



Immuno-gold Techniques in Biomedical Sciences

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Principle of Immuno-gold Staining

Antigen–antibody reactions are used widely in light and electron microscopy analyses. Among applications of light microscopy, immunocytochemistry is used in studies of cellular contents as a diagnostic tool, whereas electron microscopy is predominantly used to study locations of antigens in cellular organelles with high-resolution data. In these techniques, antibodies recognize antigens, such as proteins, carbohydrates, and lipids, that have several antibody binding sites comprising specific amino acids or polysaccharides in localized regions. For successful immuno-labeling, researchers require very specific antibodies, high-performance electron-dense probes, and specific preparation techniques that preserve the antigenicity and morphology of specimens. As antibody, both polyclonal and monoclonal antibodies can be used. Polyclonal antibodies allow strong aldehyde fixation, but

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monoclonal antibodies require lighter fixation because they are highly specific to few amino acids of the antigen. Colloidal gold is the most used probe (Ferguson et al. 1998; Singer 1959), because it provides electron density that is clear (Bendayan et al. 1987) under electron microscopy. Colloidal gold particles are 1–20 nm in diameter (10–20 nm is most common) and allow multiple labeling. Whereas large probes can be used for low magnification images, smaller probes are superior in high magnification applications. Because smaller colloidal gold particles have reduced hindrance and increased diffusion rates, particle sizes are selected on the basis of labeling intensities, efficiency, and magnification of imaging. Depending on the probe, several methods have been used for several decades (Fig. 1). In direct methods, primary antibodies are linked to ferritin or gold particles and directly bind to the antigen and can be observed immediately. These techniques allow produce the highest resolution, with a maximum of 1 gold particle per antigen. Due to their specificity, these techniques are also suited for surface labeling of sections but require well-characterized primary reagents. The most commonly used technique is a two-step method in which immuno-gold conjugates for protein A/G and secondary antibodies are used. Gold particles of various sizes are conjugated to protein A (Bendayan and Zollinger 1983), protein G, and secondary antibodies. When protein A/G is used, one gold probe is bound per primary antibody, producing the highest resolution of two-step labeling, and providing a universally applicable technique for many antibody subclasses. In contrast, when secondary antibodies are used, more gold probes can be bound per primary antibody, facilitating reactions with primary antibody fragments and

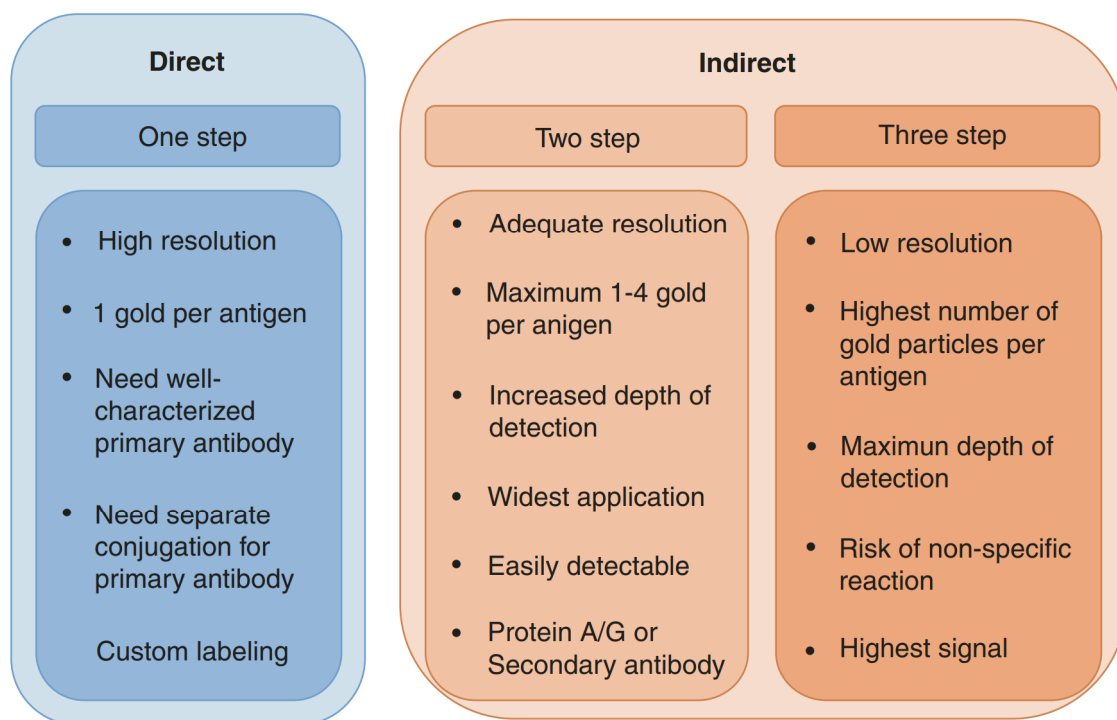


Fig. 1 Detection methods for immuno-gold techniques. For high resolution, direct method is best. And for easy detect, indirect methods is better techniques

detection at low magnification. Secondary antibodies bind more specifically than protein A/G. In these manipulations, primary antibodies are reacted with antigen, and gold particle-conjugated secondary antibodies are used to detect primary antibodies. This is the most commonly used immuno-gold staining technique. In principle, 1–4 gold particles can be bound per antigen, resulting in advantages of adequate resolution and wide application. Two-step methods are compared briefly in Fig. 1. Other indirect immuno-electron microscopy methods involve three-step processing using horseradish peroxidase (HRP) tagged antibodies, which react with 3,3'-Diaminobenzidine (DAB) signals to produce electron-dense signals. Gold can then be used to produce dot signals in immuno-electron microscopy analyses. The resolution of these methods is better suited for light microscopy, and their advantages include the highest numbers of gold particles per antigen, maximum depths of detection, and the highest signal intensities. But the increased risk of unintended reactions is a clear disadvantage. Commonly, high specificity of reactions, and hence low background signals, are the most important parameters for successful immuno-gold labeling. Because preservation of antigen and ultrastructure is different on basis of sample preparation process such as fixation, embedding media, the order of antigen–antibody reaction, and so on, researchers have to decide which technique is best for their purpose. In principle, antigens are stabilized by fixation during preparation for exposure to the antigen, antigen–antibody reactions are performed before (pre-embedding) or after plastic embedding (post-embedding), and probes are visualized for specific antigen–antibody reactions (Fig. 2). In addition, nonspecific binding sites on samples can be blocked by 1% gelatin, free aldehydes can be blocked by 0.1 M glycine, and nonspecific sites are generally blocked by 1% bovine serum albumin. Reagents and their concentrations vary depending on specimens and are generally identified using trial and error. Procedures for reducing background will be discussed in the troubleshooting section.

