

TECHSIGHT

Worth Its Weight in Gold

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Scientific progress depends on the development of analytical approaches. Cytochemistry is a tool bridging biochemistry and morphology for the identification of specific chemical components in cellular domains to better understand their functional properties. Combining immunoreagents with cytochemistry allows one to tag cellular targets with electron microscopic markers using antibodies. And because it may be possible to raise antibodies specific to most (if not all) cell and tissue components, immunocytochemistry has the potential to be extremely powerful and versatile.

Introduced in the 1940s as a direct technique (1), immunocytochemistry has evolved into an indirect approach that is sensitive and efficient (2). Whereas the direct technique uses a tissue section exposed to a specific antibody tagged with a marker, the indirect technique uses at least two antibodies—the primary antibody in its native form that generates a specific antigen-antibody complex, which, in turn, becomes the target of a secondary antibody. This secondary antibody, tagged with gold particles, is then easily detected (see figure, this page). The technique can be varied by replacing the secondary antibody with molecules, such as purified bacterial proteins A and G (2), which have pseudoimmune properties and high affinities toward immunoglobulins (see figure, next page).

To generate the meaningful molecular interactions needed for immunocytochemistry, five characteristics of an antibody molecule are essential: (i) specific recognition and binding of the antigen; (ii) strong antigen-antibody binding; (iii) selectivity of the antibody for the target epitope; (iv) avidity, recognition of different epitopes on an antigen by multiple antibodies in an antiserum; and (v) titer, the most dilute solution of antibody that still generates specific binding.

Colloidal gold is a cytochemical visualizing agent commonly linked to antibodies. Introduced in 1971 by Faulk and Taylor (3), it has attained a rank of distinction among

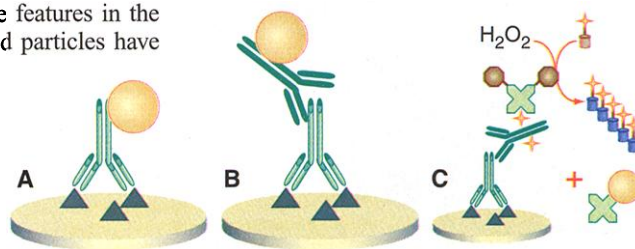
electron microscope cytochemical markers due to its versatility in adsorption to macromolecules, its electron density, its small size, and its ability to be measured morphometrically (2). Colloidal gold can be linked to antibodies, lectins, enzymes, and many other molecules with specific molecular affinities (4). Because it is particulate, colloidal gold allows for easy and accurate identification and delineation of structures without forming a dense deposit that obliterates the underlying cell structure. The level of resolution is determined by the size of the gold particles used. In general, 10- to 15-nm gold particles give the best results because they are easily recognizable under the microscope and yield discernible features in the 10-nm range. Five-nm gold particles have also been instrumental in determining the topology of membrane proteins, revealing epitopes located on the luminal, cytosolic, or transmembrane domains of proteins.

Colloidal gold can be employed in both light and electron microscopic studies, and its utility in techniques as varied as scanning electron microscopy, freeze-fracture, in situ hybridization, negative staining, and enzyme cytochemistry have made it a universal probe in cytochemistry.

The validity of immunocytochemical labeling is determined through analysis of several important control experiments, including (i) application of the primary antibody in the presence of both the tissue sample and a "free" antigen that matches the antigen on the tissue (because if the primary antibody is specific to the tissue antigen, it will bind the free antigen first and no labeling will occur); (ii) omission of the primary antibody in the labeling protocol, which will assess nonspecific adsorption of the secondary antibody-gold complex; (iii) use of pre-immune serum in place of the primary antibody to establish the specificity of labeling; (iv) use of protein-gold complexes without any immunological reagent to show that the sec-

ondary antibody-gold complex labels the antigen because of primary antibody interaction; and (v) testing the antibody on a tissue known to be devoid of the particular antigen.

Showing co-localization of various components within tissues and cells is extremely valuable for understanding their relations. Revealing the proximity of molecules within a cell compartment may also show functionally significant molecular complexes. Because gold particles can be prepared in different sizes, each bound to its own antibody molecule, multiple binding sites can be assayed simultaneously on the same section (see double labeling figure). Combination of gold immunocytochemistry with enzyme cytochemistry, autoradiography, or in situ hybridization can also simultaneously identify several components on the same tissue section (4).



