopathological specimens which were formerly not suitable for routine immunocytochemical examination.

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