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The potential of the immunogold-silver staining method for paraffin sections

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Summary. The immunogold-silver staining technique is shown to be of great value in the detection of regulatory peptide-containing nerves and endocrine cells in routinely fixed, paraffin-wax-embedded tissues. The method appears to be better for this system than peroxidase anti-peroxidase (PAP) which can yield poor or variable results.

Antibodies to regulatory peptides, including calcitonin gene-related peptide (CGRP), substance P, neuropeptide tyrosine (NPY), glucagon, pancratic polypeptide, and somatostatin 14 and 28, as well as to neurofilaments, neuron-specific enolase (NSE) and S-100, were used on sections of a variety of tissues from rat and pig including respiratory tract, skin, gut, pancreas, vagina, uterus, fallopian tube and kidney. In all cases, stronger immunostaining of nerves was obtained with the immunogold-silver technique than with PAP. The inherent density of the staining was also found to improve the visibility of endocrine cells in the section, and to permit the use of routine histological stains for counterstaining.

As immunogold-silver staining is sensitive, rapid, cheap and avoids hazardous reagents, we feel it has great potential for the immunostaining of nerves and endocrine cells that contain regulatory peptides in routinely fixed and embedded tissues and may prove useful in pathology.

Introduction

In 1983 a novel immunohistochemical procedure, the immunogold-silver staining technique, was published by Holgate and co-workers (1983). The method was a combination of immunogold staining, originally devised for use at the electron microscopic level (Faulk and Taylor 1971), but subsequently adapted for light microscopy (Geoghegan et al. 1978), and the method of Danscher (1981) for the detection of gold in biological tissues. Immunogold staining for light microscopy (Gu et al. 1981) uses relatively high concentrations of immunogold reagent (immunoglobulin adsorbed to the surface of colloidal gold particles) in order to bind sufficient colloidal gold to the tissue section to permit its visualisation under the microscope. The immunogold-silver method requires much less of this reagent to be bound, since the gold is revealed by a silver development step, and so higher dilutions of immunogold may be used.

The widespread distribution of regulatory peptides in nerves and endocrine cells throughout the body is now well-established (Polak and Bloom 1983). It is also becoming clear that these peptides play a considerable part in the manifestation of many disease processes. However, one block to furtherance of our understanding of the involvement of regulatory peptides in disease has been the difficulty of immunostaining nerves containing these peptides in routinely fixed and wax-embedded sections. In order to prevent unacceptable reduction in antigenicity or the diffusion of peptides into the extracellular compartment it has previously been necessary to use special fixatives such as p-benzoquinone (Pearse and Polak 1975; Bishop et al. 1978) or at least cryostat sections of paraformaldehyde-fixed tissue.

As Holgate and co-workers (1983) have claimed that the immunogold-silver technique has greater sensitivity than the peroxidase anti-peroxidase method of Sternberger (1979), we decided to investigate its potential for the immunostaining of regulatory peptide-containing nerves and endocrine cells in routinely fixed, paraffin wax-embedded tissues. Although endocrine cells often retain immunoreactivity in tissues so processed, we felt that immunogold-silver staining might facilitate the detection of peptides by improving the contrast of immunostaining. We also attempted various modifications in order to reduce the strong background staining which we found to occur when using the published method, and which may have been due to the different antibodies used.

Materials and methods

Tissues. A variety of tissues from rat and pig (see Table 1) were dissected and fixed in Bouin's fluid for 4 h or 10% formol saline overnight at 4° C. The former were washed thoroughly in 30% alcohol and both were processed through graded alcohols and trichloroethane to paraffin wax.

Sections (5 μm) were taken up on slides coated with poly-L-lysine (Sigma, molecular weight 540,000) to ensure good adhesion (Huang et al. 1983). Serial sections were immunostained using the immunogold-silver or PAP technique, or they were stained by haematoxylin and eosin.