Immunogold approaches. (A) Direct technique. The specific antibody is tagged with gold particles and interacts directly with the antigenic sites. (B) Indirect technique. The primary antibody in its native form is followed by a second antibody tagged to gold particles. (C) Amplified tyramide-CARD technique. The tissue section is incubated with the primary antibody and then with a secondary antibody tagged to biotin. The biotin-tyramide, once activated by peroxidase, induces deposition of avidin-gold complexes on the tissue section. Triangle, antigen; Y shape, immunoglobulin; gold cross, biotin; green X, streptavidin; octagon, horseradish peroxidase; tan cylinder, tyramide; purple cylinder, activated tyramide; circle, gold particle. Figure not drawn to scale.

In a colloidal gold sample, the number of gold particles can be counted, and densities can be correlated to concentrations of antigens in tissues or cell compartments to assess the cellular processing of a particular protein (2). Morphometric evaluations of labelings, combined with time-course experiments, can provide information about intracellular transport pathways and extracellular movement of molecules (2). Combination of techniques can reveal alterations of these functional properties for some cellular compartments, as well as loss of extracellular permeability barriers in experimental and pathological conditions (2).

Technical aspects of cytochemical techniques can limit the sensitivity of a reaction.

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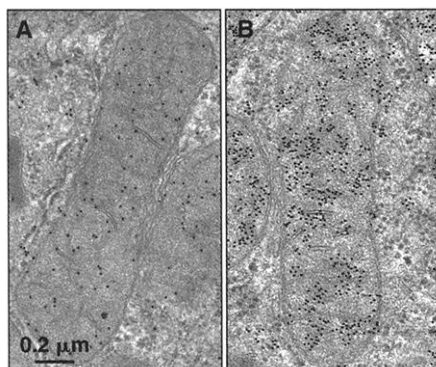
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FEATURING: IMMUNOCYTOCHEMISTRY



Ultrastructural localization of carbamoyl phosphate synthetase. (A) Rat liver hepatocyte. The specific antibody is followed by a secondary antibody-gold complex. The labeling is closely associated to the mitochondrial membranes. (B) CARD-immunogold amplification procedure. The labeling remains restricted to the mitochondria but the intensity of labeling is enhanced.

For instance, some cellular antigens are present in concentrations below current levels of detection. Damage or denaturation of antigens occurs in some tissue preparation techniques. Thus, it is essential to develop procedures that address these issues. Use of tissue processing protocols that minimize or reduce denaturation allows for retention of higher amounts of protein antigenicity. Mild fixatives in low concentration, as well as modified embedding protocols with newly designed resins, can help. Tissue handling approaches that avoid exposing the tissues to harsh chemicals, such as freeze substitution, molecular distillation, and cryo-fixation techniques, allow for detection of very labile antigenic sites through better retention of tissue components in their native conformation (5, 6). Lastly, antigen retrieval techniques, which expose antigens masked by fixation and embedding procedures, are improving tremendously the sensitivity of existing techniques (7).

The table reports the variations in labeling intensity of a given antigen by tissue processing technique. It also shows that ultrastructural preservation should also be taken into consideration when choosing the opti-

mal protocol, because antigen extraction, membrane degradation, and tissue shrinkage and swelling can occur during preparation. A compromise between labeling intensity and structural preservation should dictate the optimal protocol.

Alternatively, techniques that increase the visual signal can be used to boost the sensitivity of immunocytochemistry. The most efficient ones are as follows: (i) Use of antibodies to protein A and protein A-gold complexes, which increase the number of gold particles deposited at the antigenic sites (2). (ii) Ultra-small gold markers such as the 1-nm gold particles or the 1.4-nm Nanogold (Nanoprobes, Yaphank, NY) (8), which because of their small size accumulate in larger numbers at the antigenic site. (iii) The recently introduced catalyzed reporter deposition (CARD)-immunogold approach, which attaches biotinylated tyramide molecules to the antibody-antigen complex site. The biotinylated sites are visualized by interaction with streptavidin-gold (7).

