Thin sections of biological material, mounted on nickel or gold grids, can be labelled by floating them, section-side down, on small, 10 µl, droplets of antibody. This process is conveniently carried out on a long sheet of parafilm: only expose as much of the parafilm as it is needed and be careful not to contaminate the clean parafilm surface. All buffer washes are carried out by floating the grids on larger droplets of the appropriate solutions. When transferring grids from one solution to another (with fine forceps), care should be taken not to wet the upper surface of the grid.

SINGLE LABELLING

a] Float the grids, section side down, on PBS (pH 7.4) containing 20 mM glycine and 1% BSA for >15 min. This step will inactivate any residual aldehyde groups present after fixation and block non-specific binding sites and hence lower any background labelling. (50 mM ammonium chloride or lysine may be used in place of glycine.)

b] Place the grids on separate drops of blocking solution (PBS containing 1% BSA and 0.1% CWFS gelatin supplemented with 5% normal serum (same species as the antibody in the second immuno incubation step) for 5-10 min. This step will block non-specific binding sites and hence lower any background labelling.

c] Transfer the grids (while removing the excess liquid using a filter paper) on to drops (5-10 µl each) of the primary antibody solution.
The antibody should be diluted using PBS containing 1% BSA and 0.1% CWFS gelatin supplemented with 5% normal serum (same species as the antibody in the second immuno incubation step). It is often a good idea after diluting the antibody to centrifuge the solution for 1-2 min in a microfuge. This will remove any aggregates from the antibody solution.
Cover the grids and leave for 1 h at room temperature.
To produce adequate labelling with extremely low titre antibodies the grids can be left overnight, at 4˚C, in a moist atmosphere; this also increases the specificity of labelling and may also cut down background labelling.

d] Rinse the grids by floating on droplets of PBS or dilution buffer; 5 washes for at least a total of 15 min.

e] Transfer the grids, using forceps and removing excess liquid, to a droplet of the secondary antibody-gold probe (or a Protein A-gold solution) and incubate for 1 h at room temperature.
The secondary antibody-gold probe is diluted using PBS containing 1% BSA and 0.1% CWFS gelatin supplemented with 5% normal serum (same species as the second antibody) and centrifuged the same way as for the primary antibody.

f] Rinse 5 times with PBS for a total of 30 min.

(Some protocols have an additional fixation/washing step. After the PBS wash, the grids are floated on droplets of PBS containing 2% glutaraldehyde for 15 minutes, washed on PBS for 5 minutes and finally on several changes of distilled water for at least 15 minutes.)

g] Rinse several times with distilled water for 4 to 5 min in total.

THIS STEP IS VERY IMPORTANT. DO NOT FORGET IT!

If the PBS is not washed away then any phosphate ions present will precipitate the uranyl salts on to the sections during the staining and embedding stage.

h] The grids are now ready for staining and embedding in methyl cellulose.
This procedure is performed on-ice. Mix 9 parts 2% methyl cellulose to 1 part 3% uranyl acetate to give a final concentration of 1.8% methyl cellulose and 0.3% uranyl acetate. Transfer the grids

through 2 large puddles of methyl cellulose-uranyl acetate and then place on a small, fresh droplet. After ~10 min. lift-off each grid individually, either with forceps or with a wire loop, and remove excess methyl cellulose with filter paper. Allow grids to dry and examine in the TEM.

The final thickness of the methyl cellulose is critical for the production of contrast and fine structure preservation. The thickness is determined by the amount of excess liquid removed from the grid. Films of optimal thickness have gold to blue interference colours.

DOUBLE LABELLING

For double labelling, two primary antibodies produced in different animal species can be mixed and applied simultaneously (step c). After the washing (step d), the grids can be floated on a mixture of the appropriate gold conjugates with two non-overlapping sizes (step e). Alternatively, the grids can be labelled sequentially firstly with one antibody followed by secondary gold conjugate, and then by the second antibody – gold-conjugate combination.