Peroxidase anti-peroxidase (PAP) method. The method employed was basically that of Sternberger (1979). The optimal dilutions of primary antibodies are listed in Table 2. Sections were incubated with primary antiserum for 16–24 h at 4° C. Goat anti-rabbit Ig (Miles Laboratories) was used at 1:200 for 30 min and rabbit PAP

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Table 1. Tissues used for comparison of PAP and immunogold-silver techniques

Rat	Pig
Brain	Antrum
Genitalia	Pancreas
Kidney	Skin – snout
Pancreas	Urinary bladder
Respiratory tract	
Skin – back	
Spinal cord	
Urinary bladder	

Table 2. Optimal dilutions of primary antibodies used for immunostaining

Antigen	Dilution PAP	Dilution immunogold-silver
Calcitonin gene-related peptide (CGRP)	1:2,000	1:8,000
Neuropeptide Y (NPY)	1:2,000	1:5,000
Substance P	1:5,000	1:5,000
Somatostatin 14	1:10,000	1:10,000
Somatostatin 28	1:2,000	1:2,000
CCK39/gastrin	1:5,000	1:5,000
Neurofilament protein (triplet)	1:2,000	1:2,000
Glucagon	1:5,000	1:10,000
Bovine pancreatic polypeptide	1:12,000	1:12,000
Neuron-specific enolase	1:2,000	1:4,000
S-100	1:4,000	1:4,000

complex (UCB Bioproducts) at 1:300 for 30 min. The chromogen used was 3,3′-diaminobenzidine tetrahydrochloride. Sections were counterstained lightly with haematoxylin before mounting.

Controls used included the omission of primary antibody or its replacement with normal rabbit serum, or primary antibody absorbed with homologous antigen.

Immungold-silver method. Various modifications to the method of Holgate and co-workers (1983) were tried in an attempt to reduce background staining (Table 3). These included the use of detergents, gelatine and high salt concentrations to reduce non-specific binding of antibody, adjustment of buffer pH for the immunogold reaction to improve stability of the reagent, fixation of the sections after incubation in immunogold to prevent elution of antibodies in the low pH buffer of the silver developer, and the use of high quality commercial immunogold reagents of various gold colloid sizes.

For this method the controls used included omission of primary antibody or its substitution by antibody absorbed with homologous antigen or normal rabbit serum, or omission of the immunogold reagent to ensure that there was no reaction of the silver developer other than with colloidal gold particles.

Post-development silver reduction. In order to decrease the intensity of staining in sections which had been over-developed in silver reagent or which had a high background, a modified photographic reducer was used. After rinsing in distilled water, slides were placed in a freshly prepared solution consisting of 60 ml of distilled water, 0.3 ml of 7.5% potassium ferricyanide solution and 1.2 ml of 20% sodium thiosulphate solution. The degree of reduction was checked microscopically at one minute intervals after rinsing the slide in distilled water, until staining intensity was judged to be satisfactory.

Reagents. Immunogold (GAR G5, G10, G20, and G40) was obtained from Janssen Pharmaceutica, Beerse, Belgium; bovine serum albumin (A-7906) and citric acid (C-7129) from Sigma Chemical Company; tri-sodium citrate (10242), hydroquinone (30011) and

Table 3. Modifications made to attempt reduction of background staining in the method of Holgate et al. (1983)

Method of Holgate et al. (1983)	Modifications to steps as numbered
1. Cut 5 μm paraffin sections	2–3 Omission of Lugol's iodine/sodium thiosulphate pretreatment
2. Apply Lugol's iodine, 5 min	4. Omission of trypsin pre-treatment
3. Rinse thoroughly and remove all traces of iodine with 2.5% sodium thiosulphate	4–10 Inclusion of Triton X-100 or Tween 80 into all solutions, at concentrations of 0.2%–0.5%.
4. Treat sections 5 min with 0.1% trypsin in TBS	4–10 Addition of 0.2% gelatine to all solutions
5. Apply undiluted normal swine serum, 5 min	4–10 Use of higher sodium chloride concentrations (up to 2.5%) in all solutions
6. Treat with primary antiserum in 5% swine serum, 30 min	9. Use of commercial immunogold reagent (Janssen Pharmaceutica, Beerse, Belgium) with defined gold colloid sizes of 5, 10, 20 and 40 nm at dilutions from 1:10 to 1:500, and incubating with sections for times from 30 to 120 min
7. Wash sections in TBS, 30 min	
8. Repeat 5	
9. Apply immunogold reagent diluted 1:3 in TBS, overnight	9. Replacement of Tris-buffered saline pH 7.4 with Tris-buffered saline pH 8.2 for dilution of the immunogold reagent. The higher pH increases the stability of the gold colloid
10. Wash in TBS, 30 min. Wash distilled water, 30 min	9. Addition of bovine serum albumin in concentrations from 0.1% to 1.0% to the immungold diluent
11. Immerse in silver solution (Danscher 1981) and develop under microscopic control in darkroom	10. The use of a fixation step after the wash following the immunogold incubation to prevent any elution of antibodies in the low pH (approximately 3.5) of the silver development solution. Fixation method – 2% gluteraldehyde for 15 mins, wash in water
12. Counterstain, dehydrate and mount	11. Use of the modification by Moeremans et al. (1984) to the silver development step of Danscher (1981), and incubating sections in this reagene for times from 4 to 10 min

