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# Immunoelectron Microscopy: A Reliable Tool for the Analysis of Cellular Processes

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## 1. Introduction

Electron Microscopy is an indispensable tool to investigate the intricate structures of the cell and organelles, and also to study the cellular biological processes implicated in the responses to changes in the microenvironment. However, several cellular events may be missed if conventional ultrastructural studies are not complemented with details concerning the subcellular localization of a wide range of specific proteins which can become rearranged as part of their own dynamic processes. Thus, immunoelectron microscopy emerges as a technique that links the information gap between biochemistry, molecular biology, and ultrastructural studies, by placing macromolecular functions within a cellular context.

The present chapter is intended to describe the main scope and protocols of the immunogold methods which have been successfully utilized at our research center for the examination and analysis of intracellular and cell surface proteins in mammalian cells. Furthermore, at the ultrastructural level, we demonstrate the role of immunogold labeling in the study of biological processes induced by different stimuli from the environment.

### 1.1 General considerations concerning immunoelectron microscopy

Immunoelectron microscopy is one of the best methods for detecting and localizing proteins in cells and tissues. This procedure can be used on practically every unicellular and multicellular organism, and often provides unexpected insights into the structure-function associations. The use of primary antibodies conjugated with gold particles allows high-resolution detection and localization of a multiplicity of antigens, both on and within the cells. However, the successful application of immunoelectron microscopy depends on the preservation of the protein antigenicity, the capacity of antibodies to infiltrate throughout the cell, and finally the specificity of recognition between antigen-primary antibodies. In addition, an adequate handling of biological samples is required, which involves fixation, an

appropriate selection of an embedding resin and the ready availability of the specific antibodies for the molecules whose ultrastructural location needs to be determined.

Since 1971, when W.P. Faulk and G.M. Taylor published "An immunocolloid method for the electron microscope", colloidal gold has become a very extensively used marker in microscopy. As knowledge of protein location and distribution at the subcellular level plays a pivotal role in cell biology, this tool has been applied to detect a vast range of cellular and extracellular constituents by using *in situ* hybridization, immunogold, lectin-gold, and enzyme-gold labeling. In addition to its use at light microscopy level, colloidal gold remains the label of choice in transmission electron microscopy (TEM) for studying thin sections, freeze-etch, and surface replicas, as well as in scanning electron microscopy.

While conventional electron microscopy provides no information about specific molecules, immunogold labeling can help to connect a visible structure with a specific *in situ* localization and distribution of molecules at a high resolution. In this way, the use of the colloidal gold particles undoubtedly represents a significant event in the improvement of the immunochemistry method.

Many protocols have been developed since the introduction of colloidal gold to immunocytochemistry, with the two most widely used techniques, however, being actually based on transmission electron microscopy and consisting of either immunolabeling after embedding in resin (post-embedding immunogold labeling) or immunostaining prior to this process (pre-embedding immunogold labeling). Here, we also present some approaches from our own work, which reveal how the use of immunoelectron microscopy can offer additional insights into the structure–function relationships.

These detailed methods and notes should facilitate the selection of the best method to use for the antibody and biological material to be studied. It is our hope that this chapter will also facilitate an improved understanding of immunoelectron microscopy and its use in the biological sciences.

## 1.2 Criteria for selection of the immunoelectron microscopy technique

Before deciding to use the immunoelectron microscopy technique, researchers need to consider some relevant questions in order to select the most appropriate procedure among the methods typically used.

With the aim of guiding researchers, we propose that the following sequence of questions should be answered:

- What is the scope of immunogold labeling?
- What kind of information can be obtained by applying immunocytochemistry?
- What is the subcellular localization of the molecule of interest?
- Does the target molecule to be detected by this methodology possess a single and static location? Or does it exhibit a dynamic behaviour?
- What is the most recommended fixative mixture for the antigen under study?
- How can the antigenicity of the target molecules be preserved?
- Which is the most appropriate type of resin to be used in the immunoelectron microscopy? Does the selected resin depend on the method to be used in immunolabeling?

### 1.3 General rules of thumb in immunogold electron microscopy

- For immunoelectron microscopy, fixation is one of the most important steps in sample preparation due to the need to preserve as much biochemical reactivity as possible.
- Although there are many types of fixatives, the mixture of formaldehyde and a low concentration of glutaraldehyde is the most generally accepted one for immunoelectron microscopy.
- Immersion fixation is useful for the majority of the biological specimens (e.g. biopsy specimens, cultured cells).
- The relation between the fixative solution and size sample should be at least 40:1.
- It is critical that tissue be cut into pieces no larger than 1–2 mm<sup>3</sup> due to the penetration of the fixative and embedding resin into specimens being relatively slow.
- Immunocytochemistry at ultrastructural level can be performed on semithin and ultrathin sections of specimens embedded in epoxy or acrylic resins and also on ultrathin cryo-sections.
- Examination of the semithin sections is important to be able to assess the quality of the fixation and to select the area to be investigated by electron microscopy.
- Multiple labeling with colloidal gold conjugates of different sizes is easily performed by the post-embedding method.
- After fixation and embedding in acrylic resins, specimens without osmication show poor preservation of cellular membranes.

## 2. Protocols in immunoelectron microscopy

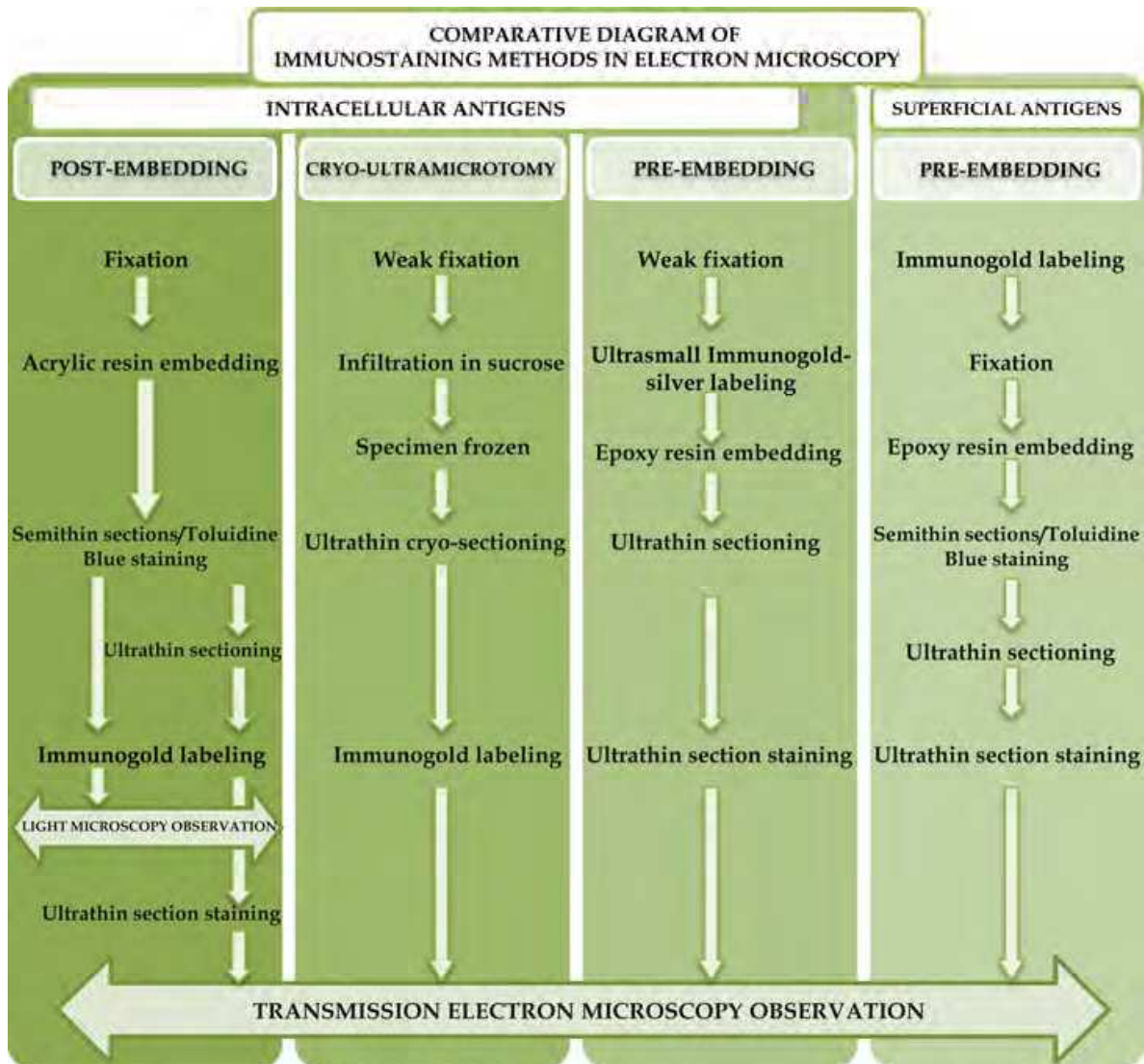
The success of immunolabeling at ultrastructural level depends on various factors, including the initial quantity and quality of antigens, and the preservation of the cell ultrastructure, to be able to finally achieve an accurate localization of the antigen within the cell. Therefore, it is necessary to attain a correct balance between antigen preservation and a good morphology at the ultrastructural level. Although it is difficult to provide a standard protocol because antigens and tissues need individual evaluations to reveal the best experimental conditions, there are many recommendations that researchers have introduced to increase the possibility of arriving at a positive result.

The two following general approaches can be applied to localize cell antigens:

- When the objective is to localize the intracellular antigens, there are at least three different protocols that can be used: *post-embedding*, after embedding in acrylic resins; *cryo-ultramicrotomy* in tissue sections obtained without embedding, and *pre-embedding* combined with membrane permeabilization.
- When the interest is focused on identifying cell surface proteins, the *pre-embedding labeling* protocol is the most convenient as antigens and ultrastructure are well preserved by this method.

However, in each case, the selection of the protocol will frequently depend on the availability of the necessary infrastructure.

Next, we propose the following graphical flowchart that can be used to compare the different approaches of immunoelectron microscopy discussed in the present chapter.



Flowchart 1. Differential procedures of immunogold methods to detect intracellular and superficial antigens.

## 2.1 Post-embedding technique

### 2.1.1 Specimen preparation

The post-embedding technique is a good alternative to produce contact between the antibodies and internal antigens exposed at the surface of thin sections obtained from resin-embedded tissues. By using this procedure, specimens are first embedded in resin and sectioned at 60–90 nm. However, post-embedding immunocytochemistry has its own limitations, with the principal drawback being that antibodies cannot penetrate into the resin, and consequently only the antigens that are exposed at thin section surfaces can be labeled. In addition, antigens are affected by fixatives, solvents, resins and heat during polymerization, thus compromising antigenicity. Nevertheless, each of these “threats” to antigenicity can be avoided, at least in part, by implementing various modifications to the conventional protocols used for electron microscopy related to fixation, dehydration, inclusion media, and temperature.



### 2.1.1.1 Chemical fixation

As a general definition, fixation is an attempt to induce the fast arrest of biological activities and to stabilize the subcellular components with a minimal distortion of the cellular structures. Chemical fixation for TEM allows biological samples to be prepared for subsequent procedures, involving washing with aqueous solvents, dehydration with organic solvents (ethanol or acetone), embedment and polymerization in plastic resins, and subsequent imaging with high-energy electron beams in an electron microscope. The speed of the penetration of the fixatives determines the success of the fixation procedures. Although small fixative molecules (formaldehyde) penetrate more rapidly than larger ones (glutaraldehyde), the latter possess more reactive sites and can thus cross-link and stabilize the cellular components more thoroughly.

The glutaraldehyde concentration should first be reduced as much as necessary. However, this may affect the ultrastructure preservation and make it difficult to locate the antigen in a specific organelle. Therefore, when a new antigen is being studied by immunoanalysis, it is desirable to use a fixative strong enough to have a good ultrastructural preservation, but reduced in glutaraldehyde concentration in comparison with a conventional fixative. For example, this fixative could have 4% of formaldehyde and 1.5% of glutaraldehyde prepared in 0.1 M Cacodylate or Phosphate buffer and used for immersion. However, if the result is poor, the next step should be to lower the glutaraldehyde concentration, with a possible reduction of up to 0.2%. Then, in spite of a poorer preservation of the ultrastructure, it may still be possible to identify the structure where the antigen localizes.

