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Letter to the Editor

Ultra-small Gold Particles and Silver Enhancement as a Detection System in Immunolabeling and In Situ Hybridization Experiments

Reading recent articles published in the Journal of Histochemistry and Cytochemistry (5,6) we became aware that the advantages offered by ultra-small gold probes as a detection system in immunolabeling and in situ hybridization experiments may still not be fully exploited. This letter summarizes the merits of ultra-small gold probes and gives some advice for their successful application.

Here, gold particles (whether colloids or not) that are smaller than 1.5 nm (usually ~1 nm) are called **ultra-small** gold particles. Ultra-small gold markers are available from several companies: AuroProbe One (Amersham; Poole, UK), GP-Ultra Small (Aurion; Wageningen, The Netherlands), 1-nm gold (British BioCell; Cardiff, UK), and Nanogold (Nanoprobes; Stony Brook, NY). It should be mentioned that some preparations also contain particles considerably larger than 1.5 nm (see Figure 3B in ref. 5) (18,19).

The main advantage of ultra-small gold particles compared with larger ones is increased detection efficiency. First, ultra-small gold probes penetrate more easily. Gold probes of different sizes (ultra-small, 7 nm or 10 nm) can penetrate into HeLa cells permeabilized with Triton X-100 to reveal the intermediate filament vimentin. In the same sample, however, a component of the internal nuclear matrix could be visualized only with the ultra-small gold probe (7). Obviously, the dense meshwork of the lamina–pore complex and/or the highly negatively charged DNA prevent larger particles from obtaining access to intranuclear antigens. Second, labeling efficiency increases with decreasing diameter of the gold probe used (11). This increase is even more prominent for the ultra-small gold particles (5,9,12). In pre- and post-embedding in situ hybridization experiments, a comparable improvement of labeling efficiency using ultra-small gold particles was found (16,17). In summary, secondary antibodies tagged to ultra-small gold particles more efficiently detect primary antibodies than those tagged to larger gold particles.

The increase in label efficiency using ultra-small gold probes is less pronounced if surfaces of resin sections (e.g., methacrylates such as Lowicryls and epoxy resins) are labeled. However, if the density of the matrix of the section is lower [e.g., thawed frozen sections (21) or cryostat sections], the ultra-small gold particles have a better chance to penetrate.

In conventional electron microscopy, the ultra-small gold particles are difficult to visualize, especially on stained ultra-thin sections (19,20). Therefore, the size of the ultra-small gold particles must be increased by controlled silver deposition (autometallography). We extensively investigated four published protocols (1,3,4,8,13) along with four commercial developers (IntenSE M, Amersham; R-Gent, Aurion; SEKL 15, British BioCell; HQ Silver, Nanoprobe). In our hands, only the enhancers containing the so-called protective colloid gum arabic (3,14) gave good results with respect to evenness of enhancement, to regularity of shape of the final particles, and to the number of particles enhanced (which determines the efficiency of detection) (see 18–20 for review). Interestingly, less efficiently working enhancers can be improved by adding gum arabic (18,19). We have not tested

