Cryosectioning and Immunolabeling

Article *in* Nature Protocol · February 2007

DOI: 10.1038/nprot.2007.365 · Source: PubMed

2 authors:

Jan W Slot
University Medical Center Utrecht
149 PUBLICATIONS **14,987** CITATIONS

SEE PROFILE

Hans J Geuze
University Medical Center Utrecht
204 PUBLICATIONS **29,984** CITATIONS

SEE PROFILE

Cryosectioning and immunolabeling

Jan W Slot & Hans J Geuze

Cell Microscopy Center of the Department of Cell Biology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands. Correspondence should be addressed to H.J.G. (h.j.geuze@planet.nl).

Published online 4 October 2007; doi:10.1038/nprot.2007.365

In this protocol, we describe cryoimmunolabeling methods for the subcellular localization of proteins and certain lipids. The methods start with chemical fixation of cells and tissue in formaldehyde (FA) and/or glutaraldehyde (GA), sometimes supplemented with acrolein. Cell and tissue blocks are then immersed in 2.3 M sucrose before freezing in liquid nitrogen. Thin cryosections, cut in an ultracryotome, can be single- or multiple immunolabeled with differently sized gold particles, contrasted and viewed in an electron microscope. Semi-thin cryosections can be used for immunofluorescence microscopy. We describe the detailed procedures that have been developed and tested in practice in our laboratory during the past decades.

INTRODUCTION

Researchers who want to know where a protein of interest is localized in a cell or tissue usually choose a fluorescent light microscopy (FLM) approach. During the past decades, FLM techniques have been developed to almost perfectionism, and the microscopes needed are widely available. Basically, a protein under study is tagged with a fluorescent group and the chimer is expressed in cells. One way to proceed is to trace the fluorescently tagged protein while it moves through the cell real-time by live cell imaging. Another strategy is to start with permeabilized chemically fixed cells, or sections of fixed cells, and treat these with antibodies bearing a fluorescent compound. The sites where the antibodies have bound to their antigen can then be studied by FLM. For a correct evaluation of the fluorescent patterns in FLM, it is crucial to apprehend that only the fluorescent signals are seen and not the morphological background in which the signals are embedded. Because researchers often want site information of the fluorescent signal, additional fluorescent antibodies are used directed against the marker proteins of relevant cellular compartments. The fluorescent pattern of the protein under study, for example, in red, is then compared with that of a given compartment marker in green. When in superposition the resulting pattern is (partly) yellow, the conclusion is drawn that the protein (partly) localizes to this compartment. It cannot be overstressed that the limited optical resolution of FLM methods, the lack of structural detail and the fact that most markers are not confined to single compartments often compromise conclusiveness. In such cases, one would prefer to resolve the intracellular structure together with the immunolabeling pattern at higher resolution than in FLM.

Immuno-electronmicroscopy (IEM) can fulfill this requirement since it has the capacity to simultaneously show the precise location of a protein and the ultrastructure of the environment wherein it is embedded. IEM allows one to conclude that a particular protein localizes to a cell structure that can be identified by morphological criteria, as for example the Golgi complex, endoplasmic reticulum, mitochondria. Preservation of ultrastructure requires fixation with chemical reagents like formaldehyde (FA) and/or glutaraldehyde (GA). In most EM studies, chemical fixation is followed by embedding in a resin in order to allow ultrathin sectioning. Another way to harden the material for sectioning is by freezing. We have recently developed an IEM method by which cells are physically fixed by high-pressure freezing (HPF) and then processed for cryosectioning and immunolabeling[1]. Because this method is not yet appropriate for routine applications, we do not detail it here. The more accessible method described below includes chemical fixation before freezing, cryosectioning and IEM.

Of evident importance in IEM is the use of a proper electron dense marker to indicate the binding sites of the specific antibodies. We routinely use electron-dense protein-A (PA)/gold particles (PAG). These are clearly distinguishable against the cytoplasmic background, are distinct, quantifiable and can be made in different sizes allowing double-, or even triple-labeling (see **Figs. 1–3**). PAG was for the first time used for section labeling in 1978 (ref. 2). Since then it was found very suitable for multiple immunostaining of cryosections[3]. Methods were worked out for controlled preparation of homogenously sized gold markers from 3 to 15 nm, which is the range of interest for IEM[4,5].

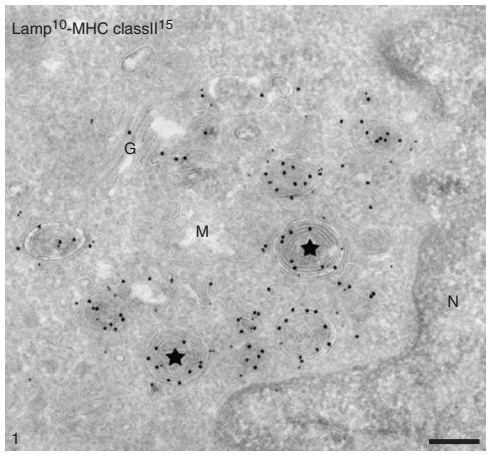


Figure 1 | Human bone marrow-derived lymphocyte. The cells were immersion fixed in a mixture of 2% wt/vol formaldehyde (FA) and 0.2% wt/vol glutaraldehyde (GA) for 1 h and then frozen. The micrograph shows excellent membrane structure and even the smallest vesicles near the Gogi complex (G) are well resolved. The section was double-labeled for the lysosomal membrane protein Lamp1, and for the antigen-presenting protein MHC class II. The sizes of the gold particles in nanometers are indicated in superscript on the figure. The presence of MHC class II in lysosomes (two of these are indicated with an asterisk) illustrates that lysosomes play an important role in antigen presentation. M = mitochondrion; N = nucleus. Bar = 200 nm.