Here, we present several examples of antigen preservation achieved with different fixatives: in Figure 1 the murine mammary tumor virus is well identified after fixation with a mix of formaldehyde and glutaraldehyde at 1.5% and included in LR-White acrylic resin [Peralta Soler, et al.; 1988]; in Figure 2, we localized galectin-1 in Müller cells on chicken retina after fixation in 2% glutaraldehyde by immersion; in Figure 3, a good antigen preservation in the lactotrophs of pituitary gland, as evaluated by labeling on Golgi cisternae as well as on secretory granules, was obtained with a high fixative concentration (3% formaldehyde and 3% glutaraldehyde). This was possible mainly due to the high concentration of antigens stored in the secretory granules of endocrine cells [Maldonado & Aoki; 1986-a].

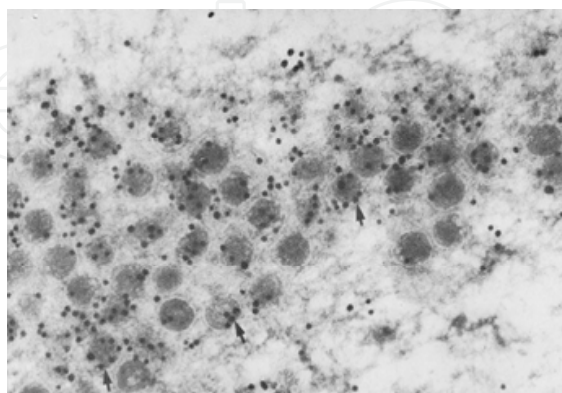


Fig. 1. Identification of intracellular viral antigens. Mammary tumor virus particles in mouse spontaneous mammary carcinoma, are identified with a specific antibody followed by immunogold on thin sections (Reproduced from Peralta Soler et al., 1988; with permission from *Micr Electr Biol Cel*). Original magnification X 120000.

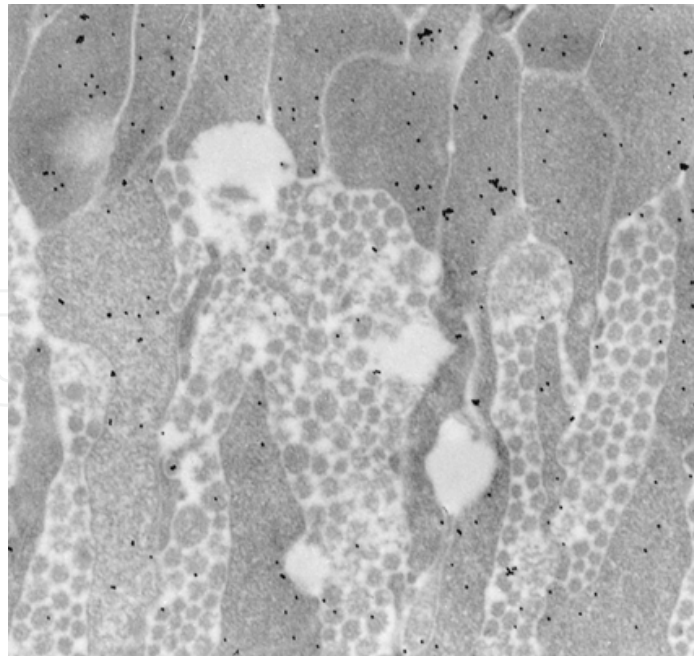


Fig. 2. Localization of galectin-1 in Müller cells of retina. Cytoplasmic projections of Müller cells, at the inner nuclear layer of chicken retina exhibit specific gold labeling. (Reproduced from Maldonado et al., 1999; with permission from Invest Ophthalmol Vis Sci). Original magnification X 25000.

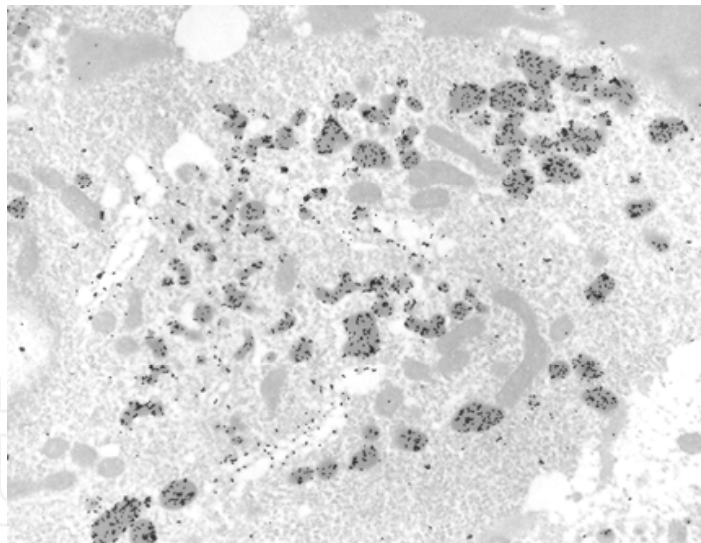


Fig. 3. Preservation of prolactin antigenicity after strong aldehyde fixation. An intense immunogold labeling of prolactin on secretory granules and Golgi complex cisternae can be seen. Original magnification X 20000.

Another way to preserve antigenicity is to omit osmium fixation, as this is a very strong fixative that cross-links proteins and polypeptide chains and consequently diminishes their antigenicity. In fact, only when there is a concentrated mass of antigens can osmium be used. Even then, before starting the immunocytochemical protocol, it requires antigens to be unmasked by means of incubation with a strong oxidant in order to reoxidize and solubilize the reduced osmium molecules.

In Figure 4, a saturated aqueous solution of sodium metaperiodate [Bedeyan & Zollinger; 1983] was applied to thin sections of osmicated pituitary gland to identify the prolactin hormone stored in secretory granules [Maldonado & Aoki; 1986-b], with the absence of immunostaining at the Golgi level being due to a decrease in the antigenicity.

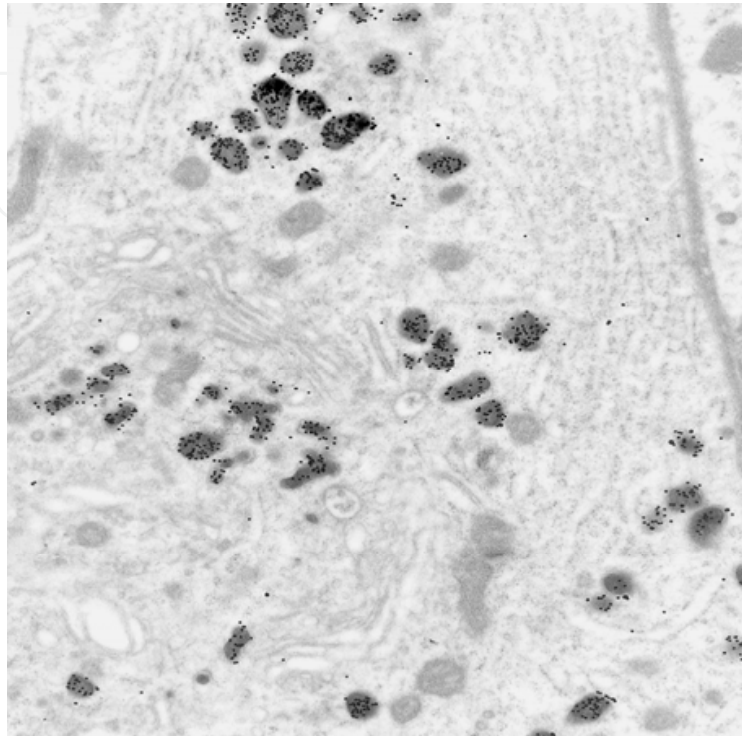


Fig. 4. Immunogold labeling on osmium fixed tissue. In contrast with Fig. 3, prolactin immunoreactivity was only preserved at secretory granules when osmium postfixation was carried out after aldehyde fixation. Immunolabeling was performed after a previous etching with sodium metaperiodate (Reproduced from Maldonado & Aoki, 1986; with permission from Histochem J). Original magnification X 20000.

In Figure 5, the identification of secretory granules positive for ANP (atrial natriuretic factor) was performed with aldehyde fixation (1.5% formaldehyde and glutaraldehyde), which were then postfixed with 1% osmium tetroxide, and removed with 10% hydrogen peroxide prepared in water [Maldonado, et al.; 1986]. The effects of not using osmium tetroxide can be partially compensated for by the inclusion of 0.2% of picric acid in the glutaraldehyde solution.

#### 2.1.1.2 Inclusion media

Epoxi resins used for conventional electron microscopy are not adequate for immunolabeling because they are hydrophobic resins that need extreme dehydration and high temperatures to be able to polymerize. For this reason, during the 1980's, experts developed water-miscible (acrylic) embedding media, which are more permeable to aqueous solutions and therefore more suitable for immunostaining at the ultrastructural level. Nowadays, various resins are available, which have been manufactured by companies specializing in electron microscopy products, with Lowicryl and LR-White being the most common. These resins have proved to be quite useful in retaining biochemical reactivity



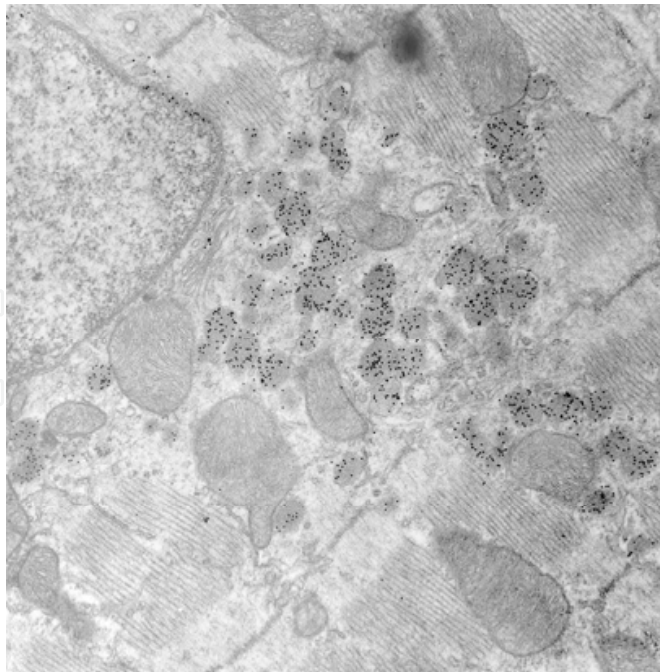


Fig. 5. Immunogold labeling on osmium fixed tissue. Immunogold identification of ANP in secretory granules of myocardocytes in thin sections of rat atrium fixed with aldehyde osmium and included in LR White. Immunolabeling was performed after a previous etching with 10%  $H_2O_2$  (Reproduced from Maldonado, et al., 1986; with permission from Anat Embryol (Berl)). Original magnification X 25000.

within samples, although the final images are structurally more poorly defined than those produced using the epoxy-embedded technique.

Lowicryl has the advantage that it polymerizes at low temperatures, which contributes significantly to antigen preservation. In Figure 6, a thin section of pituitary gland that was included in Lowicryl exhibited a strong staining for prolactin, even at the Golgi cisternae of the lactotrophs where the hormone concentration was lower than in secretory granules [Maldonado & Aoki; 1986-a]. This resin is recommended when there is scarce antigen concentration; but it has the disadvantages that a special chamber is necessary to polymerize at  $-30^\circ$  under U.V., and it is also very toxic.

LR-White polymerizes at  $50^\circ C$ , which helps to provide a good antigenicity. Furthermore, it does not need additional equipment and is much less toxic than Lowicryl. Other advantages are that LR-White tolerates partial dehydration (accepting tissue from 70% ethanol), it is beam-stable and easy to be sectioned, and osmium fixation does not interfere with polymerization as in the case of Lowicryl.