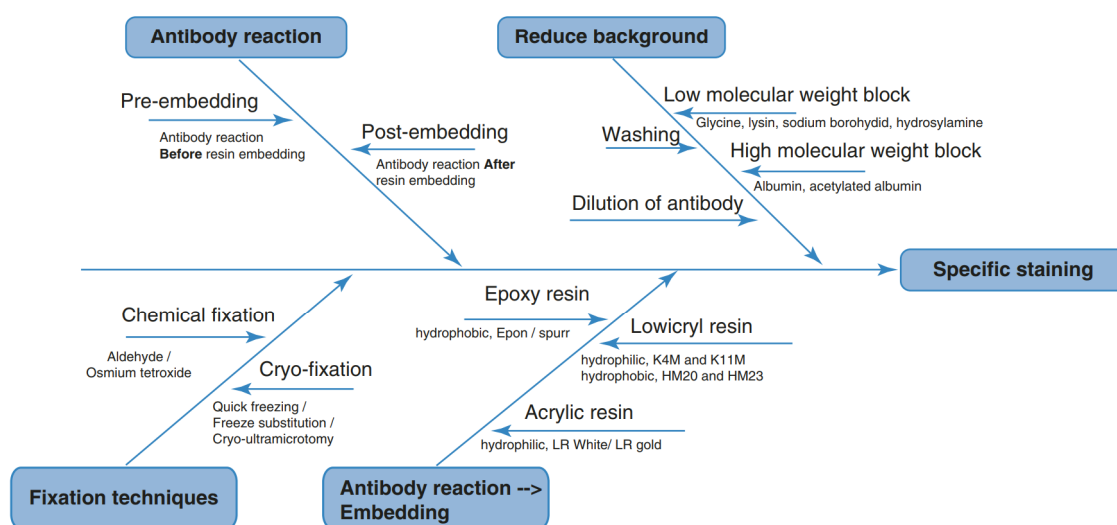


Fig. 2 Considerations for immuno-gold techniques. Techniques for fixation, reduction of background, antibody reaction, and embedding should be selected for specific purpose

Sample Preparation for Antigen Preservation

When preparing samples for antibody labeling, it is important to consider whether ultrastructure or antigen preservation is more important and whether the target is located in intracellular, extracellular, or membrane compartments. Preservation of antigen and ultrastructure is achieved using sample preparation techniques including various fixatives, antigen retrieval followed by antibody binding, and selection of appropriate embedding media. Preparation methods can be divided into pre- or post-embedding techniques depending on when the antibody reacts with antigen, as shown in Fig. 3. In pre-embedding techniques, antigen–antibody reactions are performed before resin embedding of samples, and ultrathin sections are then cut and observed under electron microscopy. In post-embedding methods, antigen–antibody reactions are performed after resin embedding and ultrathin sectioning.

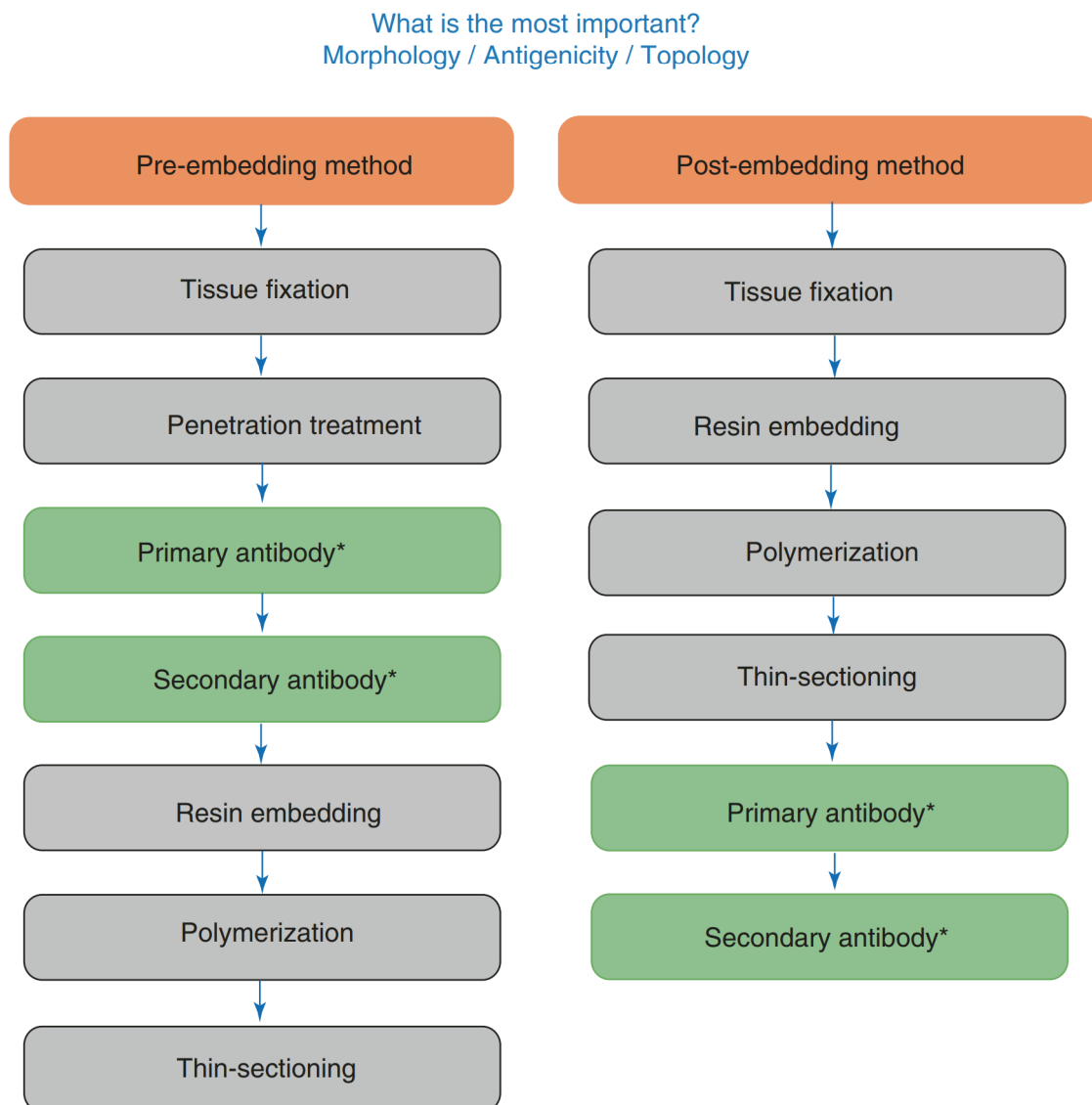


Fig. 3 Schematic workflow of pre- and post-embedding labeling procedures. Pre-embedding favors antigen preservation and post-embedding favors sample morphology

Therefore, pre-embedding methods usually have better sensitivity for antigen–antibody reactions, although weak penetration of antibodies can be a disadvantage. In contrast, post-embedding methods (Roth 1986) give better penetration of antibodies but poor preservation of antigen. These advantages and disadvantages are explained in detail in the following sections.

Pre-embedding Method

Pre-embedding immuno-gold methods have been used for a long time, but their applications are limited due to invasiveness. In general, only surface antigens of samples can be labeled using pre-embedding methods, but low concentrations of detergent or freezing/thawing can help to expose cytoplasmic antigens. Therefore, it is not easy to optimize the balance between morphology and antigen labeling. Pre-embedding has the disadvantage of weak antibody penetration but has the following advantages: First, antigens that may be destroyed during dehydration and embedding by post-embedding methods can be preserved. Therefore, pre-embedding methods are desirable when antigens are very sensitive or are present at low concentrations. Second, the use of osmium tetroxide (OsO_4) after antigen–antibody reactions can be advantageous for membrane fixation in pre-embedding method. Because most antigens are very sensitive to OsO_4 , the use of OsO_4 must be avoided or minimized in post-embedding methods. Third, pre-embedding can be applied to correlative light and electron microscopy studies (Polishchuk and Polishchuk 2019) using DAB or green fluorescent protein (GFP). Because advanced techniques show increased resolution from combinations of cryo-techniques, these will be developed with other advanced techniques for the preparation of samples and specific tags (Nisman et al. 2004).

Pre-embedding Method with DAB Reactions and Plastic Embedding

Avidin-biotin-peroxidase reactions (Hsu et al. 1981), DAB reactions, post-fixation, dehydration, plastic embedding, and ultrathin sectioning are performed after antigen–antibody reactions and prior to observations using electron microscopy (Polishchuk and Polishchuk 2019).