Here we describe a method that assures optimal accessibility of intracellular antigens, because the immunoreaction is done on ultrathin cryosections. Damage during tissue processing is minimal. The technique does not involve such denaturizing steps as dehydration in organic solvents and embedding in resins. It is compatible with very mild chemical fixation before vitrous freezing and cryosectioning according to Tokuyasu[6–8], followed by immunogold labeling. Today, the cryoimmunogold technique is probably the best option for high-resolution localization of antigens at the subcellular level. We describe in detail the preparation of PAG, chemical fixation of cells and tissues, freezing and thin cryosectioning and immunolabeling. Immunocytochemical overviews are relatively easy to achieve with semi-thin sections. Therefore, in the procedure we describe first the fluorescence labeling of semi-thin sections for FLM (Steps 27–44) as a prelude to the immunogold labeling of ultrathin sections (Steps 45–60).

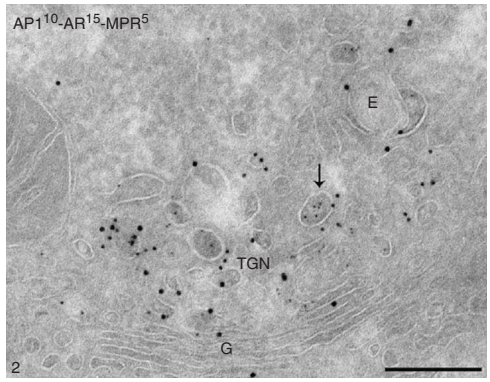
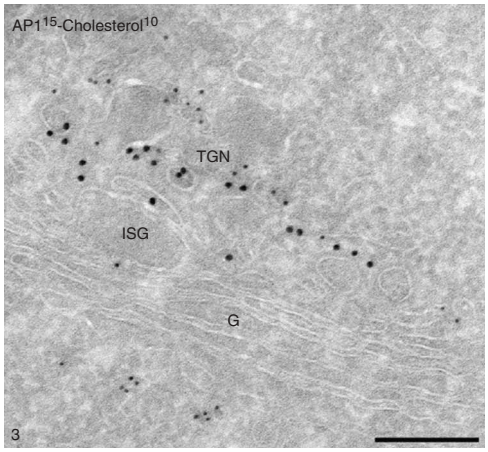


Figure 2 | Human hepatoma cell HepG2. The cells were fixed in a mixture of 2% wt/vol formaldehyde (FA) and 1% wt/vol acrolein for 1 h before freezing. The cryosection was triple-labeled as indicated. The success of triple-labeling depends very much on the types of the antibodies and concentrations of the immunoreagents. The figure shows a Golgi complex (G) and the trans-Golgi network (TGN), an important protein-sorting station in the cell. This area in the cell is very crowded with vesicles and tubules, and only at the electron microscopic (EM) level, differentially labeled vesicles and microdomains in membranes can be identified. The mannose 6-phosphate receptor (MPR) exits the TGN within clathrin-coated vesicles that are positive for the adaptor protein 1(AP1) (see arrow), while the asialoglycoprotein receptor (AR) is excluded from these vesicles. E, endosome. Bar = 200 nm.

MATERIALS

REAGENTS

- Acrolein (90% wt/vol; Sigma, St. Louis, MO, cat. no. 110221) **! CAUTION** Keep the concentrated solution in the original bottle, well sealed, in a refrigerator. Before use, dilutions should carefully be made in the hood.
- BSA (Sigma, St. Louis, MO, cat. no. A-9647) (see REAGENT SETUP)
- 4′,6-Diamidino-2-phenilindole (DAPI; Molecular Probes, Eugene, OR) (see REAGENT SETUP)
- Fluorescent antibodies (Alexa-goat antirabbit A-11008 and antimouse A11001; Invitrogen for Molecular Probes, Carlsbad, CA)
- FA (paraformaldehyde, prills 95% wt/vol; Sigma, St. Louis, MO, cat. no. 441244) (see REAGENT SETUP)
- Formvar (Merck, Darmstadt, Germany, cat. no. 12164; SPI supplies, Westchester, PA) (see REAGENT SETUP)
- Gelatin (local food brand will be satisfactory) (see REAGENT SETUP)
- GA (8% wt/vol, EM grade; Polysciences, Warrington, PA, cat. no. 111-30-8) (see REAGENT SETUP)
- Gold chloride (Sigma, St. Louis, MO, cat. no. G4022) (see REAGENT SETUP)
- Gold probes (IgG-gold, Protein A-gold; Aurion, Wageningen, the Netherlands; ProteinA-gold, subscription for 5-, 10-, 15- or 20-nm gold from UMC-Utrecht, the Netherlands. For information contact g.poshuma@umcutrecht.nl)
- Methyl cellulose (25 centipoises; Sigma, St. Louis, MO, cat. no. M-6385) (see REAGENT SETUP)



- Methylene blue (Merck, Darmstadt, Germany, cat. no. 1283) (see REAGENT SETUP)
- Mowiol (Mowiol 4-88, Hoechst, Frankfurt am Main, Germany) (see REAGENT SETUP)
- PIPES (Merck, Darmstadt, Germany, cat. no. 110220) (see REAGENT SETUP)
- HEPES (Merck, Germany, cat. no. 110110) (see REAGENT SETUP)
- Polyvinylpyrrolidone (PVP, PVP-10; Sigma, St. Louis, MO)
- Protein A (GE Healthcare Bio Sciences AB, Uppsala, Sweden, cat. no. 17-0872-50)
- Silan (3-aminopropyltriethoxysilan, Sigma, St. Louis, MO, cat. no. A3648)
- Sucrose (D+)–saccharose (VWR international bvba/sprl, Leuven, Belgium)
- Tannic acid (Aleppo tannin; Mallinckrodt, St. Louis, MO, cat. no. 8835)
- Toluidine blue O (Merck, Darmstadt, Germany, cat. no. 1273) (see REAGENT SETUP)
- Uranyl acetate (UA; SPI supplies, West Chester, PA, cat. no. 02624-AB) (see REAGENT SETUP)
- PBS (see REAGENT SETUP)
- PHEM (PIPES, HEPES, EGTA, $MgCl_2$) (see REAGENT SETUP)
- Tris (see REAGENT SETUP)
- PAG (see **Box 1**)
- Sodium borohydride
- Phosphate buffer (PB) (see REAGENT SETUP)
- Glycine (Gly)

