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Immunogold Techniques in Electron Microscopy

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Abstract

Ever since electron microscopy became an important tool in the scientific research, the focus had been mainly on ultrastructural analysis with little success in the development and application of suitable techniques for the localization of macromolecules in cells. The emergence of immunogold techniques in the 1960s managed to fill this gap in serving this function. The aim of this chapter is to equip researchers, postgraduate students, and technicians with essential knowledge to utilize immunogold techniques for ultrastructural investigations in the life sciences. The principles and factors involved have been highlighted to give researchers a quick review of the techniques before embarking on their ultrastructural localization procedures. The advantages and limitations of the four types of immunogold labeling techniques have been discussed.

Keywords: Immunogold labeling, scanning electron microscopy, transmission electron microscopy, double labeling, pre-embedding immunogold labeling, post embedding immunogold labeling

1. Introduction

Immunogold labeling is a very powerful technique for identifying active sites and the presence of biomarkers in the cells. It is probably the only technique that can probe the cells ultrastructurally because of the processes devised to attach gold probes to secondary antibodies and consecutively to primary antibody to reveal the presence of an antigen. A primary antibody is designed to bind onto a specific antigen in the cells. Thus, the gold probe with its excellent electron scattering property is an important element for immunohistochemistry in the electron microscope.

Although gold probe solutions have been in use since the mid-1800s, it was in 1961 that the gold probes became popular to be used as an ultrastructural tracer. A decade later,
Faulk and Taylor were the first to use immunogold labeling technique to identify Salmonella antigens.

Immunogold labeling techniques were initially more applicable for transmission electron microscopy (TEM) and were crucial for low-density markers. However, the emergence of high resolution SEMs has made it possible for the application of these techniques in scanning electron microscopy.

The gold probes became a popular choice over peroxidase or other enzyme marker techniques because of their localized spread around the reaction sites with minimal obscuring of ultrastructural details. Over the years, there has been a rapid development of numerous protocols for immunolabeling, including techniques for detecting multiple antigens within a cell and the selective use of different sizes of gold particles. Unlike the enzyme reaction products, gold probes can be quantified, which could be crucial for comparative studies.

The technique also allows routine EM stains such as uranyl acetate and lead citrate to be used to improve visualization of cellular details without obscuring the site of immunogold reactions.

The two most widely used immunogold labeling techniques are the “pre-embedding” and “post embedding” techniques [1-5].

2. Principles of immunogold labelling

2.1. Uses of the immunogold techniques

Immunogold labeling is being very useful in the localization of target markers in cells and tissues. The high resolution of the technique could provide excellent insight with regards to structure–function relationships in the microenvironment of cells and tissues. It can also be used in the study of protein distribution in cellular and extracellular components. However, success of immunogold labeling techniques is very much dependent on the quality of protein antigen preservation, antigen-primary antibody specificity, and the antibody’s ability to infiltrate cells and tissues. Good handling of samples which involves fixation and dehydration as well as an appropriate selection of embedding media are crucial for immunogold labelling analysis [1, 3].

2.2. Antigen-antibody reactions

Antigen-antibody reaction is an important biochemical interaction between antibodies produced by white blood cells and antigens. It is a basic reaction where the body is protected from complex foreign entities. The antibody binds with the antigen through a specific site called epitope. Immunogold labeling relies solely on this antibody-antigen reaction in order to get intended results. There are two types of antibodies: monoclonal and polyclonal antibody. Monoclonal antibodies are derived from cellular reaction of plasma cells while polyclonal antibodies are derived from the repetition of antigen stimulation. Generally, immunogold
labeling is focused more on indirect patterns (Figure 1) whereby gold conjugated secondary antibodies bind with specific primary antibodies in a microenvironment. This indirect pattern is more favorable than the direct pattern for two reasons: (a) higher density of secondary antibody albeit for longer incubation time and (b) increased sensitivity, since the secondary antibody is able to bind with multiple sites on primary antibody [3, 6-8].