1. Perfuse samples of animal tissue with 4% paraformaldehyde and 0.1% glutaraldehyde.
Caution: Aldehyde fumes are toxic. All work should be conducted in a ventilated fume hood.
All fixatives must be used on the day they are made up.
 - If the sample is not animal tissue, omit perfusion.
2. Make 100 μm thick sections using a vibratome (Piekut and Casey 1983).
3. Fix samples with 4% PFA and 0.1% glutaraldehyde.

4. Wash samples with phosphate buffer saline (PBS).
5. Use 2.3M sucrose for cryo-protection.
6. For antibody penetration, freeze and thaw by incubating for 1 min in liquid nitrogen.
 - If needed, repeat this step 3–4 times.
7. Wash samples with PBS.
8. Quench free aldehyde by incubating samples in PBS containing 1% NaBH₄ for 30 min.
9. Wash samples with PBS
10. To increase cell permeability, incubate samples in 3% H₂O₂.
11. Wash samples with PBS.
12. Block nonspecific antibody binding using 10% normal serum.
13. Incubate samples with primary antibody.
 - Aliquots of antibody must be stored at –20 °C or lower, and diluted solutions must be stored at 4 °C. Because repeated freezing and thawing destroys the antibody, it should be avoided as much as possible.
14. Wash samples with PBS.
15. Block specific antibody binding sites with 2% normal serum for 10 min.
16. Incubate samples with biotinylated secondary antibody.
17. Wash samples with PBS.
18. Incubate samples with avidin for 1 h.
19. Wash samples with PBS.
20. React samples with Ni-DAB.
 - Prepare Ni-DAB reaction solution by mixing 4.6 ml of PB, 50 µl of 0.4% NH₄Cl, 50 µl of 20% D-glucose, 200 µl of 1% Ni ammonium sulfate, 100 µl of DAB (2.5 mg) and glucose oxidase at 1-µl/ml.
21. Wash sample with PBS
22. Post-fix samples with 2.5% glutaraldehyde and 1% OsO₄ in 0.1-M PB for 30 min.
23. Wash sample with PBS
24. Incubate samples in a solution of 3:1 acetone:epoxy embedding mixture for 30 min.
25. Remove the medium and add 1:1 acetone:embedding mixture medium and then incubate for 30 min.
26. Remove the medium and add 1:3 acetone:embedding mixture medium and then incubate for 30 min.
27. Remove the medium and add 100% embedding mixture and incubate overnight at room temperature.
28. Embed samples in pure epoxy embedding mixture and incubate for 24 h at 65 °C.
29. Cut sections of 50–70-nm thickness using an ultramicrotome, and stain with 2% uranyl acetate for observation using tandem electron microscopy (TEM).
 - If cells have been grown on glass coverslips, coverslips can be removed by dropping into liquid nitrogen after the resin is polymerized.

Pre-embedding Method with GFP, Immuno-gold, and Plastic Embedding

In this procedure, sections are imaged under a confocal microscope and locations of GFP fluorescent signals are recorded by labeling with immuno-gold conjugated GFP antibody and analyzing under electron microscopy.

1. Fix sample with 2% paraformaldehyde and 0.1–2% glutaraldehyde in PB for 1 h at room temperature.
2. Wash sample three times in PB buffer for 20 min each.
3. Identify GFP fluorescent signals in sections using confocal microscopy.
4. Wash sample with PB buffer for 20 min.
5. Quench free aldehyde with 50 mM glycine in PB for 20 min.
6. Wash sample with PB buffer for 20 min.
7. Immerse samples in 15% sucrose in 0.1 M PB for 1 h.
8. Immerse samples in 30% sucrose in 0.1 M PB for 2 h.
9. Freeze and thaw samples by incubating for 1 min in liquid nitrogen. Repeat the freeze and thaw if necessary.
10. Wash samples with PB.
11. Wash samples with Tris-buffered saline (TBS).
12. Block nonspecific binding in TBS containing 10% normal serum and 1% Fish skin gelatin (FSG) for 1 h.
13. Incubate samples with primary anti-GFP antibody in TBS containing 1% normal serum, 0.1% FSG, 0.05%, and NaN_3 at 4 °C.
14. Wash samples with TBS.
15. Incubate samples with secondary antibody-conjugated nanogold in TBS containing 1% bovine serum albumin (BSA), 0.1% FSG, and 0.05% NaN_3 at 4 °C.
16. Wash samples with PBS.
17. Fix samples with 1% glutaraldehyde in PBS.
18. Wash samples with PBS.
19. Enhance nanogold signals using a nanoprobe enhancing kit.

Pre-embedding Method with GFP, DAB, and Cryo-techniques

Recently, pre-embedding methods with cryo-techniques, including cryo-fixation, have been applied to genetically engineered samples. In these studies, samples are typically frozen under high pressure and are later processed in the vitreous state directly using a focused ion beam (FIB) system or cryo-ultramicrotomy. After observation of GFP signals under cryo-light microscopy, cryo-tomography can then be applied using a Cryo-TEM. This workflow includes vitreous freezing, cryo-fluorescence microscopy, targeted sample sectioning using FIB or cryo-ultramicrotomy, and high-resolution cryo-electron tomography (Cryo-ET). The ensuing technique allows nanometer-scale 3D modeling of biological structures,

such as native proteins, intact cells, lipid rafts, and cellular spaces, in their native cellular contexts. These techniques are, however, limited by section thickness and radiation damage and can require the use of a set of equipments, including cryo-light microscopy, Cryo-TEM, cryo-FIB, and cryo-ultramicrotome instruments.

In studies of cultured cells, specific processes are employed for the preparation of grids and the selection of suitable cells. The best grids are made of gold, because other metals, such as copper and nickel, are cytotoxic and are hence unsuitable for cell cultures. Grid mesh sizes of 200 and 300 mesh are commonly used, and the mesh number should not be >300 Mesh. Although thinner but mesh maintains a sufficient transparent space between the grid bars when specimens are tilted to high angles, mesh numbers lower than 100 tend to break more frequently during handling and vitrification of specimens. As support films, formvar and carbon are most commonly used. Carbon better facilitates evaporation from both sides, leading to optimal strength, but is disadvantaged by deteriorating resolution with greater thickness. To address this disadvantage, holey carbon films have been developed. The use of these films was supported by reduced background noise during imaging of cells through the holes. Under these conditions, increased hydrophilicity of the grid also improves surface contact with the medium during cell culture. In cryo-electron tomography of whole cells, cell types such as fibroblasts and neurons with thin shapes have been used most frequently. Limits of thickness that can be imaged by TEM depend on the voltages used. In most cases, about 400 nm is considered a maximum thickness for imaging of frozen-hydrated specimens using TEM at 200 kV. However, due to limited penetration of the beam, cubic and columnar shape cells and structures near cell nuclei cannot be imaged using these techniques without sectioning. In other words, bigger cells require sectioning using cryo-ultramicrotomy or FIB.

1. Prepare formvar-coated grids using 1% solutions of chloroform or dichloromethane. Dip glass slides into the solution, dry, and then float formvar layers on water. Subsequently, place grids onto the film layer and retrieve them with a glass slide. Evaporate carbon on both grid sides. Finally, glow discharge the formvar carbon-coated grids after placing on glass slides with the carbon side facing up for 1 min.
2. Sterilize EM grids before cell growth by UV radiation for 15 min. Place cells on EM grids on the bottom of the culture dish overnight using standard cell culture protocol.
3. Use light microscopy to check for proper cell attachment to the grids, confluency, and spreading. When few cells are attached to the grid, increase confluence over further cell culture times.
4. Take images of cells with GFP signals.
5. Treat samples with low concentrations of saponin to enhance penetration of antibody.
 - This procedure can be replaced by freeze-thaw methods (See protocol 1.2)
6. Use anti-GFP antibody for pre-embedding labeling using the Fab fragments of IgG conjugated with ultra-small 1.4 nm gold particles (Hainfeld and Furuya 1992) (See protocol 1.1).