EQUIPMENT

- Copper or nickel grids (Hexagonal, 100 meshes; VECO-Stork, Zoetermeer, the Netherlands) (for coating and washing of grids see EQUIPMENT SETUP)
- Diamond knives (Element Six, Cuyck, the Netherlands; Diatome, Bienne, Switzerland) (see EQUIPMENT SETUP)

Figure 3 | Rat exocrine pancreatic cell. Tissue was obtained after whole-body perfusion with 2% wt/vol formaldehyde (FA) 0.2% wt/vol glutaraldehyde (GA) before freezing and cryosectioning. The sections were incubated with biotinylated perfringolysin O (Θ-toxin) that binds specifically to cholesterol. Toxin-binding sites were demonstrated with antibiotin and protein-A/gold[22]. In addition, the section was double-labeled for adaptor protein 1 (AP1), a marker of the trans-Golgi network (TGN). Many TGN membrane vesicles, some of which are AP1-positive are enriched in cholesterol. G, Golgi complex; ISG, immature secretory granule. This technique also allowed to show that recycling compartments and internal vesicles of multivesicular bodies contain high concentrations of cholesterol[23]. Bar = 200 nm.

BOX 1 | PROTEIN-A/GOLD MANUFACTURE

Gold sols

Prepare colloidal gold sols, containing spherical gold particles with a desirable average particle diameter (APD), by the reduction of gold chloride after method A or B. Use well-cleaned laboratory glassware in these procedures.

For the preparation of sols with APDs ranging from ~3 to 15 nm [tannic acid (TA)–citrate reduction[5]]

1. Mix in a 250-ml beaker 1 ml of 1% wt/vol $HAuCl_4$ and 79 ml distilled water.
2. Use a 50-ml beaker to make up a reducing mixture, containing 4 ml of 1% wt/vol trisodium citrate · $2H_2O$, a variable volume (0.01–5 ml) of 1% wt/vol TA, 25 mM K_2CO_3, to correct the pH of the reducing mixture and distilled water to make the final volume 20 ml. The volume of 25 mM K_2CO_3 needed is equal to the volume of 1% wt/vol TA used. When <1 ml of 1% wt/vol TA is used, no carbonate needs to be added.
3. Heat the gold chloride solution and reducing mixture to 60 °C. Then add the reducing mixture quickly to the gold chloride solution while stirring. Red sols should be formed within a second when high concentrations of TA are added. The reaction time increases gradually when lower concentrations of TA are used until, in the absence of TA, it lasts 60 min before sol formation is complete.
4. After the sol formation is finished, evident from the red color, heat the sols to boiling.

Note: When the volume of 1% wt/vol TA added in Step 2 increases from 0.01 to 5 ml, the APD of the sols gradually falls from 15 to ~3 nm. For example, if 0.015, 0.1, 0.5 or 3 ml of 1% wt/vol TA is added to the reducing mixture, APDs of ~14, 9, 6 or 3.5 nm can be expected, respectively. Such sols are sufficiently homogenous in size to use them as markers in multiple-labeling experiments. Only Aleppo tannin gives the reported results. Thus the source of TA is critical.

To make particles with APD >15 nm (citrate reduction)

1. Take 100 ml of a 0.01% wt/vol $HAuCl_4$ solution in distilled water and heat to boiling.
2. Add a variable volume (4–0.32 ml) 1% wt/vol trisodium citrate · $2H_2O$ and mix[24].
3. Let the mixture boil until the color turns bright orange-red when a high amount of citrate is added, or violet when this concentration drops to low values. The reaction time is dependent on the citrate concentration, but is always <15 min.

Note: The APD of the resulting sols is ~14 nm when 4 ml of the citrate solution is used, and increasingly larger when less citrate is added. A maximum APD of ~150 nm is reported[24]. Sols with APDs in the range of 15–25 nm are homogeneous in size, but when the APD increases further they become increasingly heterogeneous.

Protein-A (PA) binding to gold

1. Dissolve PA in distilled water at 0.2 mg ml^{-1}.
2. Adjust the pH of 100 ml of a gold sol to 6 with 0.1 N NaOH. Do not use the pH meter for unstabilized sols, because the sol will clog up the electrode. Therefore take a 5 ml sample of the sol, which is usually at a pH 5–5.5, stabilize it with excess PA (5 μg per ml sol), and determine the concentration of NaOH required to bring the pH to 6 by titrating with 0.1 N NaOH. Then add that concentration of hydroxide to the unstabilized sol.
3. Determine the stabilization concentration (gold number) of PA in small samples using Zigmondy's test[25,26]. Briefly in small test tubes, mix 250 μl samples of gold sol with samples of a protein solution in a range of 0.25–2.50 μg protein per tube. After 1 min add 25 μl 10% wt/vol NaCl. The lowest protein concentration that prevents the red to blue color change to occur (which can be judged visually) is taken as the stabilization concentration.
4. Add the stabilization concentration of PA to the rest of the gold sol at pH 6, while stirring gently.
5. After a few minutes, bring the pH to 7–7.5 by adding 0.1N NaOH.
6. Add BSA from 10% wt/vol stock solution to a final concentration of 0.1% wt/vol.