All these resins exhibit less stability under the electron beam than epoxides, and also post-stain more quickly than most of them (since they are more hydrophilic than epoxides and react more quickly with the typical aqueous post-stain). However, they will not polymerize properly if they are contaminated with acetone or have been exposed to air. For this reason, dehydration with increasing concentrations of ethanol should be followed by resin infiltration, and polymerization should be carried out in gelatin capsules (anaerobic conditions). In addition, dehydration is not as critical as with epoxide resins, since common

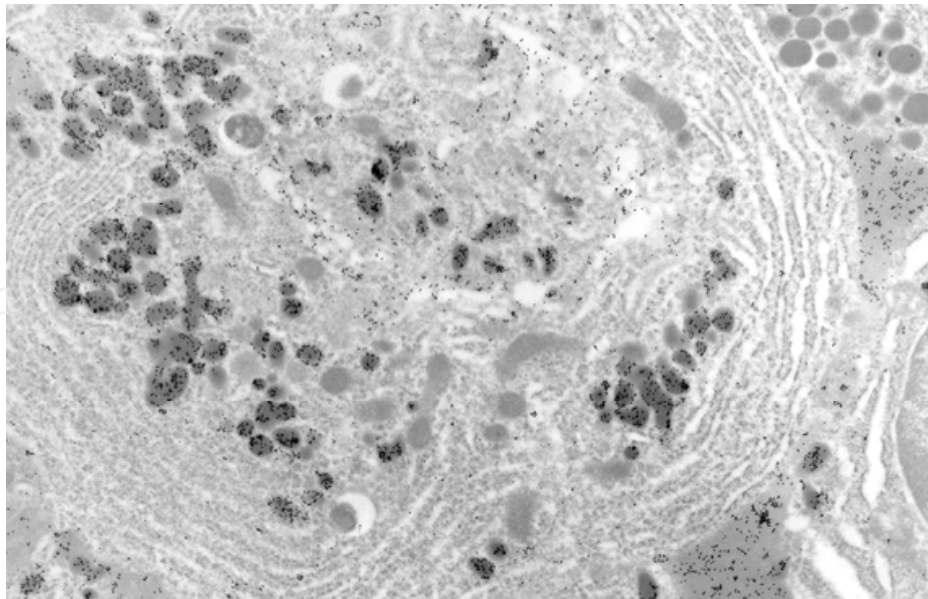


Fig. 6. High preservation of prolactin antigenicity in Lowicryl included pituitary gland. This labeling was obtained in pituitary gland fixed in 3% formaldehyde and 3% glutaraldehyde and included in Lowicryl at  $-30^{\circ}\text{C}$  with U.V. for 24 h. Original magnification X 17000.

acrylic resins can tolerate small quantities of water remaining in the sample during polymerization.

Alternatively, antigenic alterations derived from dehydration and inclusion resins can be completely avoided by using cryo-ultramicrotomy. In this technique, immunolabeling is performed on ultrathin thawed cryo-sections according to the method of Tokuyasu [Tokuyasu; 1973], thereby providing a very sensitive high-resolution localization. Its main advantages are that the antigens remain in a hydrated environment prior to immunolabeling, and that the antigen accessibility is improved compared with resin section labeling. In the case of biological samples, they need to be chemically fixed (usually with aldehydes), cryo-protected (and partly dehydrated) with high concentrations of sucrose (70-80%), frozen in liquid nitrogen and sectioned at approximately  $-115^{\circ}\text{C}$ . Dry-frozen ultrathin sections can then be picked up with a drop of methyl cellulose and/or sucrose and transferred onto an electron microscopic support grid for a subsequent immunogold procedure. There are many reviews concerning this technique such as that of Peters and Pierson [Peters & Pearson; 2008]. However, the main limitation of this technique is that it requires special laboratory infrastructure and equipment and trained personal. Therefore, it is only possible to use in specialized laboratories.

### 2.1.1.3 Immunoreactives

Gold particles have definitively replaced enzymatic markers such as horseradish peroxidase, with a disadvantage of the enzyme-based detection systems being that the reaction product formed may diffuse away from the reaction site and precipitate over a greater area. Therefore, particulate labeling with gold particles is now preferred since it provides a higher subcellular resolution. Furthermore, gold complexes have various advantages that have made them into the most used markers: the high electron density, the possibility of being prepared with different sizes, and the fact that they are multiple markers

with gold particles that can be easily bound to different proteins besides those of antibodies. Regarding the colloidal gold particle diameters usually applied, the most frequent are in a range from 5 to 25 nm when the antigens are exposed on the surface of ultrathin sections [Hagiwara, et al.; 2000]. However, these are of little use for detecting intracellular molecules with the pre-embedding method [Ferguson, et al.; 1998]. More recently, the development of ultrasmall gold (<1.0 nm) has widened significantly the scope of gold complex conjugates, thus providing a marker system with a greater labeling sensitivity. This is due to these particles being less prone to steric hindrance and able to penetrate better, even without pre-treatment with detergent [Hainfeld & Furuya; 1992].

In addition, the combination of ultrasmall gold conjugates with silver enhancement has significantly improved the ultrastructural detection of intracellular antigens by using pre-embedding procedures. This has been successfully applied by Van Lookeren Campagne to analyze distribution of the growth-associated protein B-50 in hippocampal neurons [Van Lookeren Campagne, et al.; 1992].

#### 2.1.1.4 Blocking solutions

A critical aspect to be considered when performing immunoreactions at electron microscopy level is to control the different sites that can contribute to the background in the reaction. Background is the result of "non-specific" reactions, which are due to the general physical chemical properties of both the specimen and the primary antibodies, and also result from secondary antibody/marker conjugates. For this purpose, a blocking solution should be applied immediately before incubation with the primary antibody, with the ideal blocking solution being able to bind to all potential sites of non-specific interactions, and to eliminate background or "noise" altogether without altering or obscuring the epitope for antibody binding, i.e. the "signal". Although there is no single recipe for making a blocking solution, a general recommended rule, when investigating a new target antigen or using a new antibody, is to test several different blockers in order to determine the highest signal/noise ratio of the assay.

We have long used 1% Bovine Serum Albumin or 1% normal serum of the same species as the secondary antibody. However, it is not infrequent to find non-specific markers (e.g. on the heterochromatin), which cannot be controlled by this blocking solution, particularly when IgG-gold complexes are applied. In these cases, a more robust blocker solution should be sought.

An appropriate blocking has to be able to interact with both hydrophobic and hydrophilic properties. The following three proteins are frequently used together to obtain an adequate blocking solution:

- Bovine Serum Albumin (BSA).
- Normal serum, obtained from the same species as the secondary antibody (but which must not be used when protein A is the reagent).
- Cold fish skin gelatin at 0.1%.

The two first components can be used in concentrations varying from 1-5%, and should be prepared in PBS containing 0.05% sodium azide to avoid contaminations. Sufficient washing after the blocking step is necessary in order to remove any excess protein that may prevent detection of the target antigen.

### 2.1.1.5 Control of specificity of immunogold staining

A critical point for immunoreactions, independently of whether they occur, at light or electron microscopy level or whatever marker is being used, is to validate the results obtained by including adequate controls. These can be designed to detect any unspecific interactions related to the different reagents used during the protocols.

#### 2.1.1.5.1 Controlling primary antibody

In order to determine the part of the immunolabeling that is due to unspecific binding of the IgG molecules of the primary antibody, it is necessary to replace it with the same dilution of the pre-immune serum or a serum of the same species as the primary antibody, and also to maintain the same incubation time as that used for the specific antibody. This will then reveal unspecific interactions due to the presence of hydrophobic and hydrophilic regions in the biological structures. Nevertheless, another source of unspecific label arises due to epitopes of other molecules being able to interact or cross-react with the primary antibody. This can be demonstrated by absorbing the primary antibody with the protein or peptide used to immunize, and then incubating for 24 h at 4°C in order to promote interaction with the antibody. For example, primary antibody could be combined with a fivefold excess of blocking antigen peptide [Mukdsi, et al.; 2006]. Then, after centrifugation to precipitate the antigen-antibody complex, the supernatant can be used to incubate the grid, followed by the gold complexes.

#### 2.1.1.5.2 Controlling secondary antibody

To determine whether the gold complex is contributing to the labeling through unspecific bindings to the tissue, after applying the blocking solution the grids need to be incubated with gold complex but omitting the primary antibody or the pre-immune serum.

The introduction of adequate controls, together with the introduction of blocking solution, can render a confident label that will certainly contribute to the analysis of the biological processes. Finally, when a new antigen is being studied, it is recommended that positive controls are introduced.

## 2.1.2 Post-embedding protocol

### 2.1.2.1 Fixation

- Immerse samples in a mix of 1.5% (v/v) glutaraldehyde and 4% (w/v) formaldehyde in 0.1 M Cacodylate buffer, pH 7.3, at room temperature for 5-6 h<sup>(1)</sup>. Osmium postfixation must be omitted.
- Remove fixative and wash three times in wash buffer sodium phosphate buffer (0.1M, PBS), pH 7.4.

### 2.1.2.2 Dehydration and embedding

- Dehydrate tissue pieces or cellular pellets in a series of increasing concentrations of ethanol: 50%, 70% and 90% (15 min each one)<sup>(2)</sup>.
- Remove the 90% ethanol and infiltrate with a mixture of LR-White and 90% ethanol (1:1). Shake gently in a rotor for 2 h.
- Remove the mixture and replace by 100% embedding LR-White and leave overnight at 4°C. Ensure that the tissue pieces remain completely covered in embedding resin.



- Transfer the samples to gelatin capsules filled with LR-White, and cap and polymerize in an oven at 55°C for 24 h.

### 2.1.2.3 Sectioning

- Trim the LR-White blocks to form a pyramid, and obtain semithin sections (150-200 nm) which can be examined under a light microscope after staining with the toluidine blue solution <sup>(3)</sup>.
- Trim the blocks for immunoelectron microscopy, forming a small pyramid with the region containing the tissue/cells selected from semithin sections.
- Obtain ultrathin sections (60-90 nm) with a diamond knife and mount the sections on a nickel grid (250 mesh). Store these in a grid case at room temperature <sup>(4)</sup>.
- To inactivate the residual aldehyde groups, incubate grids on drops of 0.05 M Glycine in PBS buffer for 10-20 min.

### 2.1.2.4 Immunostaining procedure

- Transfer the grids to drops of 1% PBS-BSA (blocking buffer) placed on a piece of Parafilm, and incubate in a wet chamber in a Petri dish for 30 min at room temperature in order to block unspecific sites.
- Incubate the grids in 50 µl of specific primary antibody diluted in blocking buffer, followed by an overnight incubation in a wet chamber, at 4°C <sup>(5)</sup>.
- Wash the grids with PBS under a jet using a wash bottle for 2 min.
- Incubate the grids in a drop of 50 µl protein A/colloidal gold complex (16 nm) [Maldonado & Aoki, 1986-b] or colloidal gold-IgG complex (available in 6, 10, or 15 nm) diluted in blocking buffer (1:20), for 30 min at 37°C.
- Wash the grids with PBS using a wash bottle and then with distilled water.

### 2.1.2.5 Electron microscopy contrast staining

- Incubate the grids on a drop of aqueous uranyl acetate saturate solution for 1 min, and then wash with distilled water.
- Use lead citrate if more contrast is needed <sup>(6)</sup>.
- Examine the immunolabeled ultrathin sections in a TEM.

### 2.1.2.6 Control of specificity of the immune gold staining (see 2.1.1.4.1 section)

#### Footnotes

<sup>(1)</sup> Glutaraldehyde and the other chemicals used for fixation and staining are extremely hazardous, so solutions should be prepared and used in a hood. Gloves and protective eyewear should be worn whenever handling chemicals.