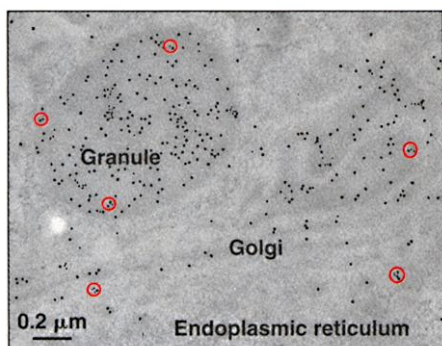
Amplification techniques may increase the amount of signal for a sample several-fold, but they can also decrease resolution because the marker becomes more distant from the original binding site with each additional step. One experiment has shown that the average distance between the exact mitochondrial membrane antigenic site and the position of the gold marker was 8.7 ± 0.69 nm with standard techniques and 11.2 ± 0.66 nm or 13.9 ± 0.55 nm with the use of amplification techniques (7).

EFFECT OF TISSUE PROCESSING ON IMMUNOLABELING

Protocol	Immunolabeling density (gold particles/ $\mu\text{m}^2 \pm \text{SEM}$)		Diameter of secretory granules ($\mu\text{m} \pm \text{SEM}$)
	Secretory granules	Mitochondria (background)	
Glut/Frozen sections	298.48 ± 16.54	6.09 ± 1.52	0.57 ± 0.01
Glut/Epoxy resins	Epon	136.11 ± 3.48	0.60 ± 0.01
	Spurr	75.57 ± 1.84	0.94 ± 0.03
Glut/Glycol Methacrylate resins	GMA 4°C	164.53 ± 7.45	0.80 ± 0.01
	GMA low-acid	525.42 ± 9.29	0.73 ± 0.01
Glut/Lowicryl resins	K4M -20°C	155.18 ± 12.40	0.77 ± 0.01
	HM20 -20°C	355.39 ± 19.82	0.68 ± 0.01
Glut/LR resins	LR White 4°C	133.22 ± 3.93	0.87 ± 0.03
	LR Gold -20°C	165.92 ± 5.05	0.93 ± 0.02
Glut/Unicryl -20°C		169.71 ± 2.35	0.93 ± 0.02
Cryo/MMD/ Paraformaldehyde/ Spurr		585.54 ± 13.02	0.40 ± 0.001
Cryo/MMD/ Paraformaldehyde/ LR White		359.28 ± 9.52	0.44 ± 0.001

Table 1. Glut, 1% glutaraldehyde; cryo, cryofixation; MMD, molecular distillation drying.

Technical improvements in colloidal gold immunocytochemistry have evolved from simply localizing antigens to providing quantitative data about cellular antigens at the molecular level. Information about cellular processing and molecular movements of antigens, their molecular conformation, and orientation within a membrane are now attainable, as are clues to the formation and composition of molecular complexes. As our understanding of cellular processes grows, the role of immunocytochemistry in unraveling molecular mechanisms is sure to increase. Developments in genetic engineering can also be expected to widen the variety of antibodies and pseudoimmune reagents available (9). An excellent example is the genetically engineered protein A/G



Double labeling. Rat pancreatic acinar cell labeled for two proteins, bile-salt-dependent lipase (BSDL) and the chaperon Grp94. BSDL was revealed by 10-nm gold particles. Grp94 was revealed by its specific antibody and the corresponding complex formed by 5-nm gold particles. Both are present in the same cellular compartments. In several instances, large and small gold particles are located near each other, indicating the existence of BSDL-Grp94 molecular complexes (some of which have been circled).

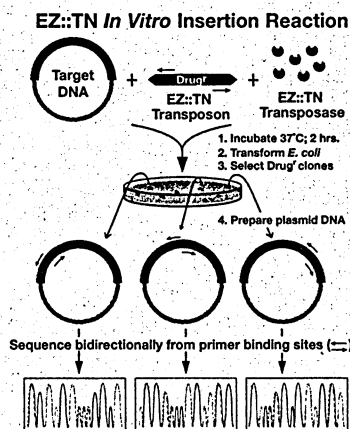
that displays the combined properties of the bacterial protein A and protein G (10). The levels of resolution in immunocytochemistry necessary for studying basic molecular processes in cellular biology have been reached. Future advances should be oriented toward developing tissue preparation methods that render the technique more reliable and free of artifacts.

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