

## Technical Notes

# Double Immunogold Staining Method for the Simultaneous Ultrastructural Localization of Regulatory Peptides<sup>1</sup>

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Recent studies have suggested that the morphological characteristics of secretory granules contained within endocrine cells and nerves may be determined largely by their chemical composition. The use of the immunogold staining (IGS) method, which is based on the adsorption of colloidal gold to immunoglobulins, has been used in our laboratory to demonstrate a wide range of intracellular antigens at both the light and electron microscope levels. In this study we have applied a modification of the IGS method for the simultaneous detection of two separate antigens in a single tissue section, using a variety of region-specific antisera to different peptides. Peptide antisera were raised in rabbits or in guinea pigs and these were applied simultaneously or sequentially to grid-mounted ultrathin tissue sections. Antigenic sites were visualized at the electron microscope level using antisera raised in goats, adsorbed to gold particles of 12, 20, or 40 nm. Using this

technique we have attempted to investigate the coexistence of multiple antigens in single tissue sections, in particular in single granules; the topographic distribution of molecular forms within one single granule or granule population; the heterogeneity of peptidergic neurons and also the heterogeneity of peptide content in morphologically similar granules. The double immunogold staining procedures described here have proved to be extremely effective for the simultaneous ultrastructural localization of two antigens (peptide-peptide; peptide-propeptide) on a single tissue section. The further development of this technique may provide useful information on neuroendocrine cell dynamics in normal and diseased states.

**KEY WORDS:** Colloidal gold; C-peptide (proinsulin); Electron immunocytochemistry; Glucagon; Insulin; Regulatory peptides; Somatostatin; Substance P; Vasoactive intestinal polypeptide.

## Introduction

In 1971 Faulk and Taylor adsorbed colloidal gold to whole sera and suggested the value this development would offer to immunological procedures at the electron microscope level (Faulk and Taylor, 1971). Since then gold particles have been linked to lectins (Horisberger and Rosset, 1977; Roth and Wagner, 1977), polysaccharides (Horisberger and Rosset, 1977), toxins (Horisberger et al., 1975), enzymes (for example, catalase (Horisberger et al., 1975)), antigens (GLAD method (Larsson, 1979)), staphylococcal protein A (Romano and Romano, 1977; Roth et al., 1978), and immunoglobulins (Geoghegan and Ackerman, 1977; De Mey et al., 1980, 1981) to localize surface or intra-

cellular antigens in different biological systems. Successive modifications (Romano et al., 1974; Horisberger et al., 1975; Geoghegan and Ackerman, 1977) have led to the development of the immunogold staining (IGS) method (De Mey et al., 1981) for the localization of antigens at both light and electron microscope levels.

We have used the IGS method at the electron microscope level in order to localize regulatory peptides and catecholamine-converting enzymes in endocrine cells and nerves of the central and peripheral nervous systems (Polak et al., 1981; Probert et al., 1981; Varn-dell et al., 1982a,b). Recent electron immunocytochemical studies have suggested that the coexistence of different molecular forms of peptides with amines (Pelletier et al., 1981; Varn-dell et al., 1982b; Terenghi et al., 1983) or with other peptides (Ravazzola and Orci, 1980; Varn-dell et al., 1982b) may determine the morphology of the secretory granules within which they are localized. Thus a reliable method for the simultaneous ultrastructural localization of regulatory peptides, their precursors, and amine-converting enzymes was sought.

Simultaneous localization procedures involving the use of gold markers have been developed at the light and electron microscope

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level. Gu et al. (1981) used the peroxidase-antiperoxidase technique in combination with gold-labeled antibodies for the localization of two regulatory peptides at the light microscope level. At the electron microscope level, lectins labeled with gold particles of two different sizes, or a lectin labeled with gold of one particle size plus another labeled with either a ferritin or a peroxidase marker have been used for the detection of surface antigens (Roth and Binder, 1978). For the localization of intracellular antigens, the GLAD (gold-labeled antigen detection) method of Larsson (1979) has shown the simultaneous occurrence of immunoreactivity to adrenocorticotrophic hormone (ACTH) and to gastrin in the G cell of the cat gastric antrum. In addition, gold-labeled protein A has been used for the double immunostaining of ultrathin frozen sections of rat pancreatic tissue (Slot and Geuze, 1981; Geuze et al., 1982). Bendayan (1982) introduced an elaborate modification to the technique that allows the double immunolabeling of two antigenic sites using gold-labeled protein A. The innovation consists of immunolabeling each side of a grid for a different antigen, thereby avoiding any cross-reaction. Recently, Roth (1982) proposed a high resolution protein A-gold double staining technique using 3 and 15 nm gold particles, however, from our experience particles of this size are not easily resolved at lower magnifications.