Note: The binding of gold particles to proteins is noncovalent, and has different characteristics for each particular protein. The strength of the binding of PA is moderate[27], but when the binding is performed under optimal conditions[28], PA and gold form a complex that is stable for months. BSA binds only weakly to the gold particles, and is therefore a suitable secondary stabilizer for protein-A/gold (PAG) probes. Other proteins with higher affinity to gold may replace the PA when mixed with the PAG preparation so that the probe will loose its reactivity. Hence, we observed a significant loss of reactivity when we mixed the PAG with gelatine, which has a high affinity to gold.

Purification of the PAG complex

1. Concentrate the preparation by centrifugation: centrifuge for 60 min in a Beckman R45 Ti rotor, at 23,000 r.p.m. for 5 nm gold, at 18,000 r.p.m. for 10 nm gold, at 12,500 r.p.m. for 15 nm gold and at 8,500 r.p.m. for 20 nm gold. Or use an appropriate speed in a different rotor. The resulting pellet should be composed of a large loose part and a small tightly packed part. If the centrifugation force is too strong, the tight pellet becomes large and an increasing amount of aggregates of gold particles is introduced into the preparation.
2. Remove the supernatant without disturbing the pellet and resuspend the loose part of the pellet in a small volume (~0.5 ml).
3. Purify the concentrated probe by layering it over a 10–30% vol/vol continuous glycerol (or sucrose) gradient in 0.1% wt/vol BSA/PBS. Centrifuge the gradient, in a SW 41 rotor (Beckman) 45 min at 41,000 r.p.m. for 5 nm gold, 30 min at 18,000 r.p.m. for 10 nm gold, 30 min at 12,000 r.p.m. for 15 nm gold) or 20 min at 9,000 r.p.m. for 20 nm gold. Adjust the speed if any other type of rotor is used. Collect the dark red band. It contains essentially a monodisperse PAG preparation without free protein-A.

Dilutions for use

The purified probe collected from the gradient is stored at 4 °C. Dilute before use in 1% wt/vol BSA/PBS so that the optical density, measured over 1 cm at 520 nm, is ~0.1, 0.2 or 0.3 for probes with APD of 5–8, 9–12 or 13–20 nm, respectively.

- Glass knives (see EQUIPMENT SETUP)
- Ultramicrotome with cryoattachment (UCT-FCS or UC6-FC6; Leica Microsystems, Vienna, Austria)
- Ionizer (For UC6: Leica Microsystems, Vienna, Austria. For other microtome types: Diatome, Bienne, Switzerland)
- Dissection microscope
- Silan-coated microscope slides (see EQUIPMENT SETUP)

REAGENT SETUP

BSA (10% wt/vol, stock solution) Add 10 g BSA to 80 ml distilled water. Stir slowly (to prevent foaming) overnight at 4 °C. Set the pH to 7.4 with

1 N NaOH. Centrifuge the solution at ~100,000*g*. Decant the supernatant carefully, add sodium azide to a final concentration of 0.02% wt/vol and distilled water to a final volume of 100 ml. Store in small aliquots in the refrigerator.

PBS, pH 7.4 stock solution Take 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4 and 2.3 g NaH_2PO_4. Add water up to 1,000 ml and solve. Dilute the stock solution ten times in distilled water before use.

PB, pH 7.4 stock solution Prepare separately a solution of 0.2 M Na_2HPO_4 and one of 0.2 M NaH_2PO_4. Mix the two solutions in a 81:19 ratio, dilute to 0.1 M and check the pH before use.

PIPES, pH 7.3 stock solution Make a 0.2 M solution by solving 6.05 g PIPES and 1 g NaOH in 75 ml distilled water. Adjust the pH to 7.3 with 1 N NaOH. Add distilled water to a final volume of 100 ml. Dilute to 0.1 M before use.

HEPES, pH 7.4 stock solution Make a 0.2 M solution of HEPES. Adjust the pH with 0.2 M NaOH. Dilute to 0.1 M before use.

PHEM (PIPES, HEPES, EGTA, $MgCl_2$), pH 6.9 stock solution Dissolve three pellets of NaOH in ~75 ml water and add 3.63 g PIPES. Set pH 7. Then add 1.19 g HEPES, 0.08 g $MgCl_2 \cdot 6H_2O$ and 0.76 g EGTA and set pH at 6.9 with 1 N NaOH. Fill out to 100 ml. Dilute with distilled water in 1:1 ratio to 0.1 M before use.

Tris, pH 7.4 or 8.5 Dissolve 2.42 g of Tris in 80 ml distilled water. Set pH with 1 N HCl and adjust to 100 ml with distilled water. For 0.1 M buffer, dilute with water in 1:1 ratio.

DAPI Store as stock solution of 2 mg ml^{-1} distilled water. Dilute 1:1,000 in PBS before use.

FA (16% wt/vol, stock solution) Dissolve 16 g paraformaldehyde powder in 90 ml of distilled water and heat to 65 °C, at which temperature it should be kept for 15–20 min. While stirring, set the pH at 7.4 with 5 M NaOH (pH paper). The pH adjustment is slow, so give it some time. Add distilled water to adjust the final volume to 100 ml. Cool the solution and filter 16% wt/vol. FA is stored in small aliquots at −20 °C.

Before use, the solution should be heated to 65 °C until it becomes clear and can be diluted in the selected buffer. Correct the buffer strength for the added volume.

GA (8% wt/vol stock solution) 8% wt/vol GA comes under nitrogen atmosphere in sealed capsules of 10 ml. Once broken, pour the contents of a capsule in a few 5-ml vials. When stored in closed vials in the refrigerator, the solution can be used for a few weeks. For longer storage, it is preferable to bubble nitrogen gas through the solution and close the vial immediately and tightly.