Figure 1. This figure shows the whole process of immunogold labeling starting from blocking the sample to secondary antibody incubation.
2.3. Gold particles as a probe

Gold became the most reliable choice for immunogold labeling due to its large specific surface area, good biocompatibility and high electron density, which offers easy detection, excellent electrical and thermal conductivity in the electron microscope. The distribution and presence of the gold particles do not hinder the viewing of the images at high resolution modes. The size of gold particles used for immunogold labeling varies from 1 to 40µm and can be chosen according to the type of labeling techniques employed [1, 4-6, 9].

Detecting multiple antigens within a cell may require the selective use of different sizes of gold particles.

2.4. Advantages of immunogold techniques

Immunogold labeling is a relatively easy technique to do and suitable for real-time monitoring of ultrastructure and antigens. It can improve the assessment of target markers on cellular microenvironment and provide good intratechnique comparison and reassessment, providing crucial information for further histochemical analysis [1].

3. Factors influencing immunogold labelling

3.1. Fixatives

Fixation of tissues in immunogold labeling should preserve both the structural integrity and antigenic components of the tissue, without being detrimental to the labelling outcome. The commonly used fixative formulations for immunogold labeling are based on glutaraldehyde and paraformaldehyde combination. Glutaraldehyde is a good ultrastructural fixative but has a lower tissue penetration rate. However, the slowness of glutaraldehyde is compensated with the addition of formaldehyde in the fixative formulations. Formaldehyde penetrates tissues at a much more rapid rate. The author uses a mixture of formaldehyde (2-4%) with glutaraldehyde (1%) for rapid stabilizing with lower cross linking and greater structural preservation [1, 7, 10-11].

It is preferable to avoid the use of osmium tetroxide as a post fixative in immunogold processing technique due to the probability of some of the gold particles being superimposed on a heavily osmicated area of the images. However, osmium tetroxide can still be used if the level of contrast in the images obtained is very poor.

3.2. Types of embedding media

Generally there are two types of resin available for use in sample processing. Epoxy resins such as Araldite, Spur's (Figure 2a), and Epon, which are hydrophobic, and acrylic resins such as Lowicryl and LR White (Figure 2b), which can be hydrophobic or hydrophilic. Although epoxy resin gives better ultrastructural preservation and stability, acrylic resins are more suited for immunolabeling because it does not need much heat for polymeriza-
tion and shows better antigen preservation. Acrylic resins can also retain water for better demonstration of protein components, while giving greater accessibility to the antibodies during incubations [1]. LR White resin is the most popular of the acrylic-based resins. It is easier to section and polymerize at moderate temperature (50°C), which is good for antigen preservation, tolerates partial dehydration, retains higher labeling density, and is much less toxic than Lowicryl [3, 12].

![Figure 2. This figure shows two types of embedding media. (a) Bottles of Spur’s resin, (b) LR White, and (c) embedding blocks of Spur’s resin (yellowish brown blocks) and LR White (white yellowish blocks).](image)

3.3. Blocking and reducing agents

Blocking solution, an important step in immunogold labeling, is applied before primary antibody incubation. Blocking solution will close the non-specific reactive sites on tissues and cell surfaces without altering the epitope for antibody binding. It will reduce the non-specific attraction of the primary or secondary antibodies to the tissue components or the resin material. Non-specific reactions or background occur because of the general physical chemical properties of both the specimen and the antibodies [3, 8].

3.4. Controls

Controls are always used to confirm the integrity of specific labeling in samples. The control should be carefully chosen as it plays a crucial role in troubleshooting the contamination of samples or poor performance of an individual reagent in any part of immunogold labeling procedure [3, 7-8]. The types of controls normally used are as follows.

Positive control

A known positive sample tested with same immunogold labeling technique to avoid false negative and test the effectiveness of the labeling procedure.