In this study we describe a new, rapid, and reliable double immunolabeling technique for the ultrastructural localization of tissue-bound antigens, based on the IGS method (De Mey et al., 1981), employing gold-labeled antisera from two different species. We have tested the method in mammalian tissues, particularly the gut and pancreas, where the distribution of peptide antigens is well established.

## Materials and Method

**Tissue preparation.** Human surgical specimens (details in text) were fixed by immersion in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Tissues (regions of gut and pancreas) excised from experimental animals (cat,  $n = 10$ ; rat,  $n = 7$ ; guinea pig,  $n = 3$ ) were fixed by immersion in the above fixative or in a mixture of 2% glutaraldehyde and 1% formaldehyde (prepared from its *para*-polymer) in 0.075 M phosphate buffer, pH 7.2, for up to 2 hr. Tissue for immunocytochemistry was not postfixated in osmium tetroxide. After fixation, the tissues were rinsed in phosphate buffer, dehydrated in alcohol, and embedded in Araldite. Silver to light-gold sections (60–100 nm) were cut and collected on uncoated 300-mesh nickel grids and allowed to dry overnight before use.

**Antisera.** Primary antisera were raised in rabbits or in guinea pigs (Table 1). Generous gifts of antisera to C-peptide (pro-insulin) were received from Professor N. Yanaihara (National Institute for Physiological Sciences, Okasaki, Japan). Anti-insulin was obtained from Miles Laboratories (Slough, U.K.). All other peptide antisera were raised at the Royal Postgraduate Medical School, London, in collaboration with Professor S.R. Bloom.

**Preparation of colloidal gold and adsorption to immunoglobulins.** Colloidal gold was produced by the chemical reduction of chloroauric acid by 1) sodium ascorbate for 5–12 nm particles (Stathis and Fabrikanos, 1958; Horisberger, 1979), and by 2) sodium citrate for large 17–80 nm particles (Frens, 1973). The linking of colloidal gold to immunoglobulins (either goat anti-rabbit or goat anti-guinea pig immunoglobulin (Ig) G) was performed as detailed elsewhere (De Mey et al., 1981; De Mey, 1983).

**Double immunogold staining method.** The grid-mounted sections were subjected to the following procedure. Fifteen microliter droplets of each reagent were used except where otherwise stated.

**Table 1.** Characteristics of the antisera used

Antiserum raised against	Antiserum raised in	Region specificity	Dilution	Absorption <sup>a</sup>
C-peptide (pro-insulin)	guinea pig	— <sup>b</sup>	1:1000	1
C-peptide (pro-insulin)	rabbit	mid	1:10,000	1
Glucagon	rabbit	N-terminal	1:2000	1
Insulin	guinea pig	— <sup>b</sup>	1:1600	0.1
Somatostatin	rabbit	— <sup>b</sup>	1:6000	0.1
Substance P	guinea pig	whole	1:2000	1
Substance P	rabbit	whole	1:8000	0.1
VIP	rabbit	whole	1:6000	0.1

<sup>a</sup>Amount of homologous antigen (nmol peptide/ml diluted antiserum) required to reduce significantly all immunostaining.

<sup>b</sup>Precise region specificity unknown or not determined in this system.

- 10% aqueous hydrogen peroxide for 5 min. This "etches" the sections and aids antibody penetration, presumably by permeabilization of the epoxy resin. The grids were then rinsed with Millipore-filtered distilled water.
- Normal goat serum (1:30 dilution) in 0.05 M Tris-saline buffer, pH 7.2 (TBS), for 10 min at room temperature.
- A mixture of the two primary antisera (one rabbit and one guinea pig antiserum) diluted to optimal titers in TBS, for 1 hr at room temperature. Subsequently the grids were "jet-washed"<sup>3</sup> with TBS and with TBS + 0.2% bovine serum albumin (BSA; Grade V; Sigma London Chemical Co. Ltd., Poole, U.K.)
- The grids were placed into droplets of TBS containing 1% BSA for 5 min at room temperature.
- The grids were drained and placed into a mixture of the two second layer antisera (20 nm gold-labeled goat anti-guinea pig IgG and 12 or 40 nm gold-labeled goat anti-rabbit IgG) at optimal titer (Varndell et al., 1982b) for 1 hr at room temperature.
- After jet-washing in TBS-BSA, TBS, and distilled water, the sections were counterstained in uranyl acetate and lead citrate (Reynolds, 1963) prior to observation with a Zeiss 10CR transmission electron microscope.