7. Vitrify samples using a plunge freezer, such as a FEI vitrobot or a homemade plunge freezer. Control temperature and humidity before and during blotting at 37 °C and 100% humidity (blot time, 1–2 s; wait time, 0 s; drain time, 0 s; blot force, 0–25; and blot total, 1s).
8. Immerse grids in liquid ethane on liquid nitrogen.
9. Use cryo-ultramicrotomy or cryo-FIB for sectioning if necessary.
10. Transfer vitrified grid from the storage box to a Gatan cryo-electron tomography holder, that has been baked and pumped to low pressure. Subsequently, transfer samples from the cryo station into the cryo-electron microscope chamber.
11. Acquire tilt series under low-dose conditions ($<100 \text{ E}/\text{Å}^2$ cumulative dose). Record images at 1° or 2° tilt increasements between $\pm 90^\circ$ or $\pm 60^\circ$.

Post-embedding Methods

In post-embedding methods, immuno-staining of ultrathin sections is performed on grids after fixation, dehydration, and plastic embedding of samples. Various fixation and embedding techniques (Fig. 4) are used to preserve antigen and ultrastructure. Cryo-fixation, low-temperature embedding using Lowicryl (Acetarin et al. 1986; Roth et al. 1981), room temperature embedding using LR white (Mandry et al. 1993), and cryo-ultramicrotomy (Bos et al. 2011) preserve antigens well, but membrane contrast is better achieved after chemical fixation and epoxy embedding.

Post-Embedding Method with Chemical Fixative, Epon Resin or Lowicryl Embedding

Post-embedding with chemical fixation is typically an excellent starting point for most projects. As embedding media, Lowicryl reportedly enhances immuno-labeling (Valentino et al. 1985) and is compatible with post-embedding techniques.

Chemical Fixation Epoxy Embedding

1. Fix samples with 1% glutaraldehyde and 1% PFA in 0.1 M sodium cacodylate solution (pH 7.0) for 1 h.
2. Wash the sample with 0.1 M sodium cacodylate buffer for 10 min three times.
3. Post-fix the sample with 2% Osmium tetroxide for 1 h at 4 °C.
4. Wash the sample three times with water for 10 min each.
5. Incubate samples with a graded acetone series (50%, 60%, 70%, 80%, 90%, 95%, 100%, respectively) for 20 min on the shaker.
6. Incubate samples with 3:1 acetone: epoxy embedding mixture for 30 min.
7. Remove the mixture, add 1:1 acetone: the embedding mixture, then incubate for 30 min.
8. Remove the mixture, add 1:3 acetone: the embedding mixture, then incubate for 30 min.

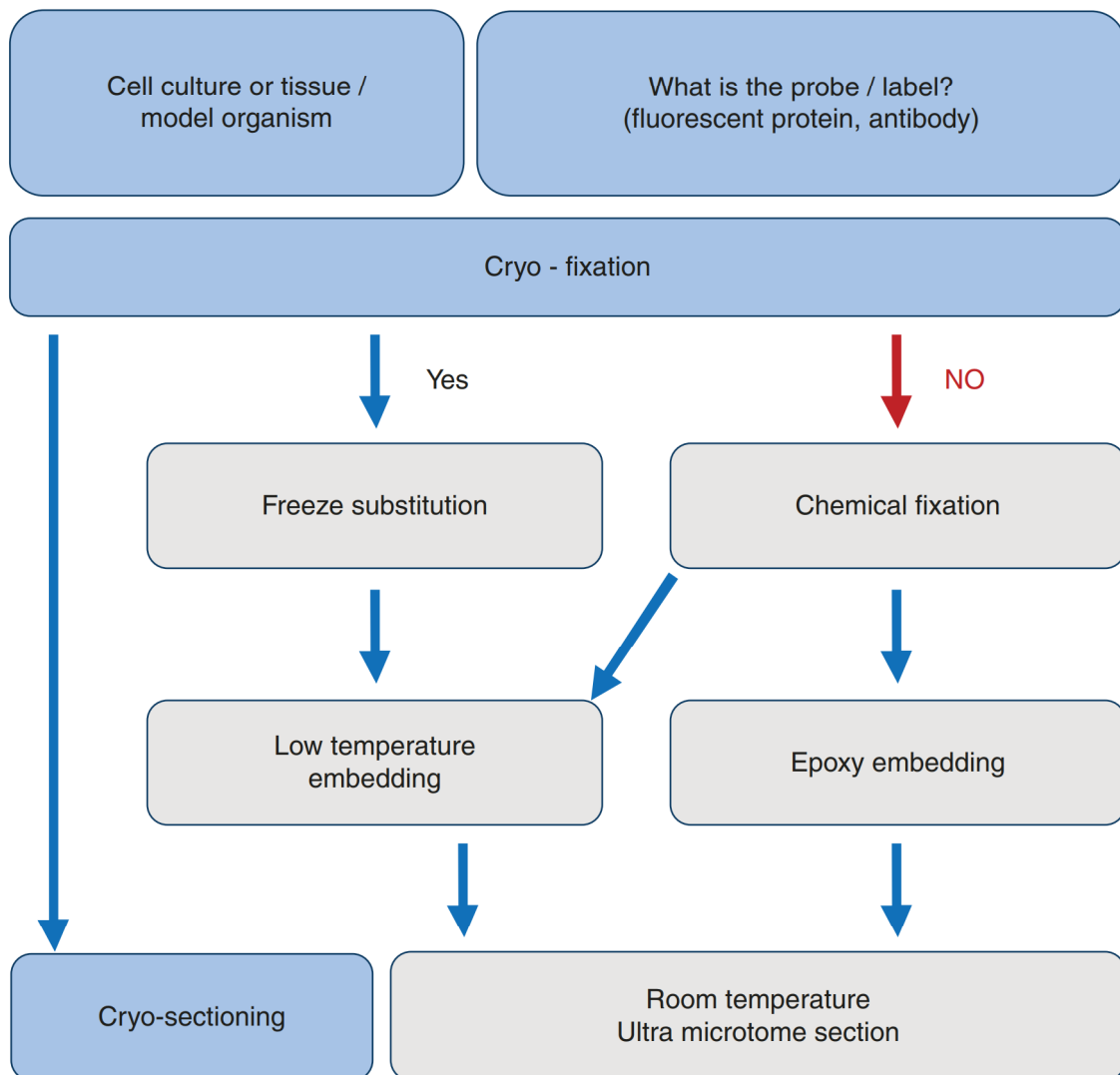


Fig. 4 Different techniques for preservation of antigen and ultrastructures. Depending on more importance among labeling efficiency and ultrastructure, adjust method should be chosen. Contrast is better in chemical fixation, and antigen preservation is better in cryo-fixation

9. Remove the mixture, add 100% embedding mixture, and incubate overnight at room temperature.
10. Embed samples in pure epoxy embedding mixture and bake for 24 h at 65 °C.

Chemical Fixation with Lowicryl HM20 Embedding

1. Fix sample with 1% glutaraldehyde and 1% PFA in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C.
2. Wash samples three times with 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min at 4 °C.
3. Post-fix samples with 2% OsO₄ in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4 °C.