Negative control

Omitting of the primary antibody step and incubating only in secondary antibody. This is to determine the secondary antibody labeling capacity.
3.5. Gold probes

Over the years, being the choice metal for immunogold labeling, gold particles have been produced to be smaller and smaller, more penetrating, and highly sensitive. The choice of gold particle size depends on the magnification at which the cells are to be studied in the electron microscopy. In principle, smaller gold particles (2 nm or 5 nm) produce a higher labeling intensity and lower steric hindrance due to their greater concentration in solution. Larger particle sizes (10 nm or more) basically reduce the potential labeling intensity due to their sheer size but are more easily seen at lower magnifications [7, 13]. Detecting multiple antigens within a cell may require the selective use of different sizes of gold particles.

3.6. Grids

The three types of grids commonly used for electron microscopy are copper grids (Figure 3a), gold grids (Figure 3b), and nickel grids (Figure 3c). For immunogold labeling, the buffers used can influence the choice of grids. Nickel grids can be a good choice for immunogold labeling for its stability and not being affected by chemical oxidation. However, handling nickel grids can be difficult due to its magnetic properties. Gold grids are stable, non-magnetic, and also a good choice for immunogold labeling but expensive. Copper grids are not suitable for immunogold labeling because Tris Buffer Saline (TBS) Tween buffer react with copper grids. A support film such as formvar, pioloform, or collodion coated on the grid may be necessary to hold the sections firmly during the immunogold labeling process [14].

Figure 3. This figure shows three types of grids: (a) copper grids, (b) gold grids, and (c) nickel grids.

3.7. Temperature and pH

The temperature and pH of reagents could influence the outcome of immunogold labeling. In most of the protocols, buffers and reagents are kept at low alkaline conditions to ensure that the gold probes could combine firmly with the target protein. This is due to the electrostatic reaction between negative charges of the colloidal gold with the positive charges of the target protein. Generally, the temperature of section incubations is kept at ambient room temperature around 16–20°C to avoid aggregation of the gold particles. The temperature of the sections can affect the stabilization of gold conjugates. Aggregation of gold conjugates can occur due to the repulsive energy forces that separate gold conjugates being lesser than the primary attraction forces (van der Waals forces). This phenomenon will eventually cause the aggregation of gold conjugates [10, 15].
4. Immunogold labelling methods

4.1. Pre-embedding immunogold technique

4.1.1. General concepts

The pre-embedding approach is to perform the immunolabeling on the sample before embedding and the sectioning process.

4.1.2. Materials

1. McDowell-Trump fixative
2. Phosphate Buffer, Sorensen 0.2M pH7.2
3. Phosphate Buffer, Sorensen 0.2M pH7.6
4. TBS Tween (0.05M TBS, 0.05% Tween 20, pH7.6)
5. Sucrose solution (0.2 M)
6. London White Resin Medium Grade
7. Ultra V Block Serum free
8. Primary antibodies
9. Secondary antibody
10. 1% toluidine blue with sodium borax
11. Ultra-antibody diluent

4.1.3. Standard protocol

Initial sample preparation steps are different for animal/human tissues and cultured cells.

a. Animal and human tissue

Cut the tissue into small pieces about 1 mm$^3$ and place in vial containing McDowell-Trump fixative for at least 24 hours.

b. Cultured cells

[Note: All centrifugation in this technique should be at 1,000–2,000g for 15 minutes.]

i. Use 6 well plates to grow adherent cells. (For suspension cells, you can proceed directly to fixation step).

ii. Incubate the cells (inside well) with 3 ml of fixative (for each well) for 10 minutes. Discard the fixative and use Trypsin to detach the cells. [Leave the plate in incubator with temperature 37°C for 10 minutes – depends on type of cell line used.]
iii. Neutralize the Trypsin with culture medium and transfer the solution into 15 ml Falcon Tube.

iv. Centrifuge the sample and discard the supernatant. [From now on, the steps are similar for adherent and suspension cells]

v. Resuspend the pellet with McDowell Trump fixative prepared in 0.1M phosphate buffer (pH 7.2) for at least 15 minutes.