CONTROLS
A number of negative controls, and where possible a known positive control, should be performed to confirm that the labelling is specific. A number of possible controls are listed below.

- Omit the primary antibody and replace with buffer only.
- Replace the primary antibody with non-immune serum from the same species.
- Use another antibody from the same species that is specific for an antigen known to be absent in the tissue.
- Replace the second antibody with a gold-BSA control at the same dilution and using the same sized gold particle.
- If labelling with cationic gold, incubate one section with unlabeled poly-L-lysine (1-10mg/ml) for one hour prior to adding the conjugate.

TROUBLE SHOOTING
There are 3 main problems encountered when immunolabelling:
- Little or no label
- High non-specific or background labelling
- Contamination on sections/grid

No label:

- Antigen absent or wrong antibody.
- Antigen destroyed, masked or extracted due to preparative procedures. Modify procedure (e.g. fixation, cryosections, resins).
- Antigen present in genuinely low amounts. Use longer incubation times, more concentrated primary antibody and/or an amplification step.
- Primary antibody not working due to wrong dilution, age, and improper storage - excessive freeze-thawing can rapidly destroy an antibody. Test the antibody with positive and negative controls.
- pH of solutions excessively acid or alkali.
- Section not exposed to solutions (i.e. grid wrong way up).
- Magnification(s) too low to detect gold particles.
- Staining/image contrast too strong so that gold label not visible. This is a particular problem when using 5-6 nm gold. Reduce counterstaining.

Excessive background staining:

- Primary antibody concentration too high - dilute by orders of magnitude.
- Gold conjugate concentration too high - dilute further.

- Inadequate washing between incubations - wash grids more thoroughly.
- Free aldehyde groups still present in fixed tissue - quench with PBS containing 20 mM glycine or 50 mM ammonium chloride for up to 1 hour before incubations.
- Non-specific binding and/or charge attraction of the antibody - use blocking agents (1-2% BSA, 5% FCS, 1% gelatin, 1% ovalbumin or 1-5% normal serum) to prevent non-specific binding and 1% Tween-20 in all solutions to reduce charge effects.

NOTE: Do not use normal serum with Protein A and Protein G.

- Ionic concentration of buffer(s) too low - use increased salt concentration (up to 2.5%).
- Inadequately fixed tissue. Necrotic and damaged cells will label non-specifically. Improve fixation conditions and use smaller tissue sections. Check the positive control.
- Background caused by blocking agents - antibody-gold probes may occasionally bind specifically to blocking agents (e.g. do not block samples with FCS if using anti-BSA antibodies). Therefore, if background labelling persists after trying the above remedies, it is advisable to try labelling without using blocking agents.

Clustering:

- Primary antibody aggregates present. Use fresh anti-sera.
- Natural amplification factor of the gold conjugate. Several antibody-conjugated gold particles may attach to the Fc component of the primary antibody, producing the appearance of clusters on the section. This does not occur with Protein A conjugates. Use higher dilution of gold conjugates if desired.
- Clumped primary antibody. Use fresh antisera.
- Clustering is sometimes caused by the natural amplification factor of the gold conjugate. For IgG gold conjugates, up to 10 conjugated gold particles may attach to the Fc component of the primary antibody, producing the appearance of clusters on the section. This does not occur with Protein A conjugates. Use higher dilution of gold conjugates if desired.

Contamination on the sections:

- Wrong grids: Using copper grids for immunoEM is not recommended. Apart from the possible inhibitory effects of Cu^{2+} ions on antibody functioning, some buffers (e.g. Tris-HCL, PBS) will react with the copper and produce a fine precipitate over the specimen. To avoid all these potential problems it is safer (and in the long run easier) to use nickel or gold grids.

- Uranyl acetate precipitation: Salt solutions are used for diluting the antibodies and for washes. If these salts are not washed out then the uranyl acetate will precipitate out during contrasting and embedding. Wash specimens with distilled water (and check there is no liquid on the top side of the grid) before incubation with uranyl acetate.