4. Wash samples three times with 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min at 4 °C.
5. Dehydrate samples using ethanol series (70%, 80%, 90%, 95%, and 100% I at 4 °C) and 100% II at room temperature, respectively for 30 min.
6. Incubate samples in 2:1 mixture of ethanol: Lowicryl for 1 h at room temperature.
7. Replace the mixture with 1:1 ethanol: Lowicryl and incubate samples for 1 h at room temperature.
8. Replace the mixture with 1:2 ethanol: Lowicryl and incubate samples for 1 h at room temperature.
9. Remove the mixture and incubate samples in pure Lowicryl mixture I for 1 h at room temperature.
10. Remove the mixture, add pure Lowicryl mixture II, and incubate overnight at room temperature.
11. Embed samples using gelatin capsules and polymerize for 24 h at 60 °C.
12. Fix samples with 1% glutaraldehyde and 1% PFA in 0.1 M sodium cacodylate buffer (pH 7.2) at 4 °C.
13. Wash samples three times in 0.1 M sodium cacodylate buffer for 10 min each.
14. Fix samples in 2% OsO₄ for 1 h at 4 °C.
15. Dehydrate samples using an ethanol series (50%, 60%, 70%, 80%, 90%, 100%) for 20 min, respectively.
16. Transfer samples to Lowicryl resin.
17. After impregnation with the pure Lowicryl, embed into the Lowicryl.

Low Concentration Glutaraldehyde Fixation with LR White Embedding

1. Fix samples in cacodylate buffer (pH 7.4) containing 4% PFA, 0.5% glutaraldehyde, and 0.3% sucrose for 2 h at room temperature.
2. Wash samples three times in cacodylate buffer for 5 min each.
3. Incubate samples for 20 min in a graded ethanol series (50%, 60%, 70%, 80%, 90%, 95%, and 100%, respectively) on a shaker.
4. Incubate samples twice with LR white hard grade for 1 h at 4 °C.
5. Embedded samples using gelatin capsules.
6. Polymerize for 48 h at 25–43 °C.

Sections, Immuno-staining, and Imaging

To prevent background staining, there are general recommendations. Nonspecific binding can be observed in not well-preserved cellular structures due to imperfections of sections such as scratches or chattering. The perfect sections should never become dry during the entire immuno-staining procedure, and grids with the sections (Fig. 5c) should be always floated on the droplets of reagents (Fig. 5d). Immersing them can result in nonspecific sticking of all labeling reagents to the supporting film, resulting in superimposition with the specific label. If buffers containing detergent are used, the washing should be done very gently to prevent detachment of sections from grids.

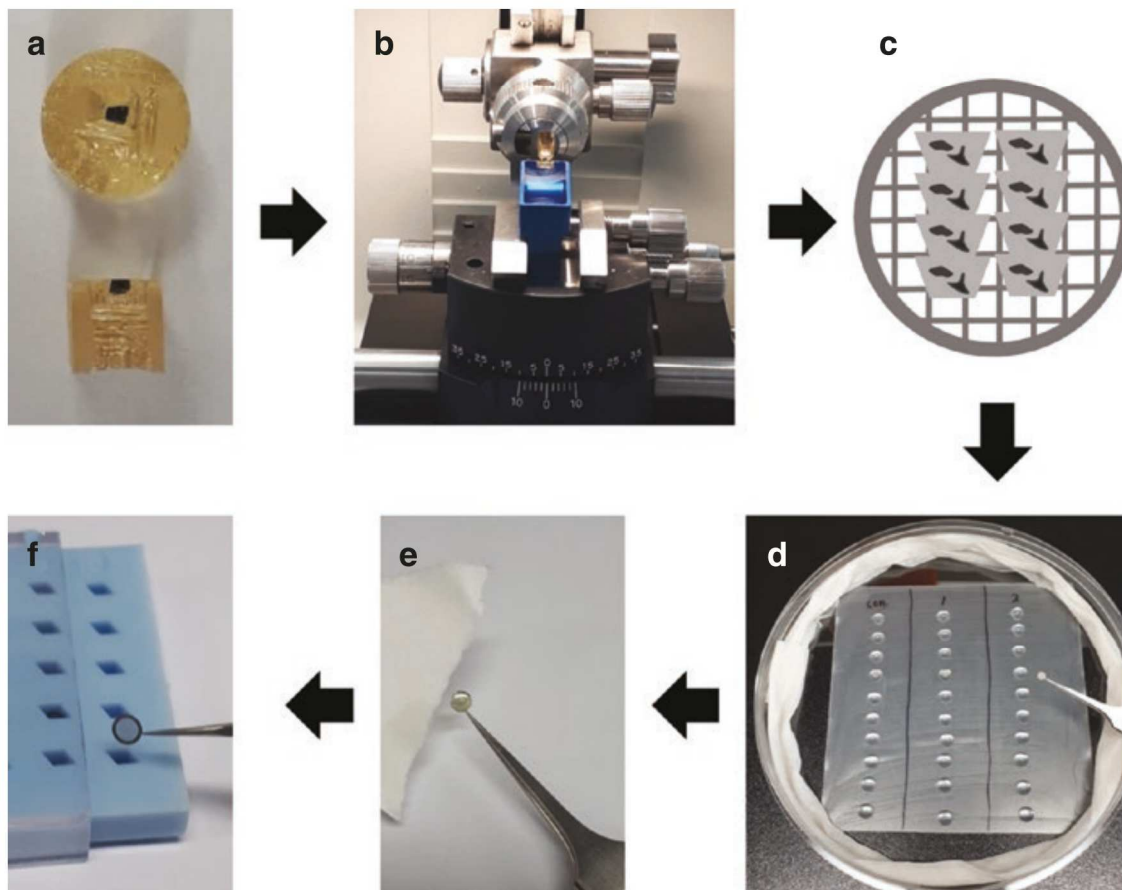


Fig. 5 Process of sample preparation and immuno-staining. (a) Samples are embedded in Epon resin. (b) Ultrathin sections are cut using an ultra-microtome. (c) Sections are mounted on a nickel grid. (d) Setup for Immuno-gold labeling. (e) Double staining with uranyl acetate and lead citrate. (f) Storage in a grid box for TEM observation

Protocol 1

1. Cut 90 nm sections from blocks using an ultramicrotome, and collect them on nickel grids (Fig. 5b and c).
2. Quench free aldehydes with PBS containing 0.1% sodium borohydride and 50 mM glycine for 10 min, with a solution containing 0.05 M glycine for 10 min, or with 0.5 M NH_4Cl for 30 min.
3. Wash grids three times with TBS containing 10% Tween.
 - Before blocking, antigen retrieval can be done by heating. Incubate 1–2 h at 95 °C (Yamashita et al. 2009).
4. Block nonspecific antibody binding sites with PBS containing 1% BSA for 1 h, with Aurion blocking solution for 15 min, or with TBS containing 5% BSA and 5% goat serum for 30 min.
5. Incubate samples with primary antibodies overnight.
 - Incubate grids in 50–100 μL drops of primary antibody in PBS containing 0.1% BSA for 1 h. Perform at 4 °C overnight if necessary.
 - Use a negative control without primary antibody (i.e., no reaction).
 - Use a positive control with primary Ab (i.e., reaction)

6. Wash grids with PBS containing 0.1% BSA for 10 min or wash three times in TBS containing 10% Tween.
7. Block nonspecific antibody binding sites using TBS containing 5% BSA and 5% goat serum for 20 min.
8. Transfer grids to secondary antibody for 1 h (antibody-conjugated with 10 nm gold particles in PBS containing 0.1% BSA).
 - To determine the locations of two different proteins, use multiple secondary antibodies of different origins against respective primary antibodies of differing origins. Double staining should be performed using differing gold sizes for each antibody–antigen complex (Hagiwara et al. 2010).
9. Wash grids once with PBS containing 0.1% BSA for 10 min or three times in TBS containing 10% Tween.
10. Stabilize reactions by incubating in 1% glutaraldehyde in PBS for 5 min.
11. Rinse grids with filtered distilled water (D.W.)
12. Allow grids to air dry.
13. Stain grids with 3% uranyl acetate for 1 min.
14. Rinse grids with filtered D.W.
15. Stain grids with lead citrate for 3 min.
16. Rinse grids with filtered D.W.
17. Observe grids using TEM.