Table 4. Results of immunostaining, comparison of Immunogold-silver method with PAP technique

Antigen	Tissue	Species	Immunostaining intensity[a]	
			Immuno-gold-silver	PAP
CGRP	Lung, bronchus	rat	+ + + +	+
	Vagina	rat	+ + + +	+
	Fallopian tube	rat	+ + + +	+
	Ovary	rat	+ + + +	+
	Uterus	rat	+ + + +	+
	Skin - nose	pig	+ + + +	+
	Skin - back	rat	+ + +	±
	Spinal cord	rat	+ + + +	+ +
	Kidney	rat	+ + + +	±
NPY	Kidney	rat	+ + +	+
	Brain	rat	+ +	−
	Uterus	rat	+ + +	−
	Vagina	rat	+ +	−
Substance P	Fallopian tube	rat	+ + + +	±
	Uterus	rat	+ + +	−
	Skin - nose	pig	+ +	−
Somatostatin 14	Antrum	pig	+ + + +	+ + +
	Pancreas	rat, pig	+ + + +	+ + +
	Spinal cord	rat	+ + + +	+
CCK/gastrin	Antrum	pig	+ + + +	+ + +
Glucagon	Pancreas	rat, pig	+ + + +	+ + +
BPP	Pancreas	rat, pig	+ + + +	+ +
Neurofilaments	Cerebellum	rat	+ + + +	±
	Hypothalamus	rat	+ + + +	±
	Vagina	rat	+ + + +	±
	Uterine cervix	rat	+ + + +	±
	Urinary bladder	pig	+ + + +	±
	Pancreas	pig	+ + + +	±
	Antrum	pig	+ + + +	±
NSE	Pancreas	pig	+ + + +	+ +
	Urinary bladder	pig	+ + + +	+ + +
	Urinary bladder	rat	+ + + +	+ +
	Vagina	rat	+ + + +	+ + +
S-100	Antrum	pig	+ + + +	+ + +
	Skin - nose	pig	+ + + +	+ +

[a] Intensity of immunostaining: − negative; ± equivocal; + weak positive; + + moderate positive; + + + strong positive; + + + + intense positive

Tween 80 (56023) from BDH Chemicals; silver lactate (85210) from Fluka AG, Switzerland (Fluorochem Ltd, Derbyshire); and photographic fixer (Amfix) from May and Baker Ltd.

Results

The immunogold-silver method was found to give superior results to those obtained using the PAP technique for all antibodies tested (Table 4). In cases where staining was acceptable when using PAP, immunogold-silver yielded a much more intense reaction in the adjacent section (Fig. 1 a–d). In many cases where very little or no immunoreaction was obtained with PAP, staining was obtained using the immunogold-silver method (Table 4). This was especially noticeable with immunostaining for regulatory peptides in nerves where different types of perikarya and fibres, visualised either weakly or not at all with the PAP technique, were seen as dark, densely stained structures standing out against the background tissue. Endocrine cells were more strongly stained than by PAP even in cases where PAP gave good staining. They were more sharply contrasted against the background, and the haematoxylin and eosin counterstain allowed their localisation to be more easily assessed. Both Bouin's fluid and formol saline gave good results.

No immunostaining was obtained with antigen-absorbed antibody or when the immunogold reagent was omitted.

Of the modifications to the original method (Holgate et al. 1983), no effect on specific or background non-specific staining was obtained by the use of gelatine in buffers or fixation following washing after the immunogold incubation. Both detergents and high salt concentration in buffers reduced the background, but not the specific staining; the lowest effective concentrations were 0.2% Triton X-100, 0.5% Tween 80 and 2.5% sodium chloride. Omission of Lugol's iodine pretreatment abolished all immunostaining, but, as usual with small peptides (Van Noorden and Polak 1984), the use of trypsin was found to have no effect either with or without Lugol's iodine.

