LETTER TO THE EDITOR


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Pre-embedding intensification procedures for double labeling in electron microscopy available to us (Görcs et al. 1986; Van den Pol and Görcs 1986; Liposits et al. 1984) are laborious and have many drawbacks, such as a few hours preincubation with thioglycolic acid (Gallyas 1982), which not only lengthens the intensification procedure but also in some cases makes it impossible to perform a second immunostaining (Buijs et al. 1990). Second, any chemical residues on the glassware or pipet influence the final outcome of the silver reaction, which is therefore difficult to control accurately. To overcome these drawbacks, we developed a methenamine silver–gold method for DAB labeling studies that allows reproducible intensification for light and electron microscopy, modified from the technique described by Rodriguez et al. (1984).

Fifty-μm-thick sections of brains fixed with 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) were treated with freshly made 0.5% sodium borohydride (NaBH₄) in Tris-buffered saline (TBS) for 10 min. This pretreatment decreased the background staining tremendously but without affecting the tissue structure. The sections were then rinsed several times in TBS until they sank. Then the sections were incubated in 50% ethanol for 30 min to increase antibody penetration, after which they were processed for the first immunostaining until they were allowed to react with 0.025% 3,3’-diaminobenzidine tetrahydrochloride (DAB) in the presence of 0.006% H₂O₂ for 15 min. DAB staining was then intensified by methenamine silver–gold reaction as follows.

Solutions Used
Stock solutions for the methenamine silver developer:

Solution A: 3% methenamine (C₆H₁₂N₄)
Solution B: 5% silver nitrate (AgNO₃)
Solution C: 1% sodium tetraborate (Na₂B₄O₇)
Gold toning stock solution: 1% gold chloride (HAuCl₄) diluted before use to 0.1%.
Rinsing solution: 2% sodium acetate (CH₃COONa)
Stabilizing solution: 3% sodium thiosulfate (Na₂S₂O₃)
All solutions are freshly made in milliQ water.

Procedure
1. The vibratome sections are rinsed in 2% sodium acetate for 3 × 10 min.
2. The silver methenamine developer is prepared immediately before use in the following way: 150 ml of Solution A is added to 20 ml of Solution B and thoroughly mixed until the white precipitate disappears. Thereafter, 20 ml of Solution C is added. The final pH of the solution is 8.7.
3. The sections are immediately put in this developer and incubated at 60°C for 5 min.
4. To stop the reaction, the sections are rinsed in 2% sodium acetate for 3 × 10 min.
5. Stabilization of the silver intensification is obtained by incubation in 3% sodium thiosulfate for 5 min.
6. Gold toning by incubation in 0.1% gold chloride for 5 min.
7. Rinse in 2% sodium acetate for 3 × 10 min.
8. Stabilization with 3% sodium thiosulfate for 5 min.
9. Rinse in 2% sodium acetate for 3 × 10 min.
10. Rinse in TBS for 3 × 10 min.

A second immunostaining can be performed with DAB after the silver intensification.

In a final step, the sections are postfixed in a mixture of 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer for 30 min, dehydrated via a graded series of ethanol, flat-embedded in Epon resin between two glass slides, and hardened at 60°C for 48 hr. Thereafter, the tissue portion of
interest is cut out. Ultrathin sections are cut with an ultramicrotome and viewed by electron microscopy without lead staining.

The methenamine silver–gold intensification method presented here does not require the use of thioglycolic acid (TGA) (Görcs et al. 1986) or periodic acid (Rodriguez et al. 1984) preincubation, which has been reported to induce partial dissociation of antigen–antibody complexes (Görcs et al. 1986) and to impair antigen detection (Buijs et al. 1990). The formation of metallic silver by DAB catalysis is rapid because of the high reactivity of methenamine at 60°C and therefore overrides the induction period of the tissue argyrophilic reaction (due to catalytic properties of the tissue), which is relatively long at the pH of the developer solution (Merchnthaler et al. 1989). Our developing time of 5 min at 60°C gave reproducible and specific intensification. The intensification can be enhanced by a longer developing time or a higher temperature, but this entails the risk of nonspecific silver deposits. Therefore, users should optimize their developing conditions according to their own materials and purposes. The gold toning should not exceed 5 min because nonspecific gold precipitation may occur. As seen by electron microscopy, gold-substituted silver grains are relatively small and their size remains the same with the same developing time. The numbers of grains depend on the local amount of DAB end-products (Figures 1B and 1C), whereas with the conventional protocol (Görcs et al. 1986; Liposits et al. 1984; Gallyas 1982) the size of the grains within a section may vary (Van den Pol and Görcs 1986), depending on several parameters such as intensification time, local amount of DAB end-products, and purity of the developer solution. The use here of sodium acetate as rinsing solution instead of distilled water (Rodriguez et al. 1984) is, in our hands, critical for obtaining reproducible results. The protocol described here provides less nonspecific background staining and has yielded, in our hands, more reproducible results than previous methenamine silver protocols (Cardno and Steiner 1985; Rodriguez et al. 1984). Because methenamine silver–gold intensification will enhance all DAB deposits, it is advisable to use a light DAB staining with low background to avoid false-positive staining. Moreover, it is easy to detect light staining after silver–gold intensification (Figures 1B and 1C). Although our silver–gold intensification procedure does not harm the ultrastructure, heavy DAB staining or overdevelopment will lead to a high concentration of silver–gold grains and therefore will impair the observation of ultrastructural details such as synaptic membrane specializations.

The method can be used to study the co-localization of two proteins in the same tissue or even in the same cell, provided that they are located in different cell compartments. Moreover, the high electron density of gold-substituted silver grains facilitates ultrastructural recognition of stained structures. We would advise this method even in the case of a single staining to decrease the chance of overlooking stained structures. In summary, this method is highly sensitive and
easy to perform and it can be ideally used for single or double histochemical and/or immunostaining at the electron microscopic level.

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Literature Cited

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