Protocol 2

1. Cut 65–70 nm sections from Lowicryl HM20 blocks using an ultramicrotome, and collect them on nickel grids.
1. Rinse twice in D.W. for 3 min each.
2. Etch sections with 1% periodic acid for 4 min.
 - Some samples can be etched in saturated sodium metaperiodate for 35 min.
3. Rinse samples twice in D.W. for 3 min each.
4. Incubate samples twice in 20 mM Tris HCl buffer (pH 8.2) containing 0.5% BSA, 0.5% normal goat serum, 0.25% Tween 20, and 0.9% NaCl (Tris washing buffer) for 5 min each.
5. React overnight with primary antibody at 4 °C.
 - Dilute primary antibodies to appropriate concentrations in Tris buffer.
6. Incubate samples six times in Tris buffer for 5 min each.
7. React samples with secondary antibody for 2 h at room temperature.
 - Dilute secondary antibody-conjugated colloidal gold particles to appropriate concentrations in Tris buffer.
8. Incubate samples six times in Tris buffer for 5 min each.
9. Rinse samples three times in D.W. for 2 min each.
10. Exclude dust and air dry.
11. Stain samples with 2.5% uranyl acetate in 50% acetone for 20 min.
 - For sections that were etched with saturated sodium metaperiodate, stain with 2.5% uranyl acetate in 50% acetone for 1 h.
12. Rinse sections with D.W. and air dry.
13. Stain sections with lead citrate for 10 min.

- For sections that were etched with saturated sodium metaperiodate, stain with lead citrate for 40 min.
14. Rinse sections with D.W. and air dry.
 15. Cover sections with 0.25% Formvar membranes on grids.
 16. Observe grids using TEM (example: Fig. 6).

Immuno-negative Staining for Virus or Vesicles (Jung and Mun 2018)

Without sectioning, small size such as vesicle or virus can be applied to immuno-staining on grid.

1. Prepare grids using formavar/carbon film and glow discharge the grids.
2. Fix the particles, such as extracellular vesicles, viruses, and bacteria, with 1 mL of 2% PFA for 5 min.
3. Load 5 μ L aliquots of fixed particles onto grids and incubate for 5 min.
4. Rinse grids three times with 100 μ L aliquots of PBS for 10 min each.
5. To quench free aldehyde groups, treat grids with 50 μ L aliquots of 0.05 M glycine for 10 min.
6. Treat grids with single drops of blocking buffer (PBS containing 1% BSA) for 30 min.
7. Incubate grids with primary antibody in PBS containing 0.1% BSA for 1 h.
8. Wash grids with five separate drops of PBS containing 0.1% BSA for 10 min each.
9. Treat grids for 1 h with single drops of 9–11-nm gold particle-conjugated anti-mouse IgG diluted at 1:100 in PBS containing 0.1% BSA.
10. Wash grids with five separate drops of PBS containing 0.1% BSA for 10 min each.
11. Wash grids with two separate drops of D.W.
12. Stain samples with 2% uranyl acetate.
13. Wash samples with D.W.
14. Observe samples using TEM (example: Fig. 7).

Post-embedding with Cryo-fixation and Low-Temperature Embedding Using Freeze Substitution

In this technique, cryo-fixed samples are processed immediately for low-temperature embedding using a freeze substitution protocol. This low-temperature embedding technique has not only provided better preservation of sensitive antigens (McDonald 1999) but also resulted in superior ultrastructure (Studer et al. 2008) and reduced background staining.

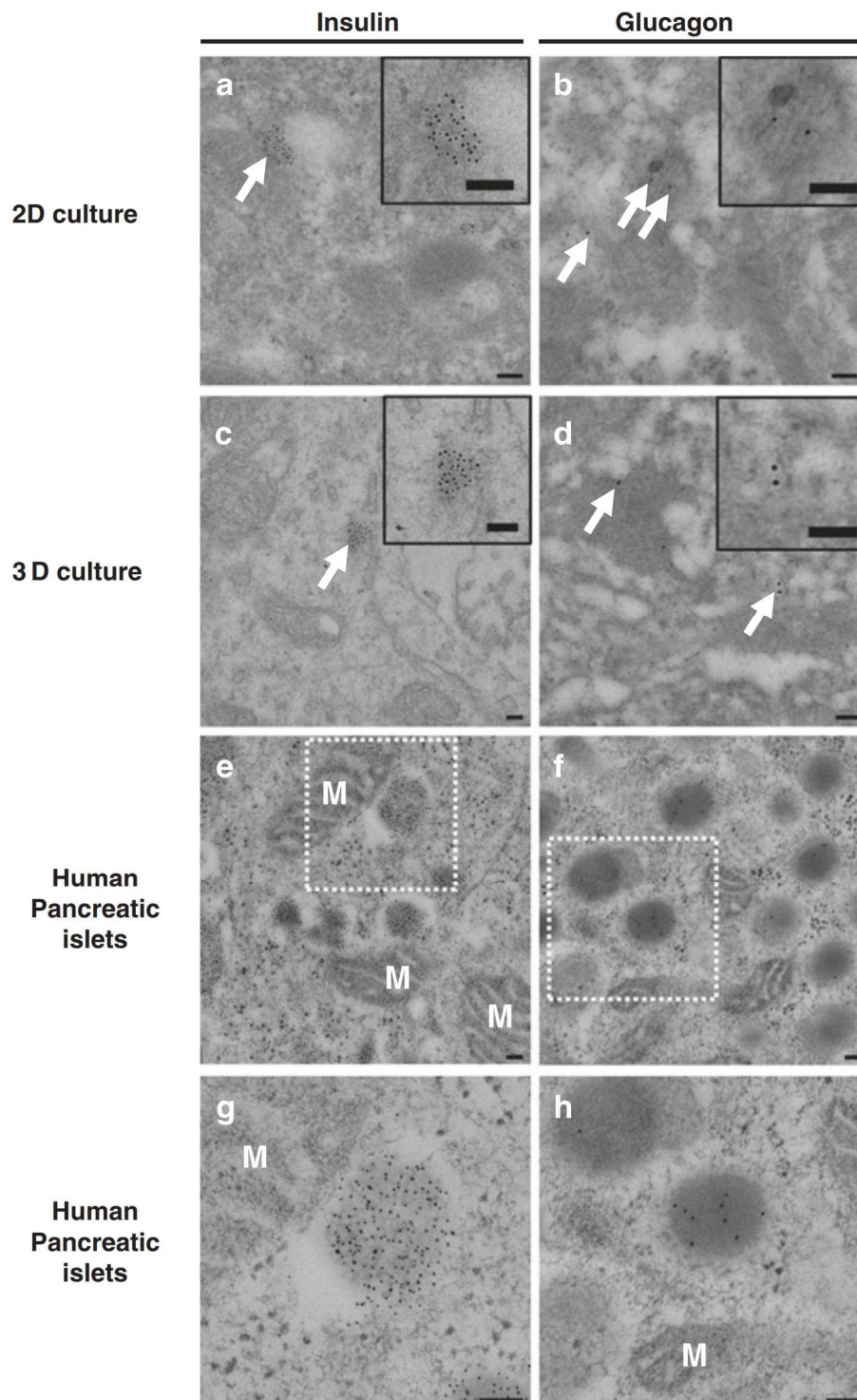


Fig. 6 Transmission electron micrograph of cultured cell and human pancreatic islet tissue. Post-embedding immune-gold labeling was performed with lowicryl HM20 embedding and insulin and glucagon were labeled using a rabbit monoclonal anti-insulin antibody, mouse monoclonal anti-glucagon antibody, 5 nm colloidal gold conjugated goat anti-rabbit IgG secondary antibodies, and 9–11 nm colloidal gold conjugated goat anti-mouse IgG secondary antibodies. Gold particles were labeled with insulin (**a**, **c**, **e**, **g**) and glucagon (**b**, **d**, **e**, **h**), and were localized in the cytoplasm of 2D cultured cells (**a**, **b**) and 3D cultured cells (**c**, **d**). Gold particles are present only on the granules of human pancreatic islets (**e**, **f**, **g**, **h**). *M* mitochondria. Scale bars: 100 nm