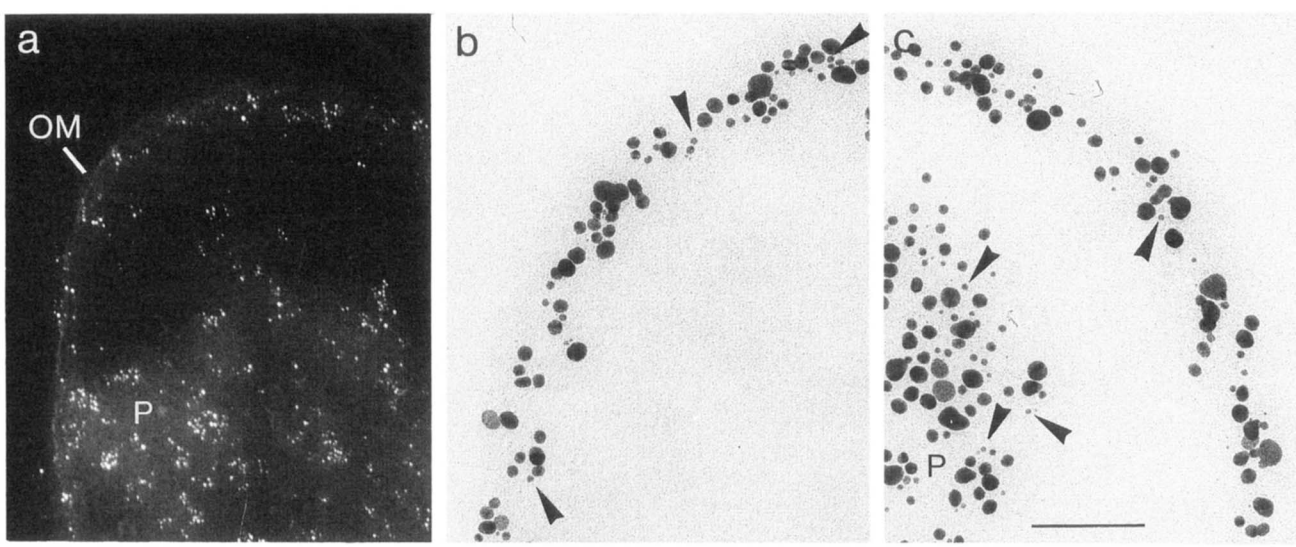

Figure 1. Unstained Lowicryl HM20 section of *E. coli* cells labeled for the outer membrane protein OmpA (which is overproduced in this strain) using rabbit serum and (**a**,**b**) GP-Ultra Small or (**c**) Nanogold conjugated goat anti-rabbit antibodies. A part of an *E. coli* cell is shown. (**a**) Unenhanced ultra-small gold particles imaged with a field emission STEM equipped with a high-angle annular darkfield detector. For details see Otten et al. (15). (**b**) GP-Ultra Small gold particles silver enhanced (Danscher protocol, 17 min, 20°C). (**c**) Nanogold particles silver enhanced (Danscher protocol, 25 min, 20°C). Arrowheads point to particles ~5 nm in diameter. OM, outer membrane; P, cytoplasmic protein (OmpA) clump. Bar = 100 nm.

the gum arabic-containing solution published by Burry and colleagues (2), but comparable results are expected. Among the protocols tested we prefer the original Danscher protocol (3,4) for its efficiency and homogeneous development and for practical reasons. First, the relatively long enhancing time of about 20 min (in EM applications) allows a reproducible and controlled enhancement process. Second, the Danscher protocol is easy to handle and has a low sensitivity to daylight. Third, the components are easily available and cheap, and the prepared solutions can be stored in a freezer. For on-section labeling, it is important to know that copper grids interfere with the enhancement process at low pH; therefore, the use of nickel (or gold) grids or, alternatively, the choice of an enhancer working at higher pH (e.g., 13), is recommended. Suitable enhancers also include the gum arabic-improved Hacker recipe (8) and the commercial HQ Silver (Nanoprobes).

Some drawbacks of silver enhancement must be mentioned. (a) Enhanced particles differ in size. It is still unknown whether the inhomegeneous size of enhanced ultra-small gold particles is caused by irregular enhancing or by the relatively wide size distribution of the ultra-small colloidal gold probes (Figures 1a and b)(18,19). Nanogold that has a constant defined diameter of 1.4 nm shows a similar inhomogeneous size distribution after enhancement (Figure 1c). This fact suggests that silver enhancing is responsible for the irregularity. The local density of gold particles might also interfere with the even deposition of silver layers (20). (b) A second drawback is merging of growing silver/gold particles. This effect is dependent on the time of enhancement (20), the gold particle density, and the quality of the enhancer used, (e.g., the longer the incubation and the larger the particles, the more they merge). Figures 1b and 1c show that particles can grow large enough for easy detection and only a few merge in densely labeled regions. The Danscher protocol proves to be so reproducible that even double-labeling experiments may be done. Either larger gold colloids can be used in combination with ultra-small gold particles or the ultra-small gold probes can be used twice in sequence. In the first case, the particles are enhanced after the labeling is completed, in the second case, silver enhancement has to be performed twice, once after each labeling step (17). In both cases the two labeled components could be clearly identified. (c) The silver layer on gold colloids is not stable after direct exposure to the electron beam and subsequent storage in air (18,19). The silver layer on gold colloids in the illuminated area disappears from on-section-labeled resin sections or relocates on methylcellulose-embedded, thawed frozen sections. This effect can be prevented by storing the illuminated samples in a desiccator. (d) Quantitation on the EM level (5) is hampered, first by the fact that unenhanced gold colloids (e.g., < 1 nm in diameter) are almost invisible in or on resin sections in conventional TEMs. Second, it is not known how many gold particles can be bound to one IgG molecule or how many secondary antibodies bind to one primary antibody. (e) The gold tag may still influence the binding characteristics of the antibodies. It has been shown that Fab fragments tagged with ultra-small gold colloids bind less efficiently than free Fab fragments (10,18). Therefore, even with this marker system, a detection efficiency of 100% cannot be expected.

In summary, ultra-small gold probes combined with an efficient silver-enhancement protocol (e.g., 3,4) form an excellent method to detect cellular macromolecules with improved efficiency.

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