- There are no standard fixation protocols, and optimal conditions must be previously established for each antigen with the essential goal being their immobilization.
- Ensure that the individual tissue pieces are fully in contact with the fixative.
- If the tissue requires fixation by perfusion, a mix of 1.5% formaldehyde-glutaraldehyde in 0.1 M Cacodylate buffer, pH 7.4, is useful [Pozzo Miller & Aoki; 1991].
- Osmolarity of the fixation vehicle is one of the most essential factors for the preservation of the ultrastructure.
- The use of distilled formaldehyde, rather than formalin, is recommended.



- Some protocols indicate incubating the specimen in 0.1 M glycine solution for 20 min to quench the free aldehyde groups.
- (2) N, N-Dimethylformamide (DMF) and methanol are other possible dehydration media.
  - (3) Thick sections can initially be cut and a light microscope used to identify the region of the block containing the sample. To carry this out blue toluidine staining applied for 30 s is useful.
  - (4) The use of nickel grids is recommended, especially if silver enhancement procedures are going to be used.
  - (5) The optimal antibody dilution and incubation conditions must be determined empirically for each antibody.
- When excessive background labeling occurs, increasing the amount of BSA in the blocking solution, lowering the antibody concentration, and/or shortening the incubation time may decrease the non-specific labeling.
  - Primary antibody dilution for immunoelectron microscopy is usually 10 times more concentrated than that for immunostaining at light microscopy level.
- (6) Other protocols recommend counterstaining the grids with 1% OsO<sub>4</sub> solution, 2% uranyl acetate for 5 min, followed by Reynolds' lead citrate solution for 1 min, and after each staining washing with distilled water.

### 2.1.3 Double labeling protocol

Post-embedding protocols allow the simultaneous localization of two antigens in the grid with two gold complexes of different gold particle sizes. Each antigen can usually be localized by two independent labels on each side of the grid, especially if the antibodies available were prepared in the same species. Otherwise, antibodies can be mixed and the reaction performed in a single step on one side of the grid. Figure 7 shows a double immunogold with a rabbit anti-prolactin antibody and a monkey anti-growth hormone in a ultrathin section of pituitary gland, with both being revealed using complexes of protein A-gold particles of 5 and 15 nm respectively. Technically, one face of the grid was labeled for PRL with the small gold particles, and after a brief drying, the other face was stained for GH with the particle complex [Pasolli, et al.; 1994]. In Figure 7, both hormones colocalized in the same secretory granule, while in Figure 8, neighbouring cells in primary pituitary cell cultures were identified as lactotrophs and somatotrophs in the same section using an identical protocol [Orgnero de Gaisan, et al.; 1997].

### 2.1.4 Immunogold applied at light microscopy level

Gold particles applied in immunolabeling have the advantage that they can also be used at light microscope level on semithin sections of the same inclusion before they are prepared. For this purpose, 1 nm gold particles (ultrasmall) offer optimal advantages mainly because their small size allows a great concentration of gold particles in a small area. However, this is not sufficient to be able to visualize the specific label in a light microscope, with it being essential to apply a silver solution to enhance the gold particles. This silver enhancement reaction is based on the gold particle catalyzed reduction of Ag<sup>+</sup> to metallic silver by using

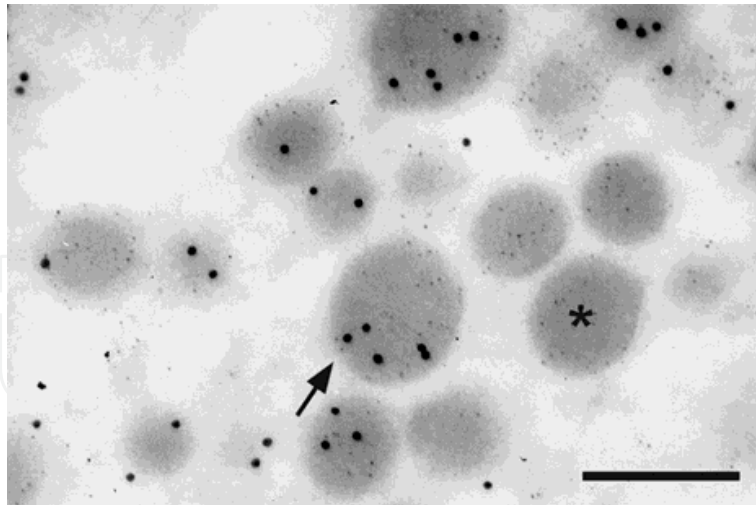


Fig. 7. Double labeling applied to identify antigen co-localization. Co-localization of growth hormone (15 nm-gold particles) and prolactin (5 nm-gold particles) in same secretory granule (arrow), and others containing only prolactin hormone are also seen (asterisk). Bar=0.25  $\mu$ m. (Reproduced from Pasolli et al., 1994; with permission from Histochemistry).

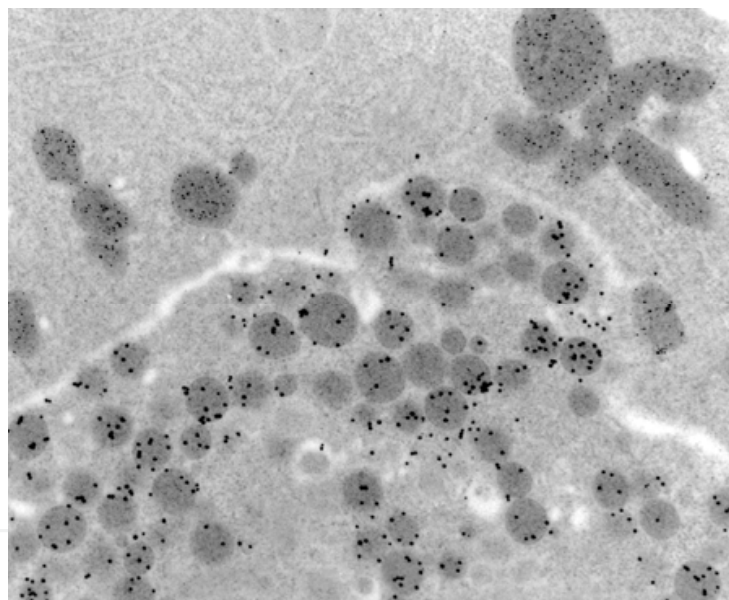


Fig. 8. Double labeling to recognize different cell types in the same section. Micrograph from normal pituitary gland in culture showing a somatotroph cell containing growth hormone (15 nm-gold particles) and a lactotroph cell expressing prolactin (5 nm-gold particles) (Reproduced from Orgnero de Gaisan et al., 1997; with permission from Ann Anat). Original magnification X 25000.

photographic developing compounds as the electron source, a system first developed by Danscher in 1981 [Danscher; 1981].

In Figure 9, silver gold enhancer was used to visualize growth hormone in sections of pituitary gland fixed in 4% formaldehyde and 2% glutaraldehyde and then included in LR-White. In this procedure, the labeling fills up the cytoplasm leaving the nuclei free and is ideal to pre-evaluate immunoreaction in a panoramic view before observation at

ultrastructural level. It is also adequate for obtaining light microscopy labeling with a better resolution than in paraffin sections, particularly when used in combination at ultrastructural level. Furthermore, it is ideal for counting positive cells at great resolution [Bonaterra, et al.;1998].

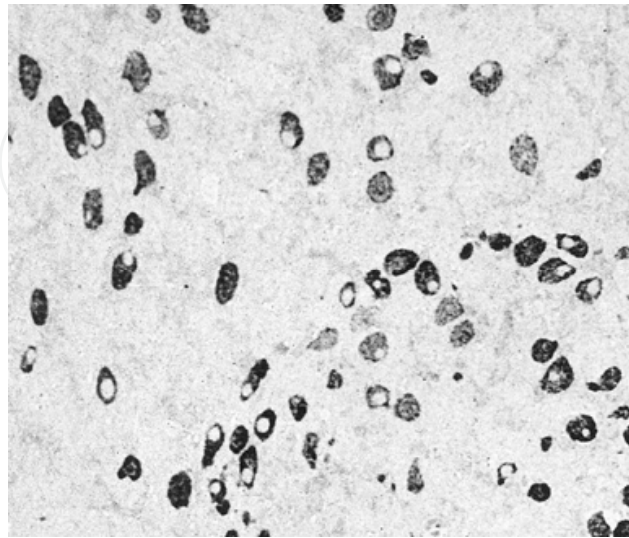


Fig. 9. Immunogold staining at light microscopy level. Growth hormone secretory cells were identified in semithin sections of pituitary gland. After gold labeled secondary antibody, a silver enhancement was performed to allow visualization by light microscopy. Original magnification X 200.

In Figure 10A, gold labeling combined with silver enhancement allowed to identify Galectin 1 (Gal-1), an immunomodulatory protein, in cytoplasmic projections of Müller cells surrounding the neuronal bodies at the inner nuclear layer in semithin sections of chicken retina. This type of staining helped to interpret the labeling obtained at the ultrastructural level (Fig. 10B) [Maldonado, et al.; 1999].

## 2.2 Pre-embedding technique

The pre-embedding method is applied to perform immunogold labeling before fixation (e.g. intact living cells) or after a weak fixation of biological samples (e.g. brain tissue), thus resulting in a greater preservation of the antigenicity of the molecules [Gutiérrez, et al.; 2008; Yi, et al.; 2001].

In this section, we address the protocols and basic applications of the pre-embedding immunogold labeling for transmission electron microscopy.

One of the main advantages of this approach is that specimens are not exposed to harmful or possibly damaging chemicals that can lead to the blocking or loss of target proteins. In addition, after performing immunolabeling, the samples are processed for conventional electron microscopy and included in epoxy resins that allow an improved preservation of the cellular ultrastructure.

In our laboratory, pre-embedding immunogold labeling for the localization of antigens on cell surfaces in culture cells has been achieved. This technique enables the immuno-

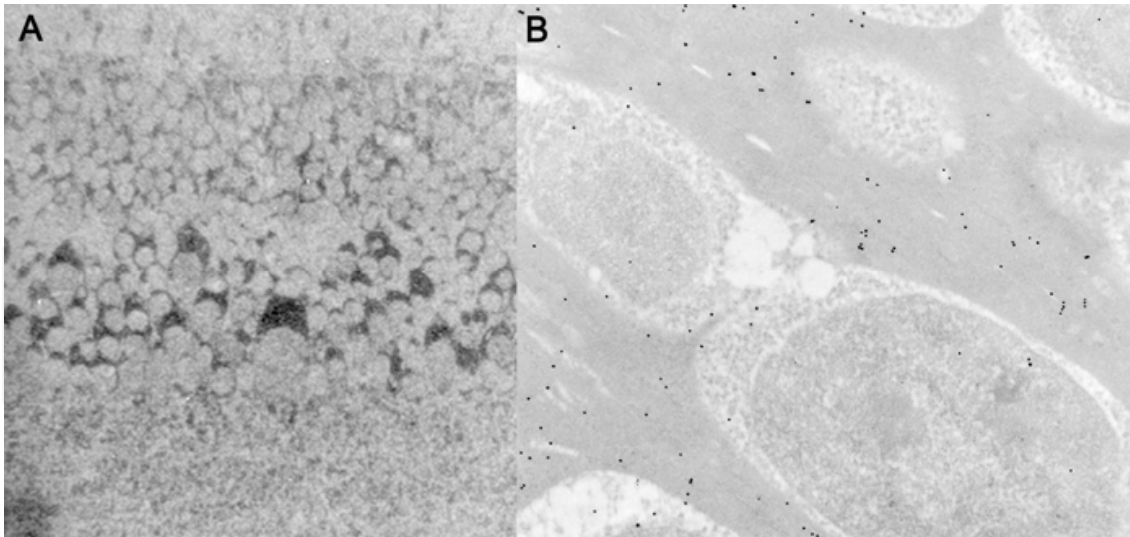


Fig. 10. Immunolocalization of Galectin 1 in chicken retina. A: Silver enhancement to localize Gal-1 in chicken retina at light level. Gold labeling combined with silver enhancement allowed identification of this protein on cytoplasmic projections of Müller cells surrounding neuronal bodies at the inner nuclear layer of chicken retina. Original magnification X 200. B: Gold labeling of Gal-1 positive structure at ultrastructural level. To verify results at light microscopy level, cytoplasmic projections of Müller cells, identified by their high electron density, exhibit specific gold stain for Gal-1 (Reproduced from Maldonado et al., 1999; with permission from Invest Ophthalmol Vis Sci). Original magnification X 25000.

detection of antigens on the surface of isolated cells, viruses or bacteria, with gold particles being localized not only on the cell surface but also on membrane extensions (pili, flagella).