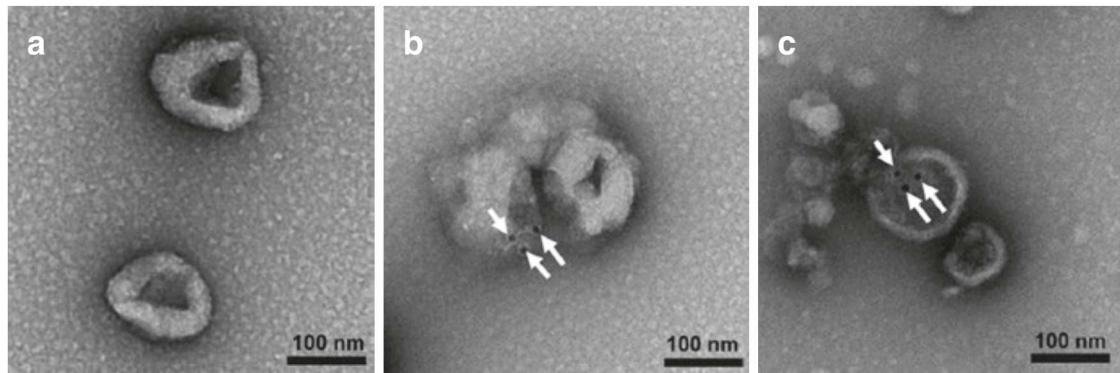


Fig. 7 Immuno-negative staining. **(a)** Representative electron micrograph of exosomes. Negative control of immuno-staining result. **(b, c)** Whole-mount immuno-negative staining shows the location of a specific protein. The white arrows show PD-L1 (CD274) expression

Low-Temperature Embedding After Chemical Fixation and Plunge Freezing

1. Fix sample in 0.1 M PB containing 4% PFA and 0.5% glutaraldehyde.
2. Wash samples with 0.1 M PB.
3. Cryo-protect samples with 10%, 20%, and 30% sucrose in 0.1 M PB for 30 min.
4. Replace solutions with fresh 30% sucrose in PB and incubate overnight at 4 °C.
5. Freeze samples at −190 °C.
6. Transfer frozen samples to an automatic freeze substitution (AFS) apparatus.
7. Infiltrate samples with 1.5% uranyl acetate in 100% methanol in the AFS at −90 °C for 30 h.
8. Increase the temperature of the AFS from −90 to −45 for 9 h.
9. Maintain samples in 100% methanol at −45 °C for 2 h.
10. Replace methanol with fresh 100% methanol twice and incubate at −45 °C for 2 h each time.
11. Place gelatin capsules into separate AFS holders and cool in the AFS.
12. Incubate samples with HM20 media in gelatin capsule containing 1:3, HM20:MeOH; 1:1, HM20:MeOH; 3:1, HM20:MeOH; and 100% HM20 for 2 h each.
13. Incubate samples in 100% HM20 overnight and replace the media with fresh media three times.
14. Polymerize with UV light at −45 °C for 24 h.
15. Polymerize by increasing the temperature from −45 °C to 0 °C for 24 h without changing the solution.
16. Polymerize with UV at 0 °C for 36 h without changing the solution.
17. Polymerize with increasing temperature from 0 °C to 20 °C for 5 h without changing the solution.
18. Cut sections of 70–90 nm and load onto nickel grids.
19. Follow the procedures detailed in the immuno-staining of sections and imaging (See section 2-2).

Cryo-fixation and Cryo-ultramicrotomy Using Tokuyasu Techniques

The following procedures were largely developed by Tokuyasu (Tokuyasu and Singer 1976). This technique is considered as a method for minimizing artifacts from ice damage and preserving antigenicity.

1. Immerse fixed tissue fragments for a few min in 10% gelatin in PB at 37 °C.
2. Allow the gelatin to solidify as a thin (0.5 mm) slab at 4 °C and repeat the fixation step for 30 min.
 - Gelatin embedding can be omitted in many cases, but occasionally helps freshly cut sections to withstand surface tension from sucrose drops. Samples embedded in gelatin become harder and darken when fully infiltrated. The optimal gelatin block size is <1 mm³.
3. Cut tissue or cell pellet in 10% gelatin after primary fixation
4. Immerse the blocks in 2.3 M sucrose for at least 30 min at room temperature or overnight at 4 °C in Eppendorf tubes.
5. Mount blocks on the specimen holder and remove excess cryo-protectant solution on the block using filter paper.
6. Plunge freeze blocks into liquid nitrogen.
 - Samples can be stored in cryo-vials in liquid nitrogen until sectioning. An alternative cryo-protectant, polyvinylpyrrolidone (PVP) can be mixed with sucrose. PVP increases the plasticity of the blocks, allowing cutting of very thin and well-stretched sections.
7. Mount frozen samples in cryo-chamber of the cryo-ultramicrotome and maintain at −90 °C while trimming semi-thin sections, and at −120 °C when performing ultrathin cryo-sectioning.
8. Trim samples for squared block of about 200 × 200 µm. Trim at 50–100 mm/s and take 200–250 nm semi-thin sections.
9. Produce 70–90 nm thin sections using a diamond knife.
10. Retrieve sections using single droplets of 1.15 M sucrose containing 1% methylcellulose.
 - 2.3-M sucrose can be used for section retrieval.
11. Place sections on formvar/carbon-coated grids after thawing.
 - Section quality is considerably dependent on specimen preparation conditions. But the time interval between thawing and attachment of sections can induce ultrastructural damage. The adhesion properties of the support film should also be optimized to eliminate wrinkles. To reduce damage, pick up gelatin embedded samples or tissues with high collagen contents without using methylcellulose (Bos et al. 2011).
12. Follow the procedure for immuno-staining (See section 2-2).

Trouble Shooting

The most important parameter for successful labeling is the specificity of interactions between antibodies and antigens. The specificity of binding can be affected by denaturation and masking of antigens, and by background noise from general

physical and chemical properties. High- or low-affinity complexes of antibody and embedding medium are typical sources of background reactions. Hence, to reduce these, low molecular weight background should be eliminated before blocking proteins. Because residual fixatives are aldehydes, amino acids (glycine, lysine), and sodium borohydride (Willingham 1983), hydrosylamine can be used to inactivate free aldehydes. Appropriate blocking steps can be used to eliminate nonspecific reactions occurring on high molecular weight compounds with multiple hydrophobic and negative charge sites. To saturate multipoint hydrophobic areas and highly positive charges with high-affinity protein binding capacities, albumin or normal serum can be used at high concentrations. Appropriate washing and incubation steps can be used to limit background noise from reactions with low molecular compounds that have a single hydrophobic and negative charge. But these procedures must be controlled carefully. To eliminate nonspecific binding of immunoconjugates due to hydrophilic interactions (positive charges), acetylated albumin can be added to blocking solution.