The use of buffer of pH 8.2 for diluting immunogold, and for washing sections before and after incubation with this reagent, gave superior results with lower background staining. It was also found that immunogold could be stored at 4° C diluted in this buffer, with 0.01% sodium azide as preservative, for periods of at least 30 days with no reduction in immunostaining quality.

The optimal concentration of bovine serum albumin in the immunogold diluent was 0.8%. Above or below that amount some increase in non-specific staining resulted. Of the immunogold reagents, best results were obtained with the 5 nm gold colloid size (GAR G5) at a dilution of 1:250 and incubating for 60 min.

The modification of Moeremans (1984) was found to give results equally as good as those obtained using the original silver development method of Danscher (1981) and was somewhat simpler.

Post-development silver reduction was found to be helpful for decreasing unacceptable background staining or for reducing intensity in over-developed sections. However, careful monitoring of the reduction is necessary to avoid removing specific staining after the background is clear. The optimum time for reduction varied between 5 and 20 min. Reduction is not usually necessary for routine work, but is useful when background must be removed.

The optimum method for immunogold silver staining was found to be as follows:

1. Dewax sections in xylene and take to water through graded alcohols.
2. Immerse in Lugol's iodine for 5 min.
3. Rinse briefly in water.
4. Place in 2.5% sodium thiosulphate solution until sections are colourless.
5. Wash for 10 min in running tap water.
6. Wash in 2 × 5-min changes of TBS 7.4 (Tris-buffered saline – 0.05 M Tris buffer, pH 7.4, containing 2.5% sodium

