Is enzyme metallography suitable for pre- and post-embedding immunogold electron microscopy?

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High-resolution detection of proteins or nucleic acids using electron microscopy provides precise spatial information of epitope distribution which gives important cues as to the function of a particular molecule within cells and tissues.

Available detection systems for electron microscopy (EM) have to deal with partially opposing factors of signal sensitivity, specificity, and resolution. Previously established methods rely on enzymatic processing of specific substrates resulting in a precipitate visible at the EM level, e.g. 3,3´diaminobenzidine (DAB) precipitation at sites of bound peroxidase coupled antibodies. This method offers good sensitivity but poor spatial resolution due to non-uniform precipitations. Furthermore, the precipitations not easily visible because they are of relatively low electron density (although visibility can be enhanced by adding metal ions). Alternatively, gold particle-coupled antibodies offer excellent spatial resolution and visibility due to the electron dense nature of gold particles but sensitivity is reduced compared to the DAB method. Strategies to improve sensitivity involved reducing the size of the colloidal gold particle (< 1nm diameter), by using discrete gold compounds with reduced surface charge, or by application of a protein shell around the gold particle thus reducing the exposed charged surface. Unequivocally, the dense absorption of antibodies and BSA at the surface of colloidal gold particles helped to overcome many of the aforementioned problems and resulted in the convenient and widespread use at the post-embedding EM level.

One apparent limitation of the post-embedding method is the fact that particle-coupled immunoreagents do not easily penetrate the plastic-embedded section, leaving essentially the epitopes exposed on the surface of the section available for binding. Obviously, this reduces sensitivity in the sense that the epitopes located within the volume of the section cannot be accessed. In addition, interpretation of results may be difficult as an essentially two-dimensional label is superimposed onto a three-dimensional ultrastructure.

The pre-embedding approach may hold the solution to these problems as it offers the possibility to bring the label directly to the cellular molecules within the thickness of the specimen representing what is seen as ultrastructure. In practice however, vigorous pretreatments such as protein extraction with detergents and enzymatic digestions are required which have a negative impact on structural preservation. Indeed, using the DAB method this goal can be approached as both the peroxidase-coupled antibodies as well as the DAB solution penetrate cross-linked proteins with only little extraction necessary whereas the adverse effects of the particle coupled antibodies are even more pronounced at the pre-embedding level. Ideally, one would like to have a method available where (1) antibodies are used that do not carry a particle load so that they can easily penetrate the fixed cells, and (2) the signal resembles that of colloidal gold, i.e. small in size and electron dense for easy detection with high spatial resolution.

One such promising method is the enzyme metallography technique [1]. The principle is that a peroxidase coupled antibody is used in the same way as for the DAB method but

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DAB is replaced by a solution with silver ions that precipitate in the vicinity of the peroxidase molecules forming small, electron dense grains. Since its introduction, the method has been successfully used in light microscopy and their principal use in EM has been demonstrated [2]. Here, we tested the usability of the enzyme metallography method for post- and pre-embedding immunogold EM to detect different antigens in the cell nucleus.

Cells and tissues were fixed for EM were incubated with antibodies against nuclear antigens (DNA, histones, lamins) followed by secondary, peroxidase coupled antibodies and by the metallography solutions (EnzMet™, Nanoprobes) for different incubation times, pH values and temperature conditions. At the post-embedding level, anti-DNA antibody on ultra-thin sections of human testicular tissue showed dense labeling over nuclei which are at various states of chromatin condensation. Because of high signal density and electron density of the signal, the label could be conveniently detected at low power magnifications (Fig.1). The uniformity of label density across sections was good and comparable to immunogold reactions. Label density, grain size, and grain uniformity were found to depend on incubation time, the ion content of the incubation solution, and, to a lower extent, on the temperature where the reaction proceeded. At higher magnifications, some background label over structures known to be devoid of DNA became evident (Fig.2). However, in the pre-embedding approach, the resolution and specificity of the enzyme metallography reaction deteriorated to a degree which in our hands became unacceptable.

In conclusion, enzyme metallography proofed to be an alternative for post-embedding immunoelectron microscopy with pros over DAB precipitation (resolution, visibility) and over immunogold detection (sensitivity) and cons (lower signal-to-noise ratio than with DAB and immunogold). In our hands, enzyme metallography did not prove to be applicable for pre-embedding immunoelectron microscopy due to very poor resolution and specificity of the signal. Further refinements of the method may improve the performance of this approach for pre-embedding applications.

[1] J.F. Hainfeld et al., Microscopy Microanal. 8 supp.2 (2002) p916.
[2] F.R. Furuya et al., Microscopy Microanal. 10 supp.2 (2004) p1210.

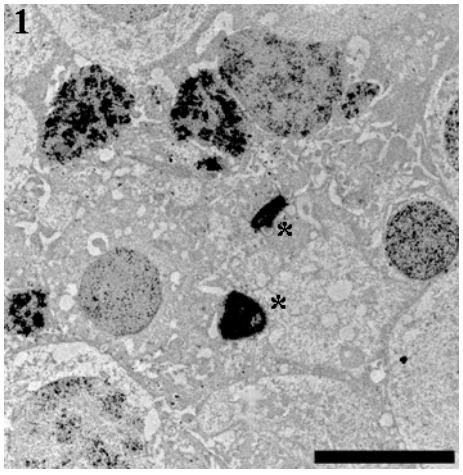

Figure 1. Low power micrograph showing DNA detected using enzyme metallography on ultra-thin sections of human testis. Dense label can be seen over nuclei at different stages of chromatin condensation (*..elongated spermatides) and scant background label is present over cytoplasmatic regions. Bar=10µm

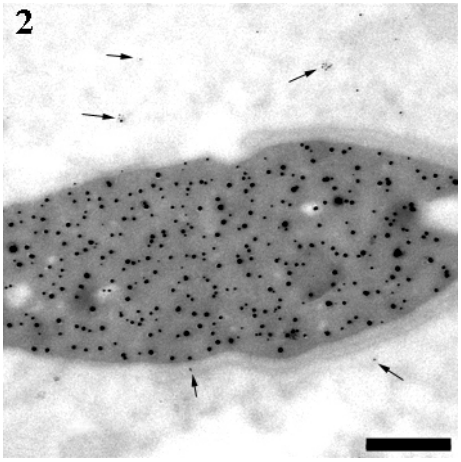

Figure 2. High-power magnification of DNA detected in a human elongated spermatide using enzyme metallography. Note dense and uniform label largely confined to the nucleus. Little background label is visible (arrows). Bar=0.5µm