Reactions of specific antigens and antibodies can be affected by antibody concentrations. Thus, antibody concentrations must be optimized in tests of antigen–antibody reactions. Antibodies are best diluted by factors of 10 (1:10, 1:100, 1:1000). Numbers of bound antibodies decrease with higher dilutions and the specificity increases with higher dilutions.

To eliminate the clustering of antibody, Fig. 8 shows trouble shooting. If colloidal gold shows aggregates, larger aggregates can be removed by centrifugation. Because colloidal gold coagulates in NaCl, quantities of protein–gold complexes have to be optimized to prevent NaCl-induced coagulation. Adjustments of pH can

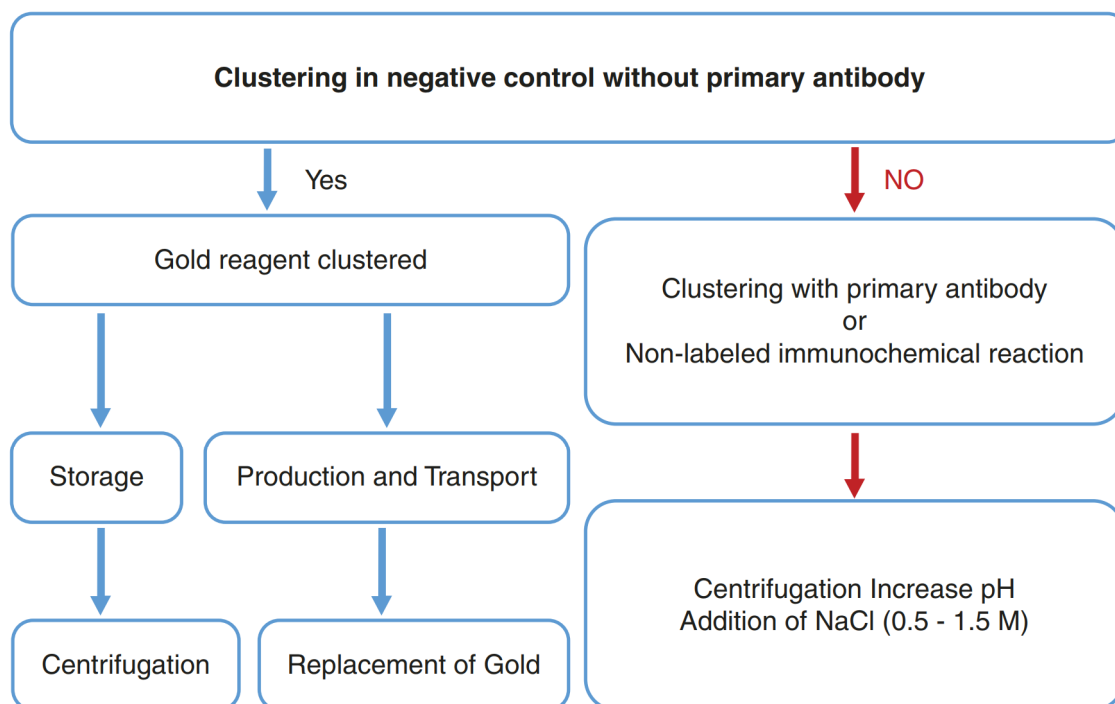


Fig. 8 Trouble shooting for clustering of antibody

prevent protein-induced flocculation and the isoelectric point (PI) of the protein is also important. The absence of aggregates and reactive sites is most important, and it depends on the electrophoretic homogeneity of the protein.

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References

- Acetarin JD, Carlemalm E, Villiger W (1986) Developments of new Lowicryl resins for embedding biological specimens at even lower temperatures. *J Microsc* 143(Pt 1):81–88
- Bendayan M, Zollinger M (1983) Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. *J Histochem Cytochem* 31(1):101–109
- Bendayan M, Nanci A, Kan FW (1987) Effect of tissue processing on colloidal gold cytochemistry. *J Histochem Cytochem* 35(9):983–996
- Bos E, Santanna C, Gnaegi H, Pinto RF, Ravelli RB, Koster AJ, de Souza W, Peters PJ (2011) A new approach to improve the quality of ultrathin cryo-sections; its use for immunogold EM and correlative electron cryo-tomography. *J Struct Biol* 175(1):62–72
- Ferguson DJ, Hughes DA, Beesley JE (1998) Immunogold probes in electron microscopy. *Methods Mol Biol* 80:297–311
- Hagiwara H, Aoki T, Suzuki T, Takata K (2010) Double-label immunoelectron microscopy for studying the colocalization of proteins in cultured cells. *Methods Mol Biol* 657:249–257
- Hainfeld JF, Furuya FR (1992) A 1.4-nm gold cluster covalently attached to antibodies improves immunolabeling. *J Histochem Cytochem* 40(2):177–184
- Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 29(4):577–580
- Jung MK, Mun JY (2018) Sample preparation and imaging of exosomes by transmission electron microscopy. *J Visual Exp JoVE* (131)
- Mandry P, Murray BA, Rieke L, Becke H, Hofler H (1993) Postembedding ultrastructural in situ hybridization on ultrathin cryosections and LR white resin sections. *Ultrastruct Pathol* 17(2):185–194
- McDonald K (1999) High-pressure freezing for preservation of high resolution fine structure and antigenicity for immunolabeling. *Methods Mol Biol* 117:77–97
- Nisman R, Dellaire G, Ren Y, Li R, Bazett-Jones DP (2004) Application of quantum dots as probes for correlative fluorescence, conventional, and energy-filtered transmission electron microscopy. *J Histochem Cytochem* 52(1):13–18
- Piekut DT, Casey SM (1983) Penetration of immunoreagents in Vibratome-sectioned brain: a light and electron microscopic study. *J Histochem Cytochem* 31(5):669–674
- Polishchuk EV, Polishchuk RS (2019) Pre-embedding labeling for subcellular detection of molecules with electron microscopy. *Tissue Cell* 57:103–110
- Roth, J. (1986). Post-embedding cytochemistry with gold-labelled reagents: a review. *J Microsc* 143(Pt 2), 125–137
- Roth J, Bendayan M, Carlemalm E, Villiger W, Garavito M (1981) Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. *J Histochem Cytochem* 29(5):663–671
- Singer SJ (1959) Preparation of an electron-dense antibody conjugate. *Nature* 183(4674):1523–1524

- Studer D, Humbel BM, Chiquet M (2008) Electron microscopy of high pressure frozen samples: bridging the gap between cellular ultrastructure and atomic resolution. *Histochem Cell Biol* 130(5):877–889
- Tokuyasu KT, Singer SJ (1976) Improved procedures for immunoferritin labeling of ultrathin frozen sections. *J Cell Biol* 71(3):894–906
- Valentino KL, Crumrine DA, Reichardt LF (1985) Lowicryl K4M embedding of brain tissue for immunogold electron microscopy. *J Histochem Cytochem* 33(9):969–973
- Willingham MC (1983) An alternative fixation-processing method for preembedding ultrastructural immunocytochemistry of cytoplasmic antigens: the GBS (glutaraldehyde-borohydride-saponin) procedure. *J Histochem Cytochem* 31(6):791–798
- Yamashita S, Katsumata O, Okada Y (2009) Establishment of a standardized post-embedding method for immunoelectron microscopy by applying heat-induced antigen retrieval. *J Electron Microsc* 58(4):267–279