Gold chloride (1% wt/vol, stock solution) Gold chloride comes in glass capsules that contain 1 g. First make a scratch around the capsule using a diamond pencil. Clean the capsule carefully, first with acetone, then with soap and finally rinse well with distilled water. Place the capsule under distilled water in a beaker. Break the capsule with a clean glass rod or tweezers. Fill up the beaker until 100 ml with distilled water. When sealed well, the solution can be stored for up to a year in the refrigerator.

Formvar (1.1% wt/vol, stock solution) Weigh 1.1 g Formvar in a 100-ml volumetric flask. Add chloroform (Merck, cat. no. 2447) to 3/4 full, stir until dissolved, fill with chloroform to 100 ml and let the solution rest overnight in a dark place at room temperature (20 °C).

Gelatin (10–12% wt/vol, stock solution) Suspend 10–12 g gelatin in 75 ml of 0.1 M PB. Stir for 10 min at room temperature. Stir 4–6 h at 60 °C. After the gelatin is solved, lower the temperature till 37 °C and add water till 100 ml. Finally, add 200 µl of 10% wt/vol sodium azide and pour the solution into 5-ml vials. Place in the refrigerator until use.

Most commercial gelatins give a precipitate (probably calcium phosphate) when made up in PB. A local food brand may be the best solution here, or use different buffers (e.g., Tris–HCl or Tris-buffered saline) in this recipe. The concentration to be used can vary for the different sources, as judged from the stiffness of, for example, gelatine-embedded cell pellets (see Step 7 of the PROCEDURE).

Methyl cellulose (2% wt/vol, stock solution) Take a 250-ml Erlenmeyer containing a magnetic stirrer and 4 g methyl cellulose. Add 196 ml distilled water, preheated to 90 °C, while stirring. Cool down rapidly on ice. Keep stirring. Add water to 200 ml, seal with parafilm and stir overnight at 4 °C. Let it stand for another 3 d at 4 °C. Centrifuge at 100,000*g* for 1.5 h. Store in the refrigerator.

Methyl cellulose–UA (for embedding and contrasting) Take 36 ml of 2% wt/vol methyl cellulose (stock solution) and mix it gently (no air bubbles) with 4 ml 4% wt/vol UA (filtered stock solution). The mixture can be stored in the dark at 4 °C for up to 3 months.

Methylene blue Solution A: 1% wt/vol Azur II in distilled water. Solution B: 1% wt/vol methylene blue in 1% wt/vol borax. Mix A and B well in a 1:1 ratio and filter before use.

Mowiol Add together in a 50-ml glass bottle with screw top: 6 g glycerol and 2.4 g Mowiol. Mix well, add 6 ml distilled water and leave for 2 h at room temperature. Add 12 ml of 0.2 M Tris–HCl, pH 8.5. Place this mixture for 2 h at 50 °C while gently agitating. Centrifuge for 15 min at 5,000*g*. Collect the supernatant and store in small units at –20 °C.

PVP–Sucrose[9,10] Weigh 0.69 g Na_2CO_3, 3 g PVP and 11.62 g sucrose. Mix the powders and add distilled water to a final volume of 20 ml. Stir for 3 h and store in the dark.

The final pH is ~10. If that is a problem, the distilled water can be replaced by any buffer.

2.3 M Sucrose Dissolve 78.73 g sucrose in a final volume of 100 ml 0.1 M PB. Stir until the sucrose is completely solved and store in 1-ml vials.

Toluidine blue Dissolve 1 g toluidine blue in 100 ml borax. Use during the next day.

UA (4% wt/vol, stock solution) Dissolve 4 g UA in 100 ml distilled water. Store in the refrigerator, protected from light. Before use, filter through a 0.22-µm Millipore membrane (Millipore, Bedford, MA).

UA (-oxalate) (2% wt/vol stock solution) Take filtered UA stock solution and mix it in a 1:1 ratio with 0.3 M oxalic acid. Add 25% wt/vol NH_4OH while stirring, drop by drop until the pH is 7–7.5 (pH paper, in the hood). ▲ CRITICAL Too high pH will give a precipitate. That is irreversible. Start again. Store like UA.

EQUIPMENT SETUP

Diamond knives Clean diamond knives thoroughly after sectioning. Further information about the cleaning can be obtained from the supplier. ▲ CRITICAL An important issue when using a diamond knife is the cleaning of the knife.

Glass knives Prepare sharp glass knives according to the balance break method[11,12]. Ultracryotomy is very dependent upon the quality of the knives. If *glass knives* are used, they should have a cutting angle that is as close as possible to 45°. Preferably, the role of the glass knife is restricted to the trimming and semi-thin sectioning. For other purposes it is best to use a *diamond knife,* provided that electrostatic problems are taken care off.

Washing of grids Take no more than 200 copper grids (preferably hexagonal, 100 mesh) in a 15-ml glass tube. Add 1 ml of distilled water mixed with 0.2 ml of 25% wt/vol NH_4OH, and rinse by shaking vigorously until the solution becomes lightly blue. Discard the ammonia solution and stir up to ten times in distilled water until no ammonia is left, which would cause brown spots on the grids. Alternately, clean (etch) during 10 s in 1% wt/vol H_2SO_4 in distilled water, immediately followed by copious rinsing with water.

Wash the grids twice in acetone and dry on filter paper in a 37 °C stove. For nickel or gold grids, washing in acetone is sufficient.

Coating of grids with Formvar film Clean the surface of a microscope slide by wiping with a chamois and next with lens paper. Blow remaining dust away with air from an air bulb. If the slide is too clean, it will be difficult to separate the Formvar film from the glass surface at later stage.