Pre-embedding immunogold labeling has been useful to label virus particles in the morphogenesis stages that imply interaction with the plasma membrane. In this particular situation, viral replication amplifies the quantity of antigens exposed in the plasmalemma, and a brief fixation with 1% glutaraldehyde for 10 min to preserve the cell ultrastructure during overnight primary antibody incubation can be tolerated. In Figure 11, gold particles decorate some virus particles being released from mouse embryonic cells infected with Togavirus [Maldonado & Aoki, 1983].

On the other hand, when the intention is to detect intracellular antigens before resin inclusion, the main problem is to internalize the primary and secondary antibodies inside the cell. However, this problem has been overcome since the development of ultrasmall gold particles. Using these in combination with posterior silver enhancement facilitates the localization of these nanoparticles at electron microscopy level.

If the biological sample is a tissue block, it is recommended to obtain slices in a vibratome (50  $\mu\text{m}$ ) after a weak fixation with 4% formaldehyde and 0.05% glutaraldehyde. Then, slices may be treated with 0.05% Triton-X-100 in PBS for 30 min in order to permeabilize the plasma membrane and allow the access of reagents.

This protocol has been applied successfully in the central nervous system by Sesack et al. [Sesack, et al.; 2006]. In the case of cultured nerve cells, detergents can be replaced by incubation with 0.05%  $\text{NaBH}_4$  and 0.1% glycine in phosphate buffer overnight at 4°C to



permeabilize cell membranes, after applying the same weak fixation referred to above for tissue blocks [Van Lookeren Campagne, et al.; 1992].

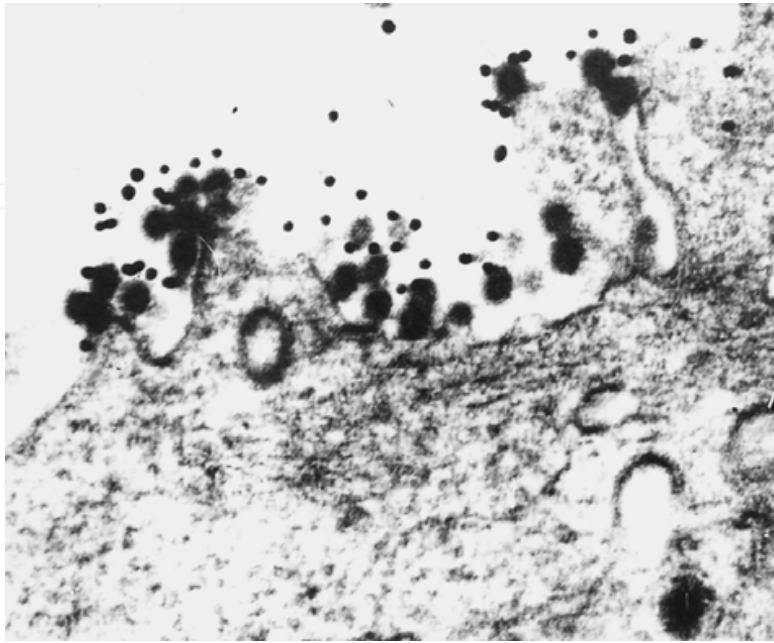


Fig. 11. Identification of superficial viral antigens. Togavirus particles budding from the plasma membrane of infected fibroblasts (Reproduced from Maldonado & Aoki, 1983; with permission from *Micr Electr Biol Cel*). Original magnification X 110000.

### 2.2.1 Pre-embedding protocol

In this section, we describe the pre-embedding immunogold labeling performed on intact living pituitary cells for the localization of antigens on cell surfaces.

#### 2.2.1.1 Immunostaining procedure

- Remove the culture medium from a 35 mm dish of 70-80% confluent cells and rinse gently with Hanks' Balanced Salt Solution, pH 7.0 (HBSS) <sup>(1)</sup>.
- Block unspecific antigens by employing 1% PBS-BSA (blocking buffer), pH 7.4, for 15 min at 37°C.
- Incubate the cell monolayers with specific primary antibodies diluted in HBSS, pH 7.0 for 1 h at 37°C <sup>(2)</sup>.
- Wash 3 times with HBSS.
- Block unspecific sites by incubating with blocking buffer for 15 min at 37°C.
- Incubate the cell monolayers with an appropriate secondary antibody conjugated to gold particles (16 nm) diluted in blocking buffer (1:20), for 60 min at 37°C <sup>(3)</sup>.
- Wash 3 times with HBSS.
- Lift the cells by applying a soft scraping in order to minimize shear <sup>(4)</sup>.
- Centrifuge the cell suspension at 1000 rpm for 5 min to pellet.

#### 2.2.1.2 Fixation

- Fix the cellular pellet with a mixture of 4% formaldehyde, 1.5% glutaraldehyde in 0.1 M Cacodylate buffer, pH 7.4 plus 7% sucrose for 2 h at room temperature.



- Wash in 0.1 M Cacodylate buffer, pH 7.4 containing 7% sucrose three times, 5 min each wash.
- Post-fix with 1% osmium tetroxide ( $\text{OsO}_4$ ) in Cacodylate buffer for 1 h, in a rotor at room temperature <sup>(5)</sup>.
- Rinse the cellular pellet twice in 0.1 M acetate buffer, pH 5.2.
- Stain in 1% uranyl acetate in 0.1 M acetate buffer, pH 5.2, for 20 min (block staining) <sup>(6)</sup>.

### 2.2.1.3 Dehydration and Embedding Sectioning

- Dehydrate with a graded series of cold (4°C) acetones: 50%, 70%, 90%, 10 min each one; followed by acetone 100% (three times, 15 min each step).
- Perform pre-inclusion in pure Araldite/acetone 100% (1:1), overnight (covered) in rotor.
- Perform pre-inclusion in fresh 100% Araldite, 60 min in rotor.
- Embed in fresh 100% Araldite using flat embedding molds (put typed or pencil-written label in order to identify the sample). Place in 60°C oven for 24-48 h.
- Obtain ultrathin sections (60-90 nm) with a diamond knife and mount the sections on a nickel grid (250 mesh). Store these in a grid case at room temperature.

### 2.2.1.4 Electron microscopy contrast staining

- Stain ultrathin sections with alcoholic uranyl acetate/lead citrate <sup>(7)</sup>.
- Examine the immunolabeled ultrathin sections in a TEM (Fig. 12).

### Footnotes

<sup>(1)</sup> An important consideration is to start from a high cellular density due to the fact that during the procedure it is possible to lose a small number of cells, mainly during the washing step. A cell confluence of 70-80% is recommended.

<sup>(2)</sup> Primary antibody incubation is carried out in the plate wells. A piece of Parafilm set on the cell monolayer ensures full access to antibodies, and gentle shaking on a rocking platform is recommended.

<sup>(3)</sup> Appropriate dilutions should be tested and prepared in the same HBSS buffer. Excessive clustering of gold particles should be avoided, and it is recommended to test the antibody dilutions.

<sup>(4)</sup> Although trypsin is widely used for cell dissociation, it is not recommended in this procedure because it can affect the immunostaining.

<sup>(5)</sup>  $\text{OsO}_4$  acts as a secondary fixative by reacting with lipids and oxidizing the unsaturated bonds of fatty acids. The  $\text{OsO}_4$  is reduced to a black metallic osmium which is electron dense and adds contrast to the biological tissues.

<sup>(6)</sup> The incubation of specimens with uranyl acetate before dehydration, also called block staining, improves the contrast of the different structures of biological samples.

<sup>(7)</sup> This staining is used to increase the electron density of the membranes and enhance the contrast.

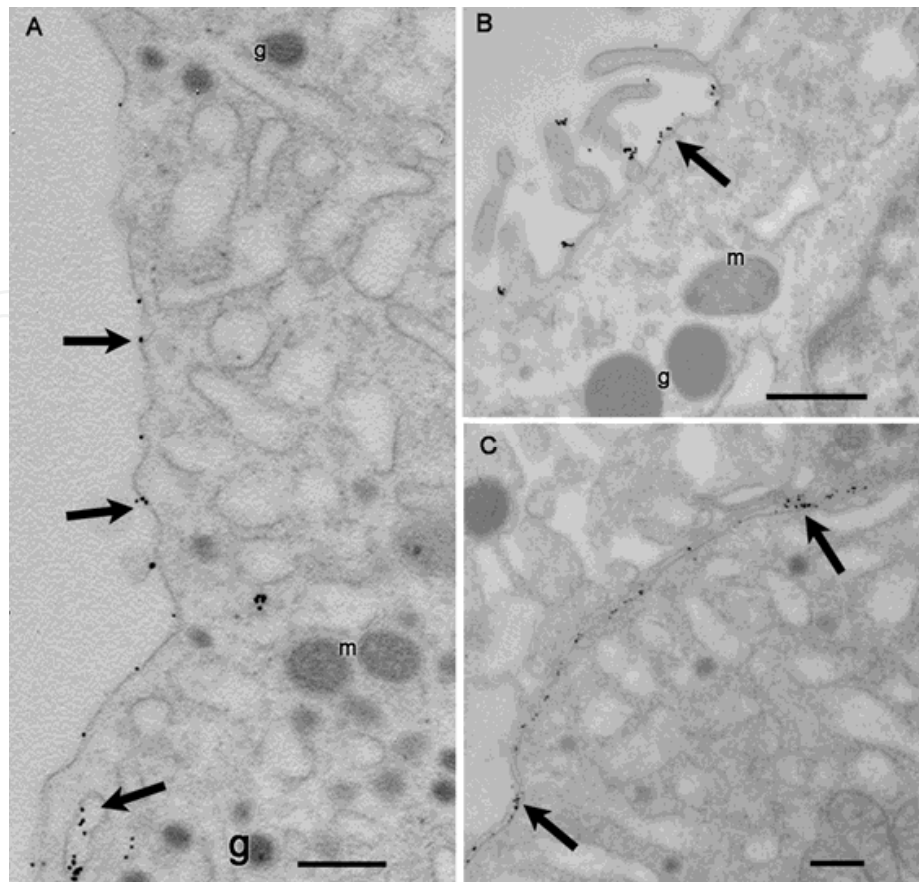


Fig. 12. Immuno detection of estrogen receptor  $\alpha$  in lactotroph cells by pre-embedding method. Colloidal gold-particles can be observed adhered to the plasmatic membrane and attached to the cytoplasmic vesicles membranes (arrows). m: mitochondria; g: granules. Bar=0.5  $\mu$ m (Reproduced from Gutiérrez et al., 2008; with permission from Steroids).

In table 1, we summarized the main differences among different protocols for immunoelectron microscopy related to the antigen cell localization explained above.

### 3. Contribution of immunogold localization of protein in revealing morpho-functional aspects of dynamic cellular processes

Immunoelectron microscopy has been an important tool in cell biology for many decades, supplying powerful, highly valuable visual evidence to help reveal the specific localization of cell proteins, which consequently allows different cellular types to be identified. In addition, the knowledge concerning the subcellular immunogold localization of a wide range of proteins has given novel information on structure-function relationships and led to breakthroughs in the understanding of the cell biology in various physiological and pathological conditions.

In this section, we report on our studies on the cell biology of different tissues, wherein the identification of key molecules by immunoelectron microscopy has provided relevant information from the static localization of proteins to their subcellular translocation, thus revealing new insights into dynamic cell processes such as metabolic activity, differentiation, transdifferentiation, proliferation and death.