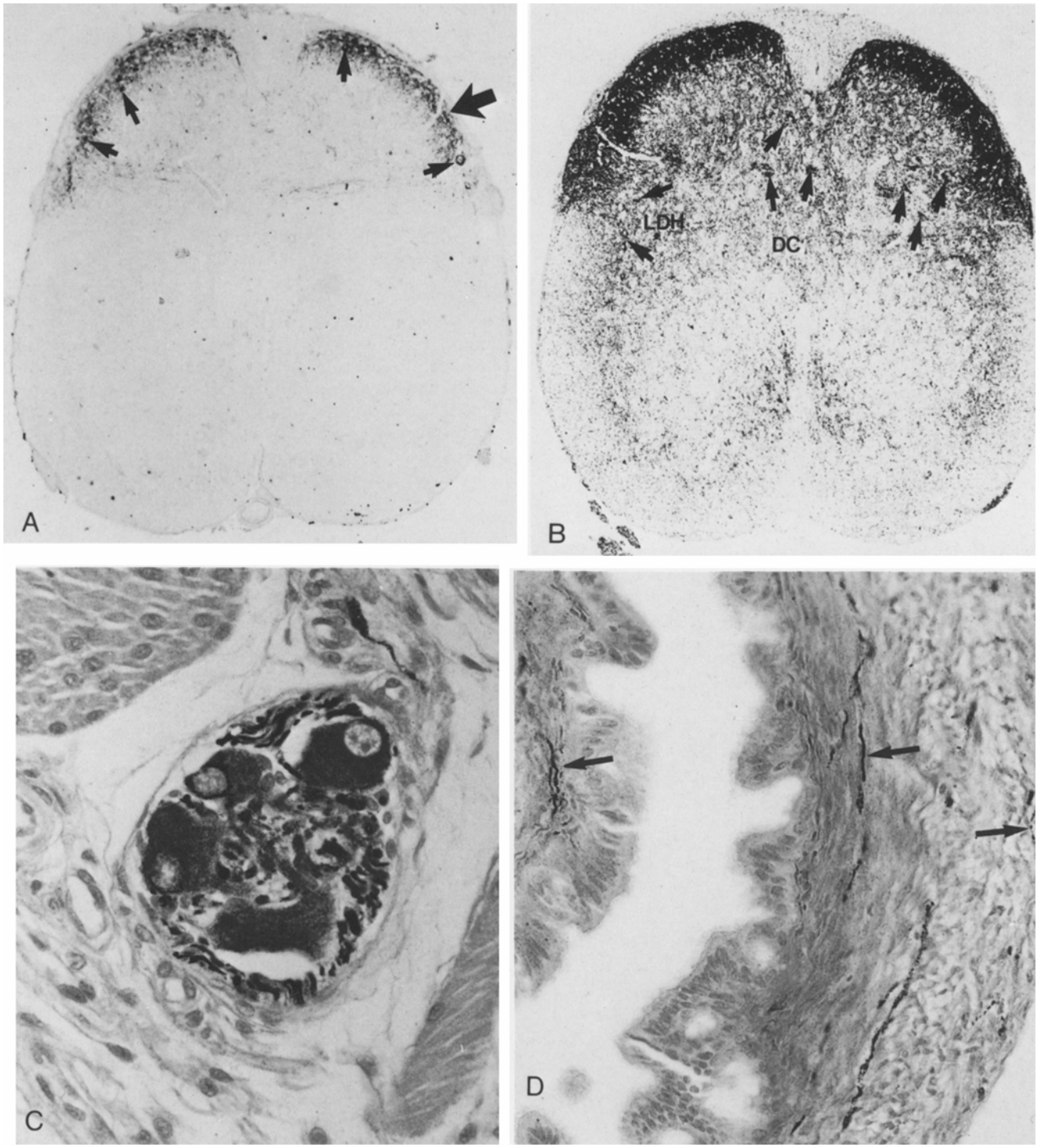

Fig. 1. **a** Rat spinal cord fixed in formol saline and immunostained with antiserum directed to somatostatin 28 (4–14) using the PAP technique. Moderately strong immunostaining is seen in cell bodies (↑), fibres and terminals in the dorsal horn (↑). 5 μm paraffin wax section, not counterstained (×70). **b** Adjacent section to that in **a** showing somatostatin-like immunoreactivity by the immunogold-silver technique using the same primary antiserum. Intense staining and more structures are revealed than by the PAP method. Note numerous cell bodies (↑) in the dorsal grey commissure (*DC*) and lateral dorsal horn (*LDH*). 5 μm paraffin wax section, not counterstained (×70). **c** Pig bladder fixed in Bouin's fluid, immunostained by the immunogold-silver technique with antibodies to neurofilaments. Intense staining is obtained in ganglion cells and nerve fibres. 5 μm paraffin wax section, weak haematoxylin and eosin counterstain (×530). **d** Immunostaining with anti-CGRP and the immunogold-silver technique in rat fallopian tube fixed in Bouin's fluid. Intense staining of nerves is seen (↑). With the PAP method staining was weak and hard to visualise. 5 μm paraffin wax section, light haematoxylin and eosin counterstain (×330)

chloride and 0.5% Tween 80 or 0.2% Triton X-100; 0.01% sodium azide may be added as preservative).
7. Apply neat normal goat serum for 10 min and drain off, but do not wash sections.
8. Incubate for 90 min in appropriately diluted primary antibody. This dilution should be determined by titration, but in our method it is up to 4 times greater than that used for the PAP method when incubation is overnight. Antibody diluent is TBS 7.4 containing 0.1% bovine serum albumin and 0.01% sodium azide.
9. Wash in 2×10-min changes TBS 7.4, followed by 2×5 min in TBS 8.2 (as TBS 7.4, but with a pH of 8.2).
10. Apply neat normal goat serum as before.
11. Incubate with GAR G6 (goat anti-rabbit immunoglobulin adsorbed to 5 nm gold colloid) diluted 1:250 in TBS 8.2 containing 0.8% bovine serum albumin, for 60 min.
12. Wash in TBS 8.2, 3×10-min changes.
13. Rinse briefly several times in deionised or distilled water followed by 6 washes (5 min each), with stirring, in the same. This washing must be very thorough to ensure that all halide is removed prior to silver development. Water purity is critical; since this is often variable, the use of glass-distilled deionised water may be necessary.
14. Transfer slides to the physical silver development solution of Moeremans et al. (1984) and allow to incubate in the dark, or under photographic safelight illumination, for 4–6 min. The development solution consists of 85 ml of citrate buffer (23.5 g of trisodium citrate dihydrate and 25.5 g of citric acid in 850 ml of glass-distilled deionised water) in which is freshly dissolved 850 mg of hydroquinone, adding just before use 110 mg of silver lactate dissolved in 15 ml of glass-distilled deionised water. Development may be checked microscopically, after rinsing the preparation in distilled water, using photographic safelight illumination. Because silver lactate and hydroquinone are photosensitive, solutions should be protected from light and used immediately.
15. Wash in photographic fixer diluted 1:4 for 1–2 min.
16. Wash in running tap water for 10 min.
17. Counterstain as desired (haematoxylin and eosin is suitable and does not affect the silver staining), dehydrate, clear and mount in DPX or Styrolite.