In a separate series of experiments each antiserum was applied independently (i.e., primary A—first gold—primary B—second gold).

**Controls.** Controls for the specificity of the reactions were performed as follows: a) using nonimmune rabbit and/or guinea pig serum as first layer; b) using the gold-labeled antisera without the presence of one, or both, primary antisera (um), and c) using the diluted antiserum absorbed with an excess of the respective antigen, antigenic fragments or fragments with known sequence homology (1–40 nmol/ml diluted antiserum).

## Results

The double immunogold staining procedure described here has proved to be relatively rapid and easy to perform. In our hands identical results have been obtained whether a mixture of antisera were used or each layer of antiserum was applied independently. This indicates that there was no appreciable cross-reactivity between the two primary and/or gold-labeled antisera tested in this study.

<sup>3</sup>Jet-washed signifies Millipore (0.45  $\mu$ m pore size)-filtered solution directed from a syringe to the tines of the holding forceps.

The three gold particle sizes (12, 20, and 40 nm) used throughout this study are easily distinguishable at the electron microscope level even after high contrast counterstaining with heavy metal salts.

Using the double immunogold staining procedure as detailed above we were able to localize insulin, together with glucagon, somatostatin

Figure 1. (a) Ultrathin section of human endocrine pancreas immunostained for insulin (guinea pig anti-insulin, goat anti-guinea pig IgG 20 nm gold; white arrows) and glucagon (rabbit anti-glucagon, goat anti-rabbit IgG 40 nm gold; black arrows). An unlabeled D cell (D) is present in the intervening space between the A and B cells. Uranyl acetate and lead citrate counterstain. Original magnification  $\times 36,800$ . Bar =  $1 \mu\text{m}$ . (b) Normal human pancreas. B cell granules (B) immunostained for insulin-like material with 20 nm gold particles and A cell granules (A) immunostained for glucagon-like immunoreactivity with 40 nm gold particles. The cell membranes separating the cells are visible (arrowheads). Uranyl acetate and lead citrate counterstain. Original magnification  $\times 115,000$ . Bar =  $0.1 \mu\text{m}$ .