vi. Centrifuge the resuspended sample. Discard the supernatant [transfer the pellet to an Eppendorf tube at this stage, if needed]. Resuspend the pellet in 0.1M phosphate buffer.

vii. Centrifuge the resuspended sample. Discard the supernatant and resuspend the pellet in 0.1M phosphate buffer.

viii. Centrifuge the resuspended sample, discard the supernatant and place this tube containing a pellet of fixed cells in a water bath at 45°C for about 15–30 minutes depending on the amount of sample.

ix. Prepare a 2% solution of agarose by dissolving the agarose in boiling distilled water. Pour the solution into a test tube while it is still molten, and place the tube in the water bath at 45°C. At this temperature the agarose remains liquid.

x. After the temperature of both the agarose and the pellet have equilibrated to 45°C, transfer a small drop of the agarose to the tube containing the pellet of cells (with a warm pipette), and stir up the pellet, just enough to break the pellet into small blocks, and to suspend these small blocks in the agarose.

xi. Immediately pour the agarose with the suspended pellet blocks on to a glass microscope slide.

xii. After the agarose has set (1–2 minutes), cut the solidified agarose containing the cells into small cubes, about 1 mm³, with a sharp razor blade and place them in a vial containing 0.1M phosphate buffer.

**Procedure for immunolabeling**

The animal/human tissue will be in small pieces while cultured cells will be held together in 2% agarose in the form of small cubes. The following steps will be similar for both tissues and cultured cells in agarose cubes.

1. Rinse the sample in 0.1M phosphate buffer for 10 minutes (3X).
2. Incubate the sample in 0.2 M sucrose solution for 15 minutes (2X).
3. Rinse the sample briefly in 0.1M phosphate buffer.
4. Incubate the sample in blocking reagents for 30 minutes at room temperature.
5. After 30 minutes, incubate the sample in primary antibody for two hours at room temperature. [Dilutions of 1:10 were used for primary antibody incubation.]
6. Wash the sample in TBS-Tween 20 buffer 5 times, for 5 minutes for each wash. After the washing step, incubate the sample in a secondary antibody with gold conjugates for one hour at room temperature with a dilution of 1:50.

7. Wash the sample again in TBS-Tween 20 solution 5 times, for 5 minutes for each wash.

8. Prepare to incubate the sample for following dehydration steps. *[Follow the steps carefully.]*
   a. 70% ethanol for 30 minutes (2X)
   b. London White Resin and 70% ethanol mixture (2:1) for 1 hour
   c. Two changes of pure London White Resin (1 hour each)
   d. Fresh London White Resin change overnight at room temperature

9. Take out the sample from vial and drop it at the bottom of BEEM capsules and fill it up with London White Resin to the brim.

10. Let the capsule to polymerize at 50°C for 72 hours.

11. Semi-thin and ultrathin sectioning of the resin-embedded samples is performed in the ultramicrotome using glass or diamond knives.

12. Collect ultrathin sections of about 60-70nm in thickness by using 200-300 mesh nickel grids. Gray, silver, or gold sections were collected.

13. Let the grids with the sections to dry.

14. Rinse the grids for 2 minutes each in two large droplets of TBS Tween 20.

15. The grids can then be stained in uranyl acetate and lead citrate for 10 and 5 minutes, respectively. If the sections stain heavily, the times can be varied. In some instances, one of the stains can be omitted to provide acceptable “gold particles to tissue” contrast levels.

4.1.4. Precautions

It is preferable to use samples of smaller size (about 1 mm³) for pre-embedding immunogold techniques, to achieve greater sensitivity and better ultrastructural preservation.

4.1.5. Troubleshooting

1. Good permeabilization of gold probes is crucial for this technique. The penetration rate can be increased by using the smallest gold particles possible, while being mindful that smaller particles would require higher magnifications in the TEM.