Pour the Formvar solution gently, without forming air bubbles, in an adapted separating funnel provided with two stop-cock valves in its outlet. The upper is used to regulate the flow, and the lower one to switch open or close. The size of the funnel is chosen so that it can hold a microscope slide in upright position. Fill the funnel until the level of the Formvar solution is approximately two-thirds of the length of the slide. Place the slide in the funnel with a pair of tweezers and cover the top of the funnel to reduce evaporation of the chloroform. Open the lower valve with one turn. The upper valve is set in such a way that the Formvar/chloroform solution flows out of the funnel in 14 s. Remove the slide from the funnel after 30 s. Loosen the film at the edges of the slide by cutting the film 0.5 mm from the edges with a scalpel or razor blade. Alternately, wipe the edges with lens paper. Fill a beaker with clean distilled water and slowly bring the slide in vertical position into the water. The film will float on the water surface. The thickness of the film should be ~50 nm, which will give it a silver-white appearance on the water surface (use a lamp). Place clean grids on the film (shiny side up) and avoid irregularities in the film. One good-quality film can carry as much as 50–60 grids. Take a microscope slide covered with a sticker. Touch it in vertical position gently at one of the short sides of the film and press the film into the water, so that the film will attach to the sticker with the grids between the film and the paper surface of the sticker. Be sure to submerge the slide far enough into the water so that the film is totally submerged. Then remove the slide from the water and let it dry.

Carbon coating of grids While the grids stay in position on the stickered glass slide, evaporate at 0.01 Pa (10^{-4} Torr) a very thin layer of carbon onto the film using standard techniques. Place a small piece of adhesive tape on the sticker with grids, which is removed after carbon evaporation. The borderline between noncoated and carbon-coated surface should be just visible.

Take a grid to check the film in the electron microscope at ×20,000 for holes and dirt. Test the strength by removing the objective aperture and focusing the beam. The film should not break. *Note:* In case of holes in the Formvar film, or when the film becomes damaged by sharp grid bars, PBS may leak through and corrosion of the grid by NaCl may occur. This may happen, for instance, when grids are stored overnight on gelatin plates (Step 46). Therefore, these are usually made up with PB, instead of PBS. However, any buffer at the backside will cause precipitates when it dries there during the immunolabeling procedure. Therefore, it is better to solve the leakage problem by finding a different brand of grids and/or to improve the preparation of Formvar films.

Silan-coated microscope slides Take clean microscope slides and place them in a mixture of silan and 100% ethanol in the ratio 1:19, for 1 min. Transfer the slides to distilled water and rinse 2 × 1 min. Take them out and let the excess water absorb into the filter paper. Let the slides air dry for 3 h at 57 °C.

Note: Further information on the preparation of cryotools, glass knives, Formvar films, colloidal gold sols and protein A-gold complexes, and the cleaning of diamond knives can be found in the laboratory manual of the UMC-Utrecht group at http://www.cmc-utrecht.nl.

PROCEDURE

Fixation

1| Make up the fixative of choice at appropriate working concentration. Fixation is accomplished by using the aldehydes, FA, GA and acrolein, either singly or in various combinations. Standard fixatives are 4% wt/vol FA and a mixture of 2% wt/vol FA + 0.2% wt/vol GA in a buffer of choice: PB (pH 7.4), PIPES (pH 7.3), PHEM (pH 6.9) or HEPES (pH 7.3). Acrolein can be used at a concentration of 1% wt/vol in a mixture with 4% wt/vol FA or 1% wt/vol GA in the same buffers. Acrolein is thought to be the fastest and most reactive, but also most toxic aldehyde. However, the crosslinking capacity is rather limited. Therefore, it is used in combination with FA or GA (**Fig. 2**).

Note: Standard buffers in the procedure are PB during the fixation, and in the sucrose and gelatine solutions and PBS during the incubations. Fixation is probably the most pH-sensitive phase in the entire procedure. The aldehydes, when these react, tend to influence the pH[11,13]. Therefore, strong buffers (0.1 M) should be used at this stage. The choice of buffer is not crucial. When needed, other buffers can be used as well.

! CAUTION These aldehydes are toxic reagents. Follow the laboratory safety rules for handling, contamination and disposal. Use them always in the hood, with relative strong airflow.

Phosphate in solutions is not compatible with uranium. If UA is used during fixation, or section pick up, the phosphate-based buffers can be replaced by PHEM buffer.

2| Fix living samples chemically using aldehydes at ambient temperature. The initial procedure to follow is different for cells (A) and tissues (B).

(A) Cell cultures

(i) Make a double-strength fixative in normal strength buffer. Measure a volume of that fixative equal to the medium and pour it gently to the medium while mixing.
Note: In case of 4% wt/vol FA fixation, do not add double-strength fixative to minimize the osmotic shock.

(ii) In case of sedentary cells, decant the medium–fixative mixture after 10 or 15 min and replace with fresh single-strength fixative. In the case of suspension cell cultures, use centrifugation to replace the medium–fixative mixture with the fresh fixative.

(B) Animal tissues

(i) Fix animal tissues preferentially by 'whole body perfusion'. Tissues with poorly developed vascular beds and biopsies should be quickly excised, immersed in fixative and cut immediately to ~1-mm thick slices or cubes.

3| After initiation, continue the fixation for at least 1 h at ambient temperature. Then replace the fixative with 1% wt/vol FA in the same buffer as the primary fixative, or in PBS.

Note: Fixation-resistant structures, like lipid-rich bodies, may need stabilization by UA during retrieval (Step 23). In such cases, the pick-up solution can be made up by a 1:1 mixture of 2% wt/vol methyl cellulose and 1–2% wt/vol UA[10]. It is also possible to add fixatives like GA, FA or even OsO_4 to the pick-up solution, making possible the cutting of sections from nonfixed frozen material for immunolabeling. Fixation occurs then by thawing the sections after retrieval in fixative. After washing, the sections can be immunolabeled and stained as in the standard procedure (Steps 46–65). This section fixation approach is described elsewhere[1,14].