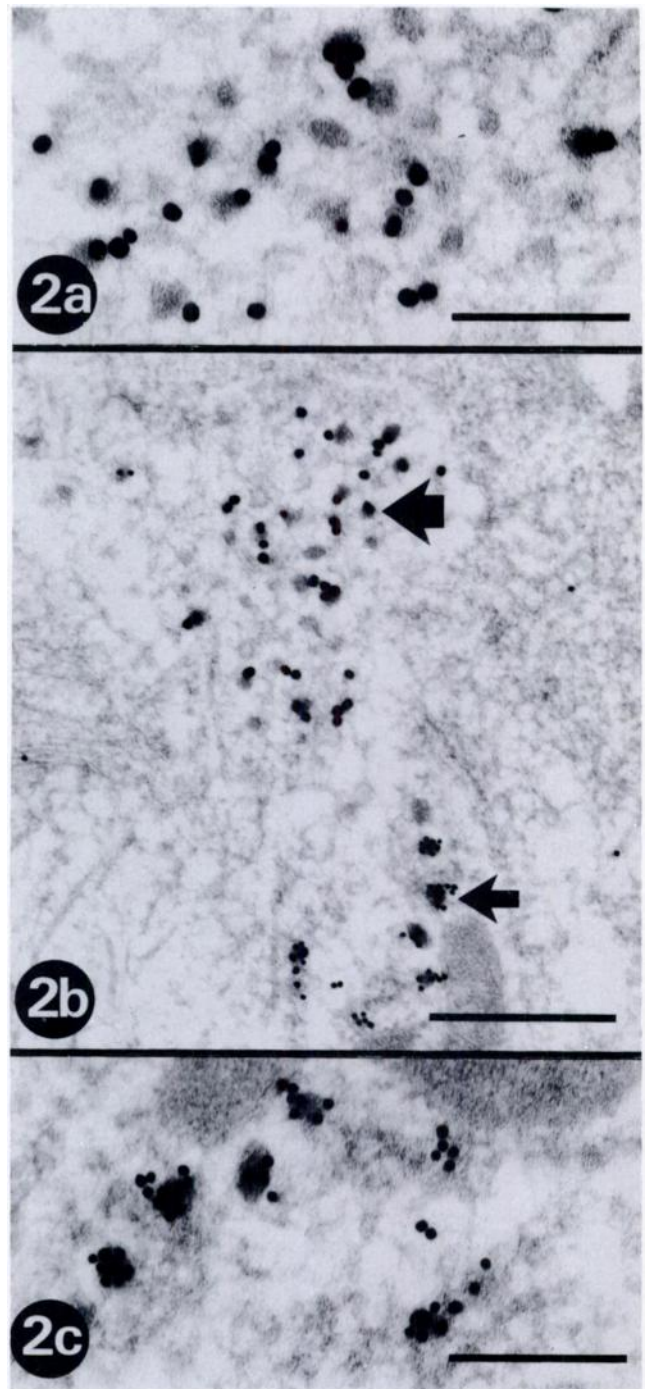
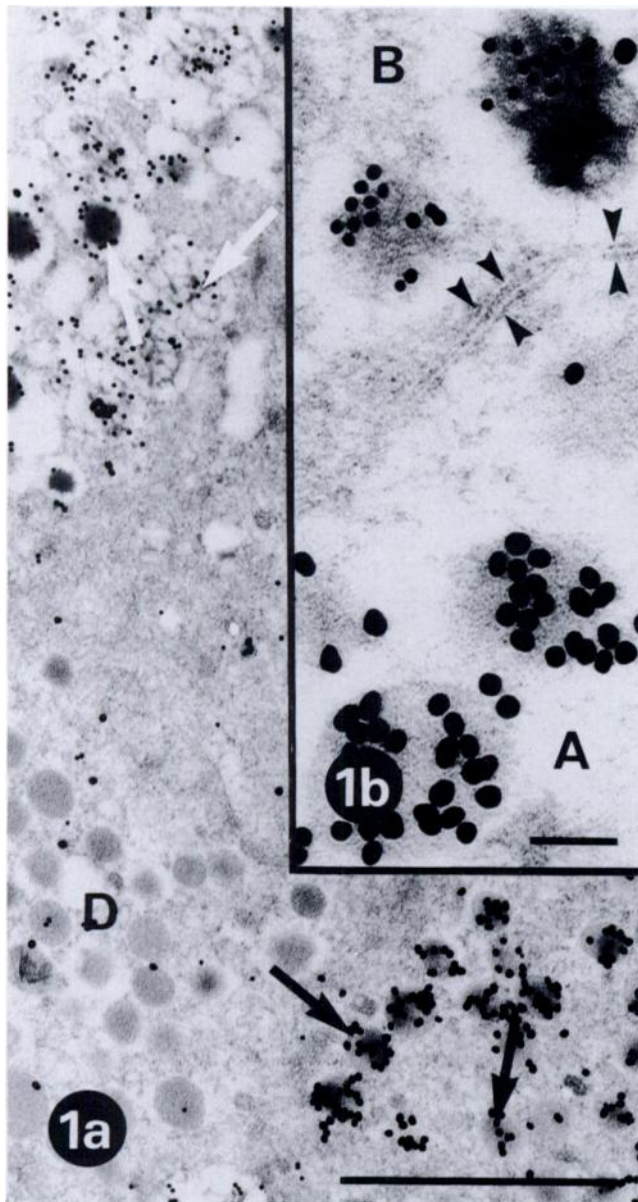


Figure 2. Nerve terminals in the myenteric plexus of guinea pig gut. Two subpopulations are evident following application of the double immunogold staining procedure. VIP immunoreactivity (a; b, large arrow) is localized to electron-dense vesicles using 40 nm gold particles. Substance P immunoreactivity (b, small arrow; c) is localized to vesicles morphologically similar to the VIP-containing vesicles using 20 nm gold particles. Uranyl acetate and lead citrate counterstain. (a and c) Original magnification  $\times 92,800$ . Bars =  $0.25 \mu\text{m}$ . (b) Original magnification  $\times 48,000$ . Bar =  $0.5 \mu\text{m}$ .

or pancreatic polypeptide to separate cells in normal pancreatic islets of man and other mammals (Figure 1). Likewise, by combining the double immunogold staining procedure with the use of ultraviolet-cured resin, we have been able to localize the neurotransmitters vasoactive intestinal polypeptide (VIP) and substance P in separate nerve terminals within the myenteric plexus of guinea pig (Figure 2).

Using non-cross-reacting region-specific antisera in combination with the double immunogold staining procedure we have localized insulin to all secretory granule types in the pancreatic B cell, whereas pro-insulin (C-peptide) has only been visualized in the non-crystalline-core secretory granules (Figure 3). This topographic distribution of

pro-insulin (C-peptide) and insulin immunoreactivities to different granule types is reported here for the first time.