Discussion

We have found that in all cases tested, immunogold-silver staining was more intense than that obtained using the PAP method. In some cases, staining was obtained with immunogold-silver when no immunoreactivity was visible with PAP. This applied principally to regulatory peptides in nerves, such as substance P or somatostatin, which were clearly visualised in routinely fixed, wax-embedded tissue using the immunogold-silver method but had previously not been strongly or consistently immunostained by the PAP method. The inconsistency with the somewhat less sensitive PAP method is presumably due to a loss of antigenicity or diffusion of the antigen during fixation or processing which leaves sufficient antigen to be detectable only by immunogold-silver staining. The technique also allowed easier visualisation of endocrine cells, largely due to the very high contrast of the immunostaining.

Our method differs from that of Holgate et al. (1983) in four main respects. Firstly, the inclusion of detergent and use of higher sodium chloride concentrations in the buffers. Secondly, the use of a higher pH buffer for the immunogold reagent. Thirdly, the use of small-sized gold colloid adsorbed to affinity-purified antibody, thus allowing better penetration of a highly specific antibody, and possibly giving smaller silver particles after development. Fourthly, the use of a modified silver development procedure (Moeremans et al. 1984) omitting the stabilising colloid used in the method of Danscher (1981) and developing at room temperature.

As well as the ability to immunostain regulatory peptides in wax-embedded tissues, this method offers several other advantages:
1. No carcinogenic or suspected carcinogenic reagents are used.
2. There is no diffusion of reaction product.
3. The modified method with highly specific immunogold reagent produces very little background staining, providing that primary antibodies are of good quality.
4. The method has a high "signal to noise" ratio. Therefore, even if specific immunostaining is weak, the inherent blackness of the silver ensures that it is still visible. With enzyme methods, a weak reaction is often difficult to see.
5. Since the method is more sensitive than PAP, higher dilutions of primary antibody may be used, even allowing for the fact that we incubated primary antibody overnight for the PAP method but only for 90 min in the immunogold-silver technique. The higher dilution reduces the concentration of any contaminating antibodies even further than for the PAP method, and provides greater economy of expensive primary antibodies. However, when titrating antibodies for use with this method it is probably better to take the disappearance of background staining as a guide to optimal dilution rather than the dilution beyond which specific staining starts to diminish. Although this will usually mean using a higher concentration of primary antibody, the detection of antigen will be maximal.
6. The intensity of immunostaining may be adjusted after development using a weak photographic reducer solution. As the reducer also removes specific staining, the process must be carefully monitored. However, in a section with strong specific immunostaining, any unavoidable background (eg that due to contaminants in the primary antibody) may be decreased whilst most of the specific staining is retained.
7. The intrinsic density of the immunogold-silver stain permits the accompanying use of routine morphological staining methods. For example, haematoxylin and eosin (rather than just haematoxylin) may be used as a counterstain, allowing better localisation of immunostaining.

In summary, the method is simple, rapid and cheap, having only two short antiserum incubations, often using the primary antibody at higher dilutions than for PAP. It permits rapid screening of tissues for a variety of antigens, and may also be useful for double immunostaining procedures, as suggested by Holgate et al. (1983). Furthermore, immunostaining with PAP in routinely processed tissues may be variable and can often be weak, particularly in peptide-containing nerves. The immunogold silver staining method, as described here, provides a valuable improvement in the immunostaining of routinely processed tissues, producing uniformly dense stains. The technique thus has great potential in the field of diagnostic pathology, and will be especially useful in the assessment of alterations in peptidergic nerves or endocrine cells.

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Note added in proof

A kit containing all reagents for immunogold-silver staining is now available from Janssen Pharmaceutica, Beerse, Belgium. This has essentially the same reagents as we have described with the exception that a light microscopy grade of immunogold (GAR G5 LM grade) is substituted for the electron microscopy grade reagents which we have used. We have tested the kit and find results to be identical to those obtained in this study.