INTRACELLULAR ANTIGENS			SUPERFICIAL ANTIGENS
Post-embedding	Pre-embedding		Pre-embedding
CELL SUSPENSIONS OR TISSUES	CULTURED CELLS	TISSUES	CULTURED CELLS
4% formaldehyde 1.5% glutaraldehyde	4% formaldehyde 0.05% glutaraldehyde	4% formaldehyde 0.05-0.1% glutaraldehyde	No fixation is required
Inclusion in <b>LR White acrylic resin</b> , and mounting of thin sections on <b>nickel grids</b>			
Grid incubation with 0,05M <b>glycine</b> in PBS buffer for 10-20 min to inactivate residual aldehyde	0,1% <b>NaBH<sub>4</sub></b> in PBS for 10 min to inactivate residual aldehyde	0,1% <b>NaBH<sub>4</sub></b> in PBS for 10 min to inactivate residual aldehyde	No required
Permeabilization is not required	Permeabilization with 0,05% Triton -X-100 in PBS for 30 min	Permeabilization of <b>vibratome slices</b> with 0,05% Triton -X-100 in PBS for 30 min	Permeabilization is not required
Blocking solution	Blocking solution	Blocking solution	Blocking solution
Incubation with primary antibody	Incubation with primary antibody	Incubation with primary antibody	Incubation with primary antibody
Gold complex of 16 nm particles, or combination of 5 and 15 nm particles for double labeling	Incubation with Ultra-small gold complex	Incubation with Ultra-small gold complex	Incubation with gold complex of 5, 10 or 15 nm particles
	Fixation with 2% glutaraldehyde	Fixation with 2% glutaraldehyde	Fixation with 1,5% glutaraldehyde
Silver enhancement if small or ultra small gold particles were applied	Silver enhancement	Silver enhancement	No required
	Inclusion in epoxy resins. Mounting of thin sections on copper grids	Inclusion in epoxy resins. Mounting of thin sections on copper grids	Inclusion in epoxy resins. Mounting of thin sections on copper grids
Counterstaining with <b>aqueous uranyl</b> and lead citrate (optional)	Counterstaining with alcoholic uranyl acetate and lead citrate (optional)	Counterstaining with alcoholic uranyl acetate and lead citrate (optional)	Counterstaining with alcoholic uranyl acetate and lead citrate

Table 1. Comparative table of protocols that can be applied to label superficial or intracellular antigens.

### 3.1 Immunocytochemical studies of cell populations are indispensable for evaluating their functional status

The anterior pituitary gland consists of several endocrine cell types which can be identified by ultrastructural features based on the size, shape, electron density, and the distribution of their secretory granules. However, under different physiological, pathological and experimental conditions, the pituitary cells display changes in the cytoplasmic organelles and in the secretory granule profiles, which make it difficult identify the various cell populations. In these circumstances, immunocytochemistry using gold particles is indispensable for the specific recognition of pituitary cell types.



We performed several studies aimed at identifying the pituitary cellular hormonal content, by applying specific antibodies against prolactin (PRL), and growth (GH), luteinizing (LH $\beta$ ), adrenocorticotrophic (ACTH) and thyrotrophic (TSH) hormones.

The typical lactotrophs, somatotrophs and gonadotrophs are easily recognized because they retain the main features which have been classically described by conventional electron microscopy studies (Fig. 13). However, other undefined groups of cells displaying small round, oval or sharply pointed secretory granules can only be identified by immunoelectron microscopy, thereby highlighting the fact that this technique is essential for identifying the hormonal content of secretory granules [Orgnero, et al.; 1997].

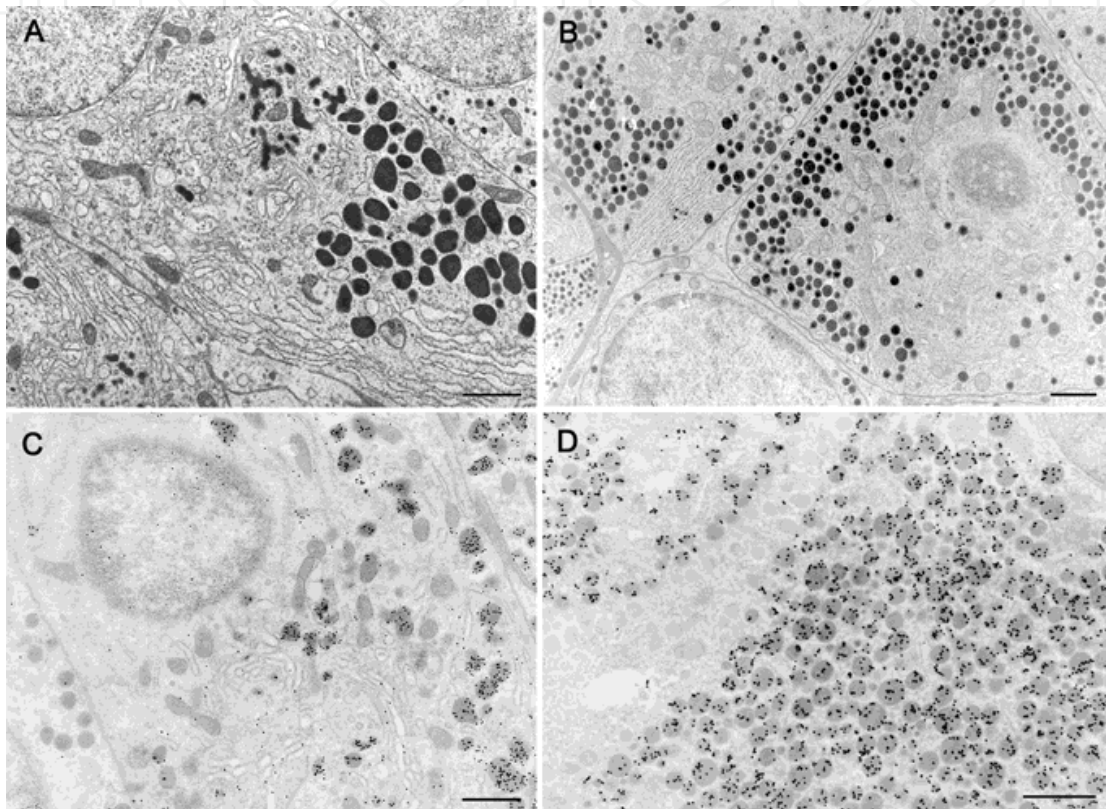


Fig. 13. Typical lactotroph and somatotroph cells identified specifically by immunoelectron microscopy. A: Typical lactotroph with immature secretory granules associated with the trans face of Golgi stacks with numerous polymorphic mature secretory granules (500-900 nm) being stored in the cytoplasm. B: Somatotroph cell with abundant round secretory granules ranging from 200 to 350 nm in diameter. C: Immunocytochemical localization of PRL in a typical lactotroph. Irregular shaped secretory granules surrounded by Golgi saccules are immunolabeled with colloidal gold particles. D: Somatotroph containing numerous spherical secretory granules immunolabeled with growth hormone antiserum. Bar=1  $\mu$ m (Reproduced from Bonaterra et al., 1998; with permission from Exp Clin Endocrinol Diabetes).

In response to different stimuli, the pituitary cell populations acquire significant ultrastructural changes related to their metabolic activity. The lactotrophs or PRL-producing cells have been extensively studied in our laboratory, and we have identified different morphological types of lactotrophs (typical and atypical) by using the specific PRL

immunogold label in secretory granules that display different shapes and sizes, distinguishable only by the application of the immunoelectron microscopy technique (Fig. 14). The proportions of each lactotroph subtype fluctuate in male and female rats, with these morphological variations appearing to be associated with changes in their secretory activities [Maldonado, et al.; 1994]. In another report, we demonstrated that the presence of morphological subtypes of lactotroph cells in rat pituitary cell cultures produced different secretory responses to neuropeptides, with the type I PRL cell population showing the highest response to angiotensin II and TRH action [De Paul, et al.; 1997].

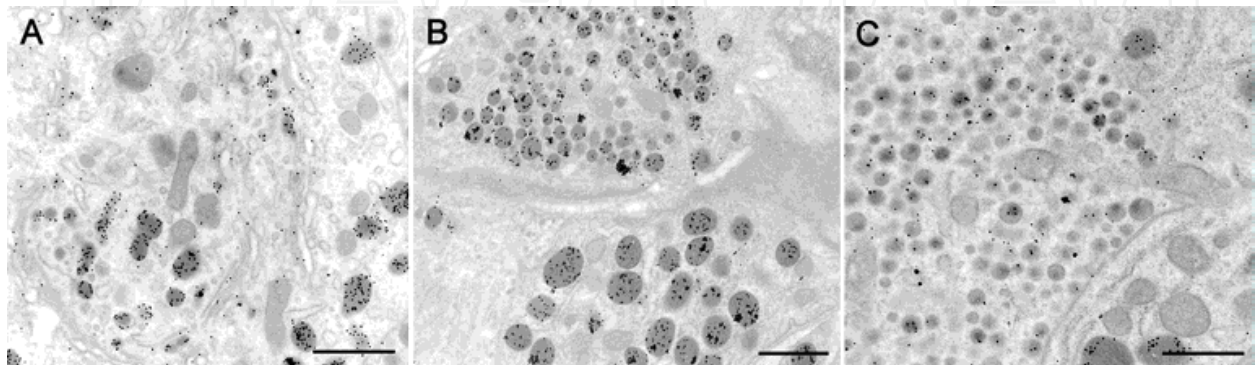


Fig. 14. Immunogold labeled lactotroph subtypes. A: Typical lactotrophs containing heavily immature and mature irregular-shaped electron-dense secretory PRL positive granules (diameter 500-900 nm). B: Atypical lactotroph characterized by the presence of spherical electron-dense granules ranging between 100-250 nm diameters atn the upper side of the picture and other typical subtypes, with bigger mature secretory granules, seen at the bottom of figure. C: Atypical lactotroph containing weakly labeled spherical granules, exhibiting an electron dense core eccentrically placed. A typical lactotroph on the right of the figure is also seen. Bar=1  $\mu$ m (Reproduced from Maldonado & Aoki, 1994; with permission from Biocell).

Classical studies on the pituitary gland performed at electron microscopy level have shown that PRL and GH are synthesized by the distinct and specific cell types, lactotrophs and somatotrophs, respectively. In addition, the application of newer techniques, with a greater resolution and sensitivity has permitted the detection of a cell that produces both PRL and GH (designated as mammosomatotroph, MS), which was able to participate as an intermediate cell in prospective functional interconversions between somatotroph and lactotroph cells in the adult pituitary gland [Beresford; 1990]. In a previous work, we reported that the colocalization of PRL and GH by double immunogold labeling in the same cell was rarely observed in the pituitary of adult rats. Moreover, oestrogen treatment, which is implicated in the transdifferentiation of GH cells into PRL cells, had no effects on the MS population (Fig. 15). Thus the data obtained do not support the suggested role for MS, as transitional cells in the presumptive interconversion of PRL and GH producing cells [Pasolli, et al.; 1994].

In our laboratory, it has been demonstrated that the various endocrine cells constituting the pituitary gland do not occur at fixed proportions and undergo extensive changes depending on the physiological and experimental conditions. Each type of cell proliferates in response to sustained stimuli induced by trophic factors and hypothalamic hormones, with this situation being reversed by a degeneration of surplus cells after the interruption of a specific



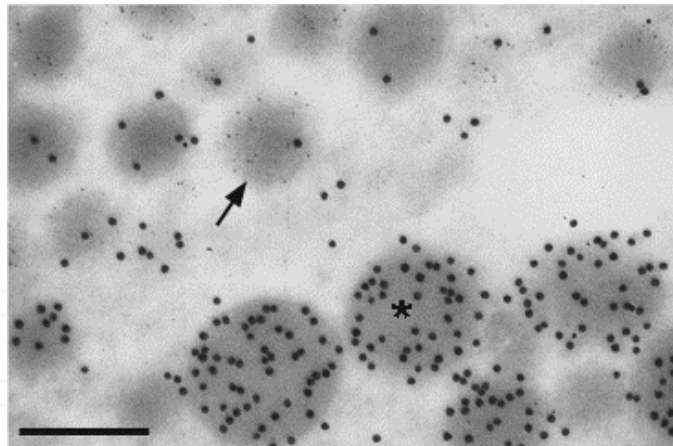


Fig. 15. Mammosomatotroph cell double immunogold labeled in pituitary rat. Co-localization of growth hormone (20 nm-gold particles) and prolactin (5 nm-gold particles) in a mammosomatotroph cell (arrow). A portion of a somatotroph labeled only for growth hormone (asterisk) is at the bottom of the figure. Bar=0.25  $\mu\text{m}$ . (Reproduced from Pasolli et al., 1994; with permission from Histochemistry).

stimulus [Haggi, et al.; 1986; Pasolli, et al.; 1992; Torres, et al.; 1995; Bonaterra, et al.; 1998]. Even though the involuted pituitary cells can be recognized by just using conventional electron microscopy, specific immunogold labeling of their hormonal secretory content allows the cell type involved to be identified with complete confidence (Fig. 16). Also, the presence of an increased number of dead cells activated the phagocytosis of the cell remnants and debris through the stellate cells immunolabeled to S-100 protein [Orgnero, et al.; 1993].