■ **PAUSE POINT** When in 1% wt/vol FA, samples can be stored for more than a year in the refrigerator. If storage in the frozen state is preferred, add >1.8 M sucrose to the storage solution. This will prevent freezing damage. One may also consider adding sucrose during overseas transport, because sometimes samples become frozen while underway. For efficient packing, cells cultured on plates should go first through Steps 4 and 5, followed by washes in PBS for 5 min (three changes) before they are transferred to the storage solution.

? TROUBLESHOOTING

Sample (block) preparation and mounting

4| Rinse the material three times in PBS, 5 min for each rinse.

5| Cover sedentary cells that are cultured on plates with 1% wt/vol gelatin in PBS. Then scrape them carefully at ambient temperature and transfer them to an Eppendorf tube for further handling. For cells cultured on filters, the filters can be included in the blocks during sectioning. In that case, cut the filters in ($\sim 1 \times 1$ mm^2) pieces that are further treated as tissue pieces.

6| Embed the sample in gelatin. Follow option A for cells and option B for tissues:

(A) Cells

(i) Spin cells down.

(ii) Suspend the pellet in 12% wt/vol gelatin in PBS at 37 °C for 1–5 min. Make sure that the cells are well mixed in the gelatin.

(iii) Centrifuge cells down and place on ice for 10–20 min to solidify the gelatin. After solidification, Steps 7–10 must be performed at 4 °C, preferably under a dissection microscope in the cold room. Use ice if there is no cold room available. The danger then is the build-up of excess water on the specimen table (Step 10), which introduces unwanted water in the sucrose.

? TROUBLESHOOTING

(B) Tissues

(i) Cut the fixed tissue in small blocks (~ 1 mm^3) or, if possible, in thin slices (thickness ~ 1 mm) using a sharp razor blade or scalpel.

(ii) Immerse the pieces in 12% wt/vol gelatin in PBS at 37 °C for 15–30 min, with occasional mixing.

(iii) Take tissue fragments with a drop of adhering gelatin, place on a microscope slide covered by a piece of parafilm. The amount of gelatin should be reduced so that the cover does not float, but rather gently rests on the tissue, exerting a mild pressure. Transfer to ice for solidification. From here on, tissue blocks are, like cells, handled at 4 °C (see Step 6A(iii)).

▲ CRITICAL STEP The gelatin embedding is important to equalize the cutting conditions between intra- and extracellular space. Sometimes, for example, in spongy tissues like lung, better results are achieved when the immersion is performed more gradually in 3, 6 and 12% wt/vol gelatin, successively.

7| For cell preparations, cut off the tip of the tube that contains the solidified cell pellet. Cut the tip in half and place it in ice-cold buffer for another 15 min before carefully removing the gelatin cells from the plastic in a drop of cold buffer. Cut the cell pellet into $\sim$1-mm thick slices. If problems occur with the removal of the gelatin from the excised tips, try placing the tip in cold 2.3 M sucrose for 20–30 min before removing the gelatin cells pellet from the tip. This may facilitate the separation of the pellet and the plastic tip, but further handling of the pellet in the sucrose is difficult. Therefore, one may like to transfer the pellet back to the cold buffer before cutting slices.

For the embedded tissue pieces, remove the parafilm cover by adding a drop of cold PBS at the slide-cover junction.

8| Cut the gelatin slices containing cells or tissue into cubic blocks of ~ 1 mm^3. If the specimen has to be sectioned in a specific orientation, for example, cross-sections of muscle fibers or kidney medullary tubules, it is advisable to cut the blocks in such a way that the orientation can be recognized (e.g., elongated in the direction, perpendicular to the proposed plane of sectioning. Transfer the blocks to small vials containing 2.3 M sucrose in PBS and rotate (~ 3 r.p.m.) overnight.

9| Prepare aluminum, 2 mm rivets with a 3 mm flat head (table) to be specimen holders. These rivets make good specimen holders that fit into the ultracryotome. Roughen the surface of the table by scratching with sandpaper, scalpel or diamond pencil. In particular, when sandpaper is used, make sure to wash away any grainy contamination. Clean the pins in acetone. Dry and inspect the table surface under the dissection microscope. Avoid any further contamination, such as grease from fingers.

10| Remove the specimen blocks from the 2.3 M sucrose with forceps or a tiny wire loop. Place them on the table of a clean specimen holder. Be careful not to touch the table surface with anything except the specimen. Remove most of the excess sucrose with a piece of filter paper, but leave enough to glue the block at the basal side to the specimen holder. Mount the specimen in a good position for sectioning.

Note: Specimens of cells grown on filters are best mounted with the filter perpendicular to the block face from which sections will be cut. The cutting direction should then be parallel to the filter. In this way, the basal to apical axes of the cells are in the sections. Also this is the best position to prevent complications during sectioning due to the difference in density between the material in the gelatine-embedded sections and that in the filter. For block preparation of flat specimens that have to be cut parallel to both length axes, we refer to Oorschot and colleagues[15]. They designed a method to section neuronal cells grown on a flat substrate.

11| Freeze the blocks mounted on specimen holders by immersing them in liquid nitrogen (LN). Leave them under LN in an aluminum can. Freezing is not a critical step. In 2.3 M sucrose, ice crystals will never be formed[16]. It is even possible to bring the specimen unfrozen in the cryochamber, where it will freeze relatively slowly. This may be advantageous when cracks tend

to form between specimen and holder during fast freezing in LN. This occurs occasionally when large specimen blocks are mounted, or when too much sucrose is left behind after the mounting.