## Discussion

In this study we have used gold-labeled antibodies prepared by the method of De Mey et al. (1980, 1981), which is based on the procedures described by Geoghegan and Ackerman (1977) and Geoghegan et al. (1978). This technique has been used successfully for the ultrastructural localization of many different intracellular antigens (De Mey et al., 1981; Probert et al., 1981; Varndell et al., 1982a,b) as a sensitive and reliable alternative to the protein A-gold technique of Romano and subsequent workers. The absolute electron-density of the gold particles, the possibility of counterstaining with uranyl acetate and lead citrate, the high sensitivity and specificity of the procedure, and the ease of the postembedding methodology convey considerable advantages over alternative electron immunocytochemical techniques. One further advantage that has been exploited in this study is the feasibility of producing homogeneous populations of different gold particle sizes (Horisberger, 1981; Slot and Geuze, 1981). The double immunogold staining procedures described here are based on the adsorption of goat immunoglobulins to different gold particle sizes for the simultaneous demonstration of two antigens in the same tissue section.

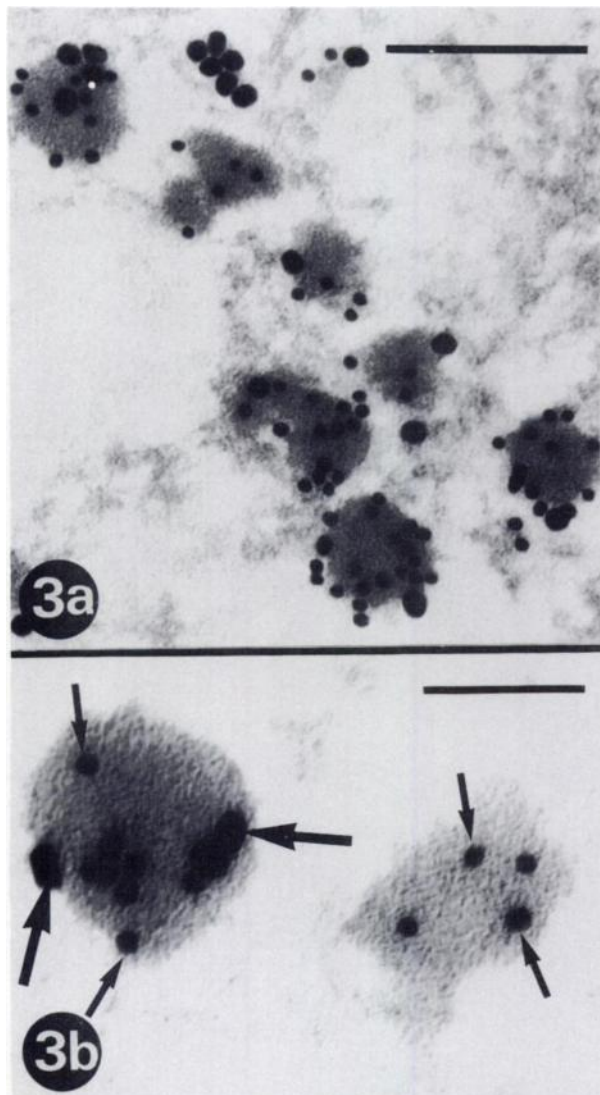
Using these methods we have been able to demonstrate two bioactive peptide antigens in adjacent cells (insulin and glucagon in the normal pancreas) and, with the use of region-specific antisera, two molecular forms of gastrin to specific granule subpopulations within antral G-cells (data not shown; see Varndell et al. (1983)). Similarly, insulin and its precursor molecule pro-insulin have been shown to coexist in some, but not all, B cell granules. In addition, the combination of ultraviolet-cured epoxy resin with region-specific antisera and the double immunogold staining procedure has facilitated the investigation of peptide-containing nerve and endocrine cell heterogeneity. No immunostaining localized exclusively to secretory granules was observed in sections subjected to any of the controls.

The use of immunoglobulins from two different species visualized by the simultaneous or sequential application of two colloidal gold particle sizes adsorbed to immunoglobulins from a third species offers a new technique with which to demonstrate dual localization of antigens in single tissue sections. The further development of this technique will provide valuable information on the biosynthesis, storage, and secretion of regulatory peptides.

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Figure 3. (a) Normal human pancreatic B cell. Insulin and C-peptide (pro-insulin) immunoreactivities are visualized using the double immunogold staining procedure (20 and 40 nm colloidal gold particle sizes, respectively). Uranyl acetate and lead citrate counterstain. Original magnification  $\times 100,000$ . Bar =  $0.25 \mu\text{m}$ . (b) Insulin-like immunoreactive material is present in both B cell granule types (small arrows), whereas C-peptide (pro-insulin) is restricted to the homogeneous core granules (large arrows). Uranyl acetate and lead citrate counterstain. Original magnification  $\times 200,000$ . Bar =  $0.1 \mu\text{m}$ .



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