4.2. Post embedding immunogold technique

4.2.1. General concepts

The post embedding labeling technique is the more widely used technique in comparison to the pre-embedding technique. For post embedding, samples are first fixed, embedded, and
sectioned before being subjected to immunolabeling. The indirect labelling method is generally preferred for this technique whereby the primary antibody is unlabeled and the gold is conjugated to a secondary antibody.

4.2.2. Materials

1. McDowell-Trump fixative
2. Phosphate Buffer, Sorensen 0.2M pH7.2
3. Phosphate Buffer, Sorensen 0.2M pH7.6
4. TBS Tween (0.05M TBS, 0.05% Tween 20, pH7.6)
5. Sucrose solution (0.2 M)
6. London White Resin Medium Grade
7. Ultra V Block Serum free
8. Primary antibodies
9. Secondary antibody
10. 1% toluidine blue with sodium borax
11. Ultra-antibody diluent

4.2.3. Standard protocol

Please refer to 4.1.3 Section A and B [for initial sample preparation for animal/human tissue and cultured cells].

Procedure

1. Rinse the sample in 0.1M phosphate buffer for 10 minutes (3X).
2. Incubate the sample in 0.2 M sucrose solution for 15 minutes (2X).
3. Rinse the sample briefly in 0.1M phosphate buffer.
4. Prepare to incubate the sample for following dehydration steps. [Follow the steps carefully.]
   a. 70% ethanol for 30 minutes (2X)
   b. London White Resin and 70% ethanol mixture (2:1) for 1 hour
   c. Two changes of pure London White Resin (1 hour each)
   d. Fresh London White Resin change overnight at room temperature
5. Take out the sample from vial and drop it at the bottom of BEEM capsules and fill up with London White Resin to the brim.
6. Let the capsule to polymerize at 50°C for 72 hours.

7. Semi-thin and ultrathin sectioning of the resin-embedded samples is performed in the ultramicrotome using glass or diamond knives.

8. Collect ultrathin sections of about 60–70nm in thickness by using 200–300 mesh nickel grids.

9. Let the grids with the sections to dry.

10. Rinse the grids for 2 minutes each in two large droplets of TBS Tween 20.

11. Incubate the grids in blocking reagents for 30 minutes at room temperature.

12. After 30 minutes, incubate the sample in primary antibody for 2 hours at room temperature. [Dilutions of 1:10 were used for primary antibody incubation.]

13. Wash the sample in TBS-Tween 20 buffer 5 times, for 5 minutes each time. After the washing step, incubate the sample in a secondary antibody with gold conjugates for 1 hour at room temperature with a dilution of 1:50.

14. Wash the sample again in TBS Tween 20 solution 5 times, for 5 minutes for each wash.

15. The grids can then be stained in uranyl acetate and lead citrate for 10 and 5 minutes, respectively. If the sections stain heavily, the times can be varied. In some instances, one of the stains can be omitted to provide acceptable “gold particles to tissue” contrast levels.

4.2.4. Precautions

1. Resin preparation should be performed carefully with the 70% ethanol added slowly, drop by drop to the London White Resin and gently shaken to avoid the resin mixture becoming milky (cloudy).

2. Sections tend to overlap during the embedding process so it is important to maintain the tissue as flat as possible during the embedding procedure, in order to obtain uniformly labelled sections on grid.

3. It may be preferable to omit osmium tetroxide to preserve better antigenicity.

4.2.5. Troubleshooting

Gold conjugates tend to aggregate, making it difficult for the localization of markers. Generally, the temperature of section incubations is kept at ambient room temperature around 16–20°C to avoid aggregation of the gold particles. The temperature of the sections can affect the stabilization of gold conjugates. Aggregation of gold conjugates can occur due to the repulsive energy forces that separate gold conjugates being lesser than the primary attraction force (van de Waals force). This phenomenon will eventually cause the aggregation of gold conjugates [10, 15].
4.3. Double immunogold labeling technique

4.3.1. General concepts

Double immunogold labeling is a technique that reveals the distribution of multiple antigens simultaneously on the same sample section. This is made possible with the availability of different sizes of gold probes. Post embedding, labeling is the preferred method for this technique.