- Methyl cellulose: When using methyl cellulose to support cryosections during drying, a slightly opaque, globular precipitate sometimes appears. This may be due to either the methyl cellulose being (a) uncentrifuged, (b) stored too long, or (c) warmed prior to use. Make a fresh solution of methyl cellulose and centrifuge prior to use but prevent any warming.

Low Molecular Weight Block Step
To inactivate residual aldehyde groups present after aldehyde fixation incubate for EM: with 0.05 M glycine or lysine in PBS buffer for 15 minutes for LM: with 0.1% $NaBH_4$ in PBS for 15 minutes
High Molecular Weight Block Step
PBS buffer with 5% BSA and 0.1% CWFS gelatin supplemented with 5% normal serum, for 30 minutes (same species as the antibody in the second immuno incubation step).
Wash steps
Incubation buffer for 5 minutes
Primary antibody incubation (see also *)
Incubate with a dilution of specific primary antibody, preferably affinity-purified, 1-5 µg/ml in incubation buffer, (or a high dilution of a high titre antiserum), for 30 minutes to 1 hour. Antibody concentration and incubation time may have to be adapted according to the specific characteristics of the primary antibody and the specimen. If longer incubation times are required (e.g. with low titre antisera) the procedure should be carried out at 4ºC overnight.
Wash steps
Incubation buffer for EM: 3 x 5 minutes for LM: 4 x 10 minutes
ImmunoGold conjugate incubation
Incubate with the gold conjugate reagent, dilution 1/20-1/40 (EM-grade reagents) or dilution 1/50-1/100 (Ultra Small reagents) in incubation buffer for 2 hours.
Wash steps
Incubation buffer for EM: 6 x 5 minutes for LM: 4 x 10 minutes
Further wash steps
PBS, 3x5 minutes, postfix in 2% glutaraldehyde in PBS, 5 minutes, PBS for 5 minutes and finally distilled water for 5x2 minutes.
Wash steps
Distilled water for 5x2 minutes.
Contrasting or Staining
*Remark: when performing pre-embedding labelling the time involved with incubation and washing may have to be prolonged to warrant complete penetration of reagents to internal antigens and removal of unreacted reagents!

DOUBLE LABELING
For double labeling using secondary antibody immunogold EM-grade conjugates, two primary antibodies produced in different animal species are mixed and applied simultaneously. After the washing step, a mixture of the corresponding immunogold EM-grade reagents with two non-overlapping sizes is applied.

Labelling procedure:

a] Place the grids on separate drops of 5% FBS in PBS for 10 min. This step will block non-specific binding sites and hence lower any background labelling.

b] Transfer the grids to drops of 20 mM glycine in PBS (pH 7.4) for 5 min. The glycine binds to free aldehyde groups from the fixative that may still be available for cross-linking antibodies and lead to background labelling. Ammonium chloride (50 mM) or lysine may be used in place of glycine.

c] Using forceps lift the grids off the glycine solution and remove the excess liquid using a filter paper. Place the grids on to drops (5-10 µl each) of the antibody solution. The antibody should be diluted using 5% FBS in PBS and after mixing centrifuged for 1-2 min in a microfuge. This will remove any aggregates from the antibody solution. Cover the grids and leave for 30 min - 2 h at room temperature. To produce adequate labelling with extremely low titre antibodies the grids can be left overnight, at 4˚C, in a moist atmosphere; this also increases the specificity of labelling and may also cut down background labelling.

d] Rinse with PBS; 5 washes for at least 15 min.

e] Transfer the grids, using forceps and removing excess PBS, to a droplet of the secondary antibody-gold probe (or a Protein A-gold solution). The secondary antibody-gold probe is diluted using 5% FBS in PBS and centrifuged the same way as for the primary antibody. Incubate for 30 min - 2 h at room temperature.

f] Rinse 5 times with PBS for 30 min.

g] Rinse several times with distilled water for 4 to 5 min in total.

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