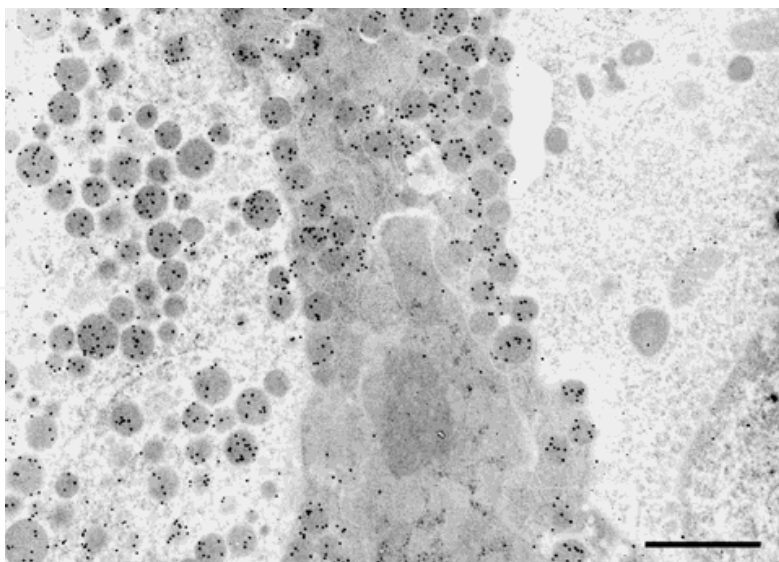


Fig. 16. Immunoelectron microscopy of somatotroph cells in different functional states. Two immunogold labeled somatotrophs in a section from pituitary male rats. A degenerated somatotroph can be seen in the centre and an active growth hormone positive cell on the left, both containing characteristic spherical secretory granules immunolabeled with specific antiserum. On the right, an unlabeled cell serves as the negative control. Bar=1  $\mu\text{m}$  (Reproduced from Torres et al., 1995; with permission from Histochem J).

The size of the different pituitary cell populations was assessed by morphometric analyses of the specific immunostaining of each endocrine cell type, using light microscopy immunocytochemistry by applying immunogold complexes followed by silver enhancement. A comparative analysis of the variations in each pituitary cell population from rats submitted to different experimental and physiological conditions, revealed marked fluctuations in the size of their cell population, which was closely related to the levels of secretory activity. The response of endocrine cells exposed to increased hormonal demands was expressed not only by a release of secretory granules but also by hypertrophy and hyperplasia occurring in proportion to the levels of stimulation. In contrast, cessation of the stimulus activated regressive processes that restored the various pituitary cell populations to their initial values.

Summing up, the evaluation of the pituitary cell types by immunocytochemistry correlated with their ultrastructural changes and the serum hormone levels, which permit the metabolic cell activities and the homeostatic regulation of pituitary gland to be inferred.

### **3.2 Subcellular translocation of kinases evidenced by immunoelectron microscopy is indicative of biological cell activities**

In order to study the mechanisms involved in modulating the proliferation and cell death of normal and tumoral pituitary cells, we investigated the intracellular translocation of protein mediators which are activated in response to different stimuli. It is well known that variations in the intracellular localization of proteins are related to their different biological functions, thus revealing dynamic processes in the cells. Immunoelectron microscopy is a key technique that can be applied to identify the specific distribution of these proteins, thereby contributing to the understanding of their functions. It has been widely demonstrated that the same protein can display opposite functions, depending on its subcellular localization, with a common example of this being the family of protein kinases C (PKC) [Akita; 2002]. These are normally inactive in the cytosolic, but once activated translocate to different cellular compartments.

In our laboratory, we investigated the regulation of cell proliferation by specific PKC isozyme translocation in normal and tumoral pituitary cells. This dynamic process was studied by using an ultrastructural immunocytochemical technique with post-embedding immunogold labeling, following protocols described previously [Petiti, et al.; 2009]. Immunoelectron microscopy demonstrated that in unstimulated normal lactotroph cells, PKC $\alpha$  and PKC $\epsilon$  were evenly distributed throughout the cytosol and nucleus, but were not associated with any specific organelles. Interestingly, after a mitogenic treatment, the PRL cell population showed an association of gold particles, thus indicating the presence of PKC $\alpha$  at the plasma membrane, while PKC $\epsilon$  was mainly localized to the rough endoplasmic reticulum (RER) and Golgi networks [Petiti, et al.; 2008].

In tumoral pituitary GH3B6 cells, the proliferative stimulus induced the specific translocation of both PKCs to the plasma and nuclear membrane. These results indicate the existence of a close correlation between the subcellular localization of PKC isozymes with different biological functions, revealing that the localization in the plasma and nuclear membranes is associated with cell proliferation and that their immunolabeling in Golgi networks might be linked with vesicular trafficking (Fig. 17A-C) [Petiti, et al.; 2009]. Our investigation illustrates

that the specific subcellular targeting of PKC $\alpha$  and PKC $\epsilon$  is indicative of the role of these enzymes in the regulation of the activity of normal and tumoral lactotroph cells.

Bearing in mind that different PKC isozymes are implicated in modulating almost all aspects of pituitary tumorigenesis, it is important to explore the participation of these kinases in cell proliferation, survival and cell death. In contrast with the role of PKC $\alpha$  and PKC $\epsilon$  in promoting pituitary cell growth, it has been demonstrated in GH3B6 pituitary adenoma cells that PKC $\delta$  activation plays a crucial role in programmed cell death [Leverrier, et al.; 2002]. Also, in a recent study, we identified the non-apoptotic mechanism paraptosis as being the predominant cell death type involved in the regression of pituitary tumors after bromocriptine treatment.

In tumoral pituitary cells, we determined the fine localization of PKC $\delta$  by means of immuno-electron microscopy and observed immunolabeling in the cytosolic matrix, which was associated with RER and some isolated mitochondria in intact male rats. Furthermore, bromocriptine treatment was able to enhance the immunogold labeling to PKC $\delta$  in the nuclear compartment of pituitary cells at different involutive and dying stages (Fig. 17D). These results are indicative of PKC $\delta$  intracellular translocation, inferring that a dynamic process occurs in pituitary cell death [Palmeri, et al.; 2009].

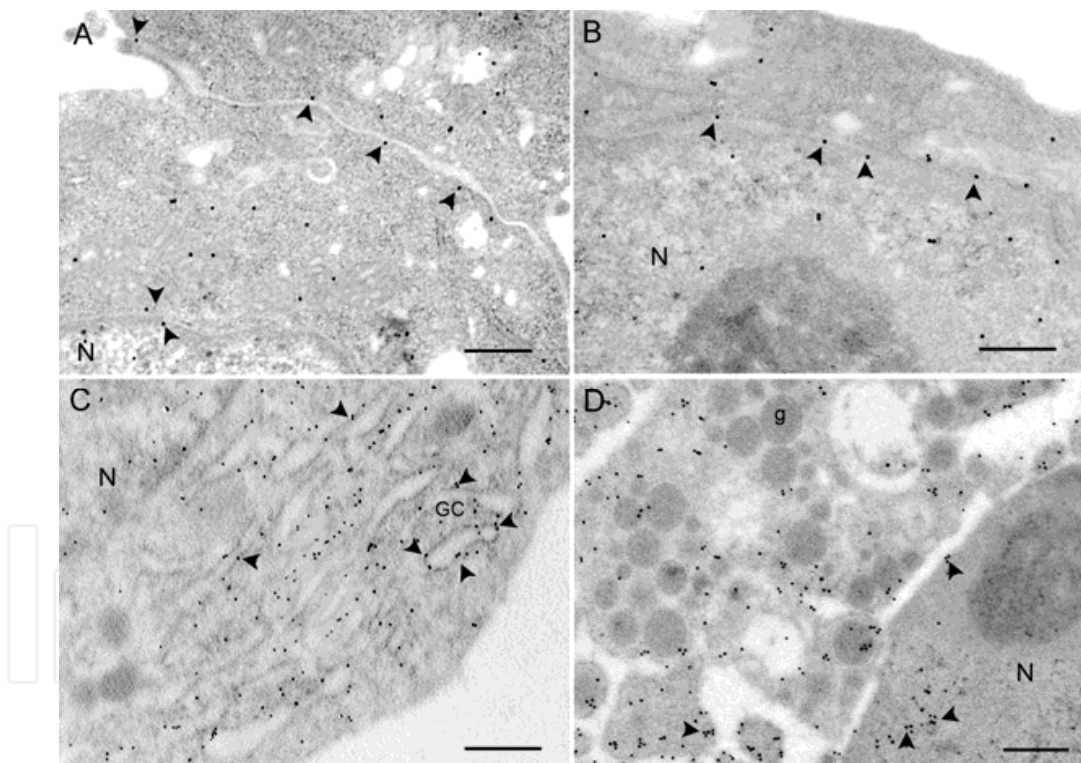


Fig. 17. Immuno-electron labeling for PKC isozymes in pituitary cells. Immunogold particles for PKC $\alpha$  attached to the plasma membrane (A) and PKC $\epsilon$  associated with nuclear envelope (B) (arrowhead) after mitogenic stimulation in pituitary GH3B6 tumoral cells. PKC $\epsilon$  was mainly localized to the Golgi complex (GC) (arrowhead) in proliferating lactotroph cells (C). Bc treatment enhanced PKC $\delta$  labeling in the nuclear compartment associated with euchromatin and nuclear envelope (arrowhead). N: nucleus; g: granules. Bar=1  $\mu$ m. (Reproduced from Petiti et al., with permission from Mol Cell Endocrinol 2008 and J Mol Histol 2009, and Palmeri et al., with permission from Toxicol Appl Pharmacol 2009).

### 3.3 Immunoelectron microscopy emerges as an indispensable tool to understand the differentiation status of the cell

Diverse changes in the cell microenvironment, such as those generated by inflammatory stimuli, can induce significant alterations in both the epithelial and stromal cells, thus leading to modifications in cell biology and function. However, immunogold labeling at the ultrastructural level is necessary in order to reveal and characterize these processes, by evaluating cytoskeleton components, secretory products, growth factor receptors, etc.

In lung epithelium, we have reported changes induced by allergy inflammation in bronchiolar Clara cells (which are strongly involved in several key homeostatic mechanisms). The apical cytoplasm of these polarized secretory cells is filled with polymorphic mitochondria and scarce secretory granules under the plasma membrane (Fig. 18A). Nevertheless, even under normal conditions, the immunogold technique was able to differentiate between these two organelles: mitochondria stained positive to CYP1E2 (P450 cytochrome), while the secretory granules were easily identified by their immunoreactivity for CC16, the main secretory protein of Clara cells (Fig. 18B). After applying a short allergic stimulus, these cells hypertrophied and filled up with big secretory-like granules of moderate electron density (Fig. 18C). Then, immunogold helped us to identify the mitochondria as scarce CYP1E2-positive structures intermingled among the granules and to observe that the numerous secretory granules strongly gold-labeled for CC16 (Fig. 18D). Following chronic allergic exposition, the cells continued to be hypertrophied, but their secretory granules were bigger and fused with very low electron-densities (Fig. 18E), with immunogold indicating that they were not storage sites for CC16 (Fig. 18F). In this way, immunoelectron microscopy confirmed that a short allergy stimulus induced Clara cells to respond with hypersecreting CC16 as a protective mechanism against inflammation, while a chronic inflammatory microenvironment caused their transdifferentiation to mucous secreting cells that lost their CC16 storage in secretory granules [Roth, et al.; 2007].