4.3.2. Materials

1. McDowell-Trump fixative
2. Phosphate Buffer, Sorensen 0.2M pH7.2
3. Phosphate Buffer, Sorensen 0.2M pH7.6
4. TBS Tween (0.05M TBS, 0.05% Tween 20, pH7.6)
5. Sucrose solution (0.2 M)
6. London White Resin Medium Grade
7. Ultra V Block Serum free
8. Primary antibodies
9. Secondary antibody
10. 1% toluidine blue with sodium borax
11. Ultra-antibody diluent

4.3.3. Standard protocol

Please refer to 4.1.3 for initial sample preparation and 4.2.3 (Step 1 until 12) for sample preparation and continue with following steps:

1. After incubating the sample in blocking reagent for 30 minutes, incubate the grids in a mixture of two primary antibodies for 2 hours at room temperature.

2. Wash the sample in TBS-Tween 20 buffer 5 times for 5 minutes each. After the washing step, incubate the sample in a mixture of secondary antibody with gold conjugates (different sizes) for 1 hour at room temperature with a dilution of 1:50.

3. Wash the sample again in TBS Tween 20 solution 5 times for 5 minutes of wash each.

4. The grids can then be stained in uranyl acetate and lead citrate for 10 and 5 minutes, respectively. If the sections stain heavily, the times can be varied. In some instances, one of the stains can be omitted to provide acceptable “gold particles to tissue” contrast levels.
4.3.4. Precautions

1. Ensure that the primary and secondary antibodies are compatible with each other.
2. Ensure that the size of gold conjugates for each antigen can be differentiated during observation in the TEM. Do not choose gold probes that are similar in size.

4.4. Immunogold technique for scanning electron microscopy

4.4.1. General concepts

Immunogold labeling in the SEM allows the localization of antigens on the surface areas of samples. The technique can also be performed on internal surfaces of samples after the removal of the external layers to expose the targeted regions. Some prior knowledge of the general areas where the antigen is likely to be located could be useful in obtaining specific results.

4.4.2. Materials

1. McDowell-Trump fixative
2. Phosphate buffer, Sorensen
3. 1% osmium tetroxide
4. Graded alcohols
5. Hexamethyldisilazane (HMDS)
6. Ultra V Block Serum free
7. Primary antibodies
8. Secondary antibody
9. Ultra-antibody diluent

4.4.3. Standard protocol

Initial sample preparation

a. Animal and human tissue

Fix the animal/human tissue in McDowell-Trump fixative prepared in 0.1M phosphate buffer (PBS) (pH 7.2) at 4°C for 2–24 hours.

b. Cultured cells

i. Prepare a solution of 0.1% poly-L-lysine in phosphate-buffered saline (pH 7.2).

ii. Place a drop of the poly-L-lysine solution on a coverslip and place it in a 6-well plate for about 1 hour.
iii. Rinse the poly-L-lysine coated coverslip with distilled water and fill the well with suitable medium for adherent cells to grow. Let the cells attain 80–90% confluency.

iv. Carefully discard the medium from the well and immediately fill the well with fixative to replace the medium. (Do not let it dry in between the step). Leave it alone for about 10 minutes.

The following steps are similar for tissues and cells grown on coverslip in the 6-well plate.

1. Wash tissue/coverslip with cells (SAMPLE) in PBS (3 x 10 minutes)
2. Incubate the SAMPLE in blocking reagents for 30 minutes at room temperature.
3. Incubate the SAMPLE in primary antibody for 2 hours at room temperature.
4. Wash the SAMPLE in TBS-Tween 20 buffer 5 times, for 5 minutes each time.
5. Incubate SAMPLE in a secondary antibody with gold conjugates for 1 hour at room temperature with a dilution of 1:50.
6. Wash the SAMPLE in TBS Tween 20 solution 5 times, for 5 minutes each time.
7. Wash in distilled water (2 x 10 minutes).
8. Dehydrate as follows:

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>35% ethanol</td>
<td>15 minutes</td>
</tr>
<tr>
<td>50% ethanol</td>
<td>15 minutes</td>
</tr>
<tr>
<td>75% ethanol</td>
<td>15 minutes</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>15 minutes (x2)</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>20 minutes (x3)</td>
</tr>
</tbody>
</table>

9. The SAMPLE can be subjected to either the “air drying protocol” or the critical point drying (CPD) protocol for drying.

**HMDS method**

Immerse the SAMPLE with cells in 1–2 ml of hexamethyldisilazane (HMDS) for 10 minutes.

Decant the HMDS and leave the SAMPLE in a desiccator to air-dry at room temperature.

**CPD method**

Transfer the SAMPLE into the CPD specimen holder. The specimen holder should contain (or be immersed in) enough acetone to cover the SAMPLE (15–30 minutes).

Critical point drying (refer to SEM preparation chapter).

10. The dried SAMPLE is then mounted on to a SEM specimen stub with a double-sided sticky tape (or silver paint).

11. View in the SEM
4.4.4. Precautions

1. Times recommended are for small pieces of tissues (smaller than 1–2 mm³). For larger pieces, times may have to be varied accordingly.

2. Buffer: normally 0.1M of pH 7.2, but requirements may vary for different tissues. Refer to published papers.

3. Never let the tissues to dry at any stage (till the samples are immersed in the HMDS).

4. ADVANTAGES AND LIMITATIONS OF THE IMMUNOGOLD TECHNIQUES

Pre-embedding immunogold labeling

Advantages
- All antigenic site throughout the specimen are potentially accessible for staining.
- Has superior sensitivity for the localization of sparse antigens.
- Produces more discrete and nondiffusible markers indicating the precise subcellular localization of antigens which are also readily quantified.

Limitations
- The labelling procedures require extended incubations in buffers to increase the penetration of probes. As a result, preservation of adequate ultrastructure is often a problem.
- Potential sources of false-positive and false-negative results resulting from
  - Cross-reaction of the primary antibody with unknown proteins
  - Cross-reaction of secondary antibodies with inappropriate species
  - Nonspecific immunolabeling
  - Erroneous transport of tract-tracing agents

Post-embedding immunogold labeling

Advantages
- Since immunostaining is done after the sample has been fixed, embedded, and sectioned, these preparation steps will preserve antigenicity and reduce problems regarding ultrastructural preservation.
- Better reactivity due to the contact between the antibodies and internal antigens exposed at the surface of thin sections obtained from resin embedded tissues.
- Better visualization of antigenic sites with high resolution allows good quantitative evaluations of labeling intensity and morphometrical determinations.

Limitations
- Restriction of antibody staining to epitopes present on the section surface.
• Antibodies cannot penetrate into the resin, and consequently only the antigens that are exposed at thin section surfaces can be labelled.

• Antigens could be affected by fixatives, solvents, resins, and heat during polymerization, thus compromising antigenicity.

**Double immunogold labeling**

**Advantages**

• Ability to be bound to several molecules makes marker being extremely versatile.

• Provide better understanding on relationship and localization of different types of molecules.

**Limitations**

• Chances for section contamination are higher than single labeling procedure.

• Increase in background noise.

**Immunogold with scanning electron microscopy (SEM)**

**Advantages**

• Reveals the surface immunoreactions on a sample that cannot be otherwise resolved with light microscopy techniques.

• A good depth of field in the SEM provides additional information on undulated surfaces.

• Ability to specifically mark and examine one structure and be able to relate with other structures for better understanding.

**Limitations**

• Certain information other than the lateral position of gold particles in the backscatter image is lost.

• Most biological samples need to be dehydrated before being placed in the SEM. This can distort cellular features or create artifacts.

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