Stromal cells are other important targets of microenvironmental modifications. In particular, fibroblasts and smooth muscle cells have proved to be very sensitive to cytokines and growth factors, with the transforming growth factor beta being a well-known stromal remodeling agent. A clear example of this remodeling was observed in prostatic smooth muscle cells after inflammation; under normal conditions. These cells are fully differentiated to a contractile phenotype containing mainly cytoskeleton components, as can be shown by immunolabeling smooth muscle  $\alpha$ -actin (ACTA2), and scarce synthesis organelles (Fig. 19A and B). In experimental prostatitis, we observed phenotypic changes in these cells, including an increase in the cisternae of rough endoplasmic reticulum and mitochondria and the loss of cytoskeleton fibers (Fig. 19C), with the contribution of immunogold staining in this process having been focused on confirming the muscular nature of these cells and corroborating the reduction of the ACTA2-positive cytoskeleton compartment within the cells (Fig. 19D) [Quintar, et al.; 2010].

Another important example of remodeling in response to microenvironmental changes is the transformation of fibroblasts (constant components of the stroma) into myofibroblasts by myodifferentiation, a process that could be observed by immunolabeling smooth muscle  $\alpha$ -actin at electron microscopy level. In a prostatitis model, we detected cells with morphological features of myofibroblasts, including a well-developed rough endoplasmic reticulum and signs of nuclear activation (Fig. 19E). As these cells are associated with reactive stroma in



cancer, it was important to verify that they were present in the stroma in the prostatitis model, which was carried out by applying immunogold labeling to ACTA2 (Fig. 19F).

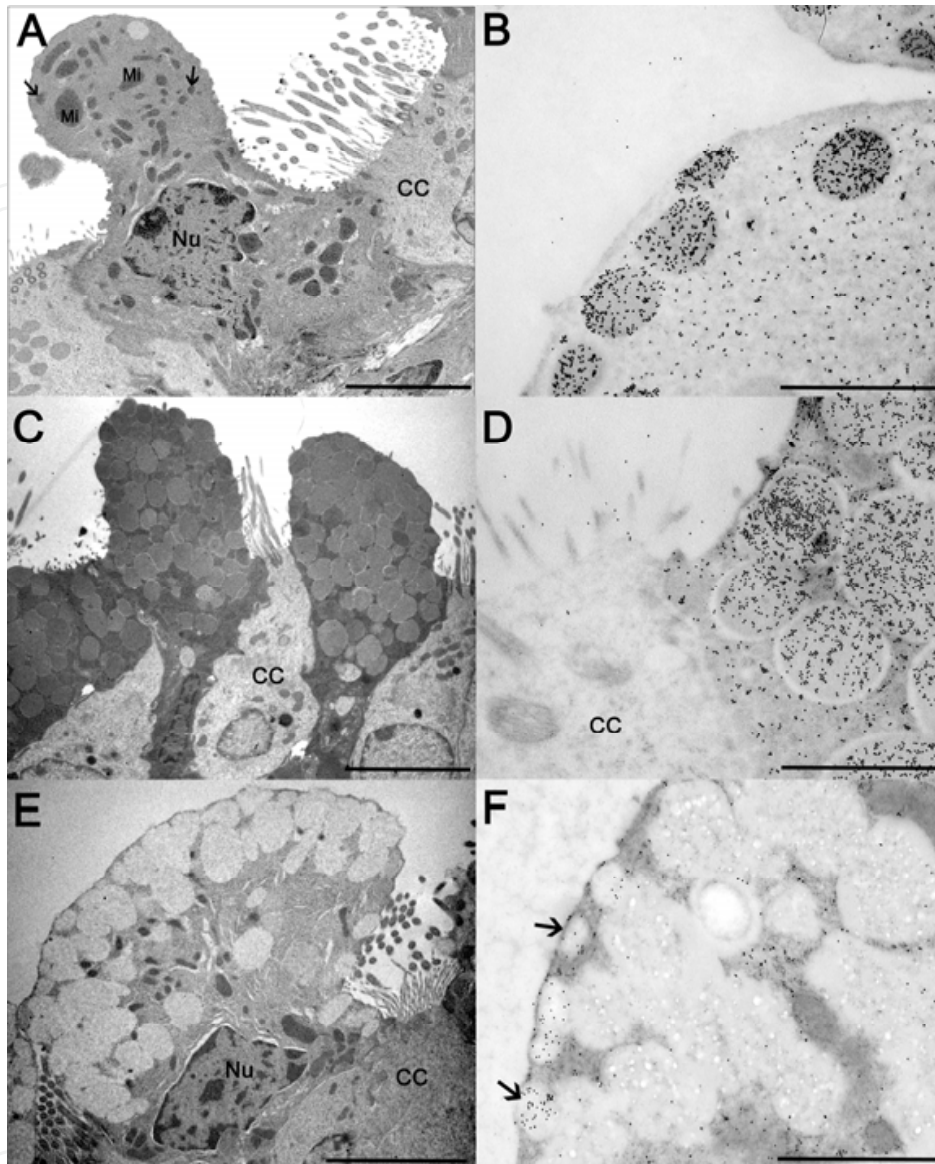


Fig. 18. Immunogold for CC16 to analyze effects of allergic inflammation on bronchiolar Clara cell secretions at ultrastructural level. A: Normal bronchiolar Clara cells with numerous mitochondria (Mi) and few small secretory granules with round profiles and moderate electron density. B: By immunogold labeling, the protein CC16 was shown to be intense at the secretory granules, while it was moderate in the cytoplasm. C: After acute OVA challenge, Clara cells exhibit numerous large secretory granules filling the whole apical cytoplasm. D: Large and electron-lucent secretory granules, appearing after acute OVA challenge, are heavily decorated for CC16 protein. Also, free cytoplasmic labeling appears around the granules. E: After chronic OVA, mucous transformed Clara cells show characteristic large fused electron-lucent granules. F: Mucous transformed Clara cells exhibit scarce CC16 label restricted to a few positive small granules under the plasma membrane with the cytoplasm portions intermingled. CC: ciliated cell; Nu: nucleus; OVA: ovalbumin. Bar=1  $\mu\text{m}$  (Reproduced from Roth et al., 2007; with permission from Histochem Cell Biol).

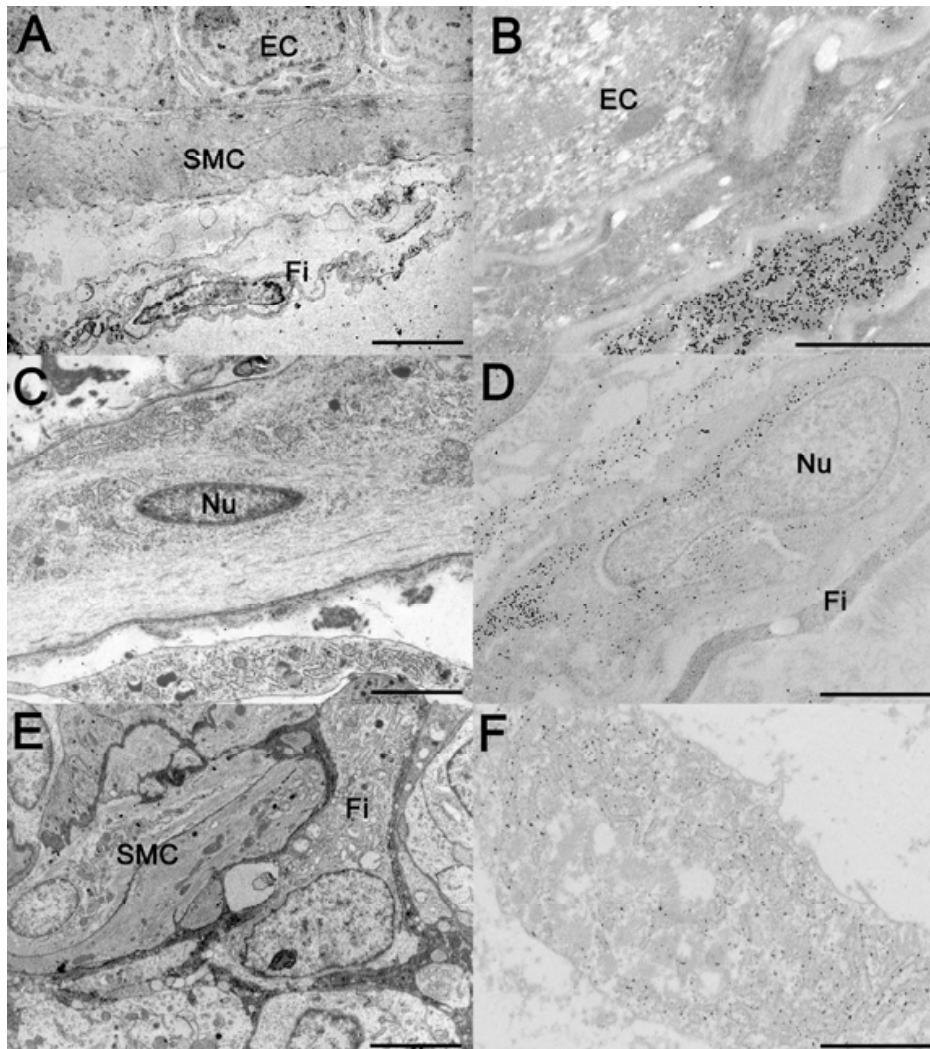


Fig. 19. Immunogold for ACTA2 to analyze the effects of prostatitis on cytoskeleton components of prostatic stromal cells at ultrastructural level. A: Periacinar smooth muscle cell (SMC) in normal prostate gland. B: ACTA2 immunogold labeling indicates a homogenous distributed actin cytoskeleton in periacinar smooth muscle cells on normal prostate gland. C: Smooth muscle cell in prostatitis. Note the large development of cytoplasmic organelles accompanied by loss of contractile filaments. D: ACTA2 immunolabeling on smooth muscle cell indicates that actin filaments are restricted to peripheral zones of these periacinar cells in prostatitis. E: In prostatitis, a periacinar stimulated fibroblast exhibits well developed rough endoplasmic reticulum; smooth muscle cells exhibit signs of dedifferentiation as described in figure C. F: ACTA2 immunolabeling exposes the myodifferentiation process occurring in stimulated periacinar fibroblast in prostatitis. Nu: nucleus; Fi: fibroblast; EC: epithelial cell. Bar=1  $\mu\text{m}$ . (Reproduced from Quintar et al., 2010; with permission from Prostate).

#### 4. Conclusion

This review was written with the main purpose of emphasizing the great potentiality of immunolabeling as an indispensable tool to combine the ultrastructure with the specific identification of the molecules involved in biological processes, thus contributing to the understanding of the structure-function relationships. In order to encourage scientists from different fields of cell biology, the challenge of immunocytochemistry at ultrastructural level has been treated using a wide approach, thereby providing the knowledge and criteria necessary to implement various techniques and to suggest solution the problems inherent to this methodology. Studies performed by our research group have been described, with the identification of key molecules by immunoelectron microscopy permitting dynamic cell processes in different tissues to be inferred.

#### 5. Acknowledgment

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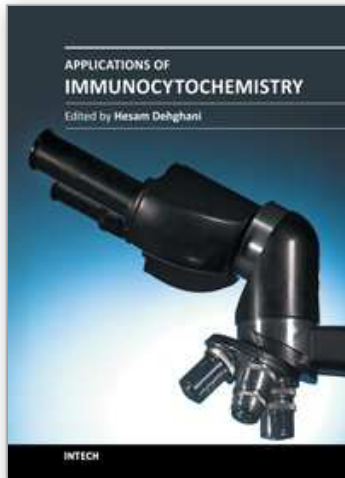


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