■ **PAUSE POINT** Frozen specimens can be stored for years under LN even if they have already been used for sectioning.

12| Follow the instructions in the microtome manual to cool the cryochamber down to the desired temperature and install the knife. Before freezing, the tissue or cell blocks are immersed in 2.3 M sucrose (Step 8). This high-concentration sucrose renders plasticity to the frozen samples. For that reason it was selected[17] from the wide range of concentrations originally suggested by Tokuyasu[7,8]. Later, it was also found that >2 M sucrose concentrations always freeze vitreously[16]. At higher temperatures (−100 to −80 °C), the material is soft enough for block trimming and semi-thin (100–500 nm) sectioning. When the temperature drops further, the material becomes harder and brittle so that large fragments or entire blocks may be lost when trimming or semi-thin sectioning is done at those temperatures. This is especially true if there is a lot of gelatine at the cutting face of the block. Ultrathin (~50 nm) sections are best cut at temperatures ~−120 °C. At higher temperatures, the material is too soft and ultrathin sections become increasingly compressed.

Block trimming and semi-thin sectioning at −80 to −100 °C

13| Get the following tools ready: (i) Several 'brushes' (eyelashes glued on the tip of a thin wooden stick of ~15 cm). (ii) A 3-mm loop made of stainless 0.3 mm steel wire (ruthanium wire used by dentists), mounted on a wooden stick. (iii) A 2.3 M sucrose solution in PBS, or a 1:1 mixture of 2.3 M sucrose in PBS and 2% wt/vol methyl cellulose. (iv) Microscope slides (uncoated and coated with Silan) and cover slips. (v) Toluidine blue or methylene blue solutions. (vi) Sharp glass knives.

14| Transfer the specimen in the LN-filled can to the cryochamber of the microtome. Take it out with long precooled tweezers, fix the specimen holder properly in the microtome arm, and wait a few minutes to allow the temperature to equilibrate. Approach the semi-square block surface carefully with the knife and align its shortest side parallel to the knife edge. If the block shape is too irregular, use a precooled scalpel to trim it into a semi-square or rectangle. Usually, this is sufficient for semi-thin sectioning. Block surfaces up to ~1.5 mm square can be used for semi-thin sectioning.

15| Section at semi-thin setting, that is, 100–500 nm, until the entire block surface is included in the sections. Initially they may have a snowy appearance, which is due to the sucrose that covers the block. Shiny, transparent (parts of) sections tell that the specimen is being cut, that is, the gelatine or the tissue. Remove section remnants aside from the knife edge using the eyelash and place some perfect ones on a clean spot somewhere further down on the knife.

16| Pick the selected sections up with a sucrose droplet in the wire loop. When the loop enters the cryochamber, some vapor starts leaving the droplet. Immediately after that stops, press the almost frozen droplet gently on the sections and they will stick to the sucrose. Withdraw the loop from the chamber. The sections will thaw and spread over the sucrose. Usually, semi-thin sections are strong enough to resist surface tension on the sucrose drop after thawing. However, if stretching damage occurs, the sucrose solution may be replaced by the sucrose/methyl cellulose mixture (Step 23).

17| Touch the droplet, with the section on the bottom, on a microscope slide for light microscopical inspection (A) or for FLM (B).

(A) LM observation

(i) For LM observation, place a drop of the toluidine blue or methylene blue on the sucrose-covered section and gently rock the slide for 10–20 s to dissolve the sucrose.

(ii) Rinse gently with distilled water and dry. If desired, leave some water on the stained sections and place a cover slip in place before viewing. Observations on these sections can give a quick impression on the quality of the block and the histological image may tell which part of it should be selected for ultrathin sectioning and EM observation.

(B) FLM

(i) For FLM, take a silan-coated glass slide and, using a diamond pen, scratch three lines of ~1.5 cm perpendicular to the long axis of the slide at ~2-cm intervals. Remove all glass splinters with a paint brush or compressed air.

(ii) Place three or four drops with semi-thin sections on the scratched side of the slide, along one side of a scratch mark. Sections of two different specimens that need identical immunostaining can be placed along each side of the scratch. For different labeling procedures, place sections along different scratches. Use a fine wax pen to demarcate the area with sections around a scratch. The wax line prevents the incubation drops from spreading out of the demarcated area.

■ **PAUSE POINT** The sections covered with sucrose (or sucrose/methyl cellulose) can be stored in a cool place, which is sealed against drying for several days until immunolabeling.

Fine trimming and ultrathin sectioning at −120 °C

18| Get the following equipment ready for use: (i) Several 'brushes' (eyelashes glued on the tip of a thin wooden stick of ~15 cm). (ii) A 3 mm loop made of stainless 0.3 mm steel wire (ruthanium wire used by dentists) mounted on a wooden stick. (iii) A 2.3 M sucrose solution in PBS, or a 1:1 mixture of 2.3 M sucrose in PBS and 2% wt/vol methyl cellulose.

(iv) Carbon-coated Formvar films on copper or nickel grids affixed to the stickered surface of a microscope slide (see EQUIPMENT SETUP). (v) A sharp and well-cleaned cryodiamond knife and (vi) a regulated ionizer mounted on the cryoultramicrotome.

19| After the area of interest is located by LM (Step 17A), or FLM (Steps 17B, 27–44) trim the block face further to 0.4–0.6 × 0.3–0.4 mm rectangles by using either a 45°-glass knife or a diamond trimming tool at a chamber temperature of −80 to −100 °C. First trim the specimen material at one side of the planned block face with a sharp corner of the knife: cut ~0.2-μm thick sections with the instrument at very high cutting speed (100 mm s^{-1}). When the sectioning is deep enough (50–100 μm), turn the specimen 90° and repeat the procedure until all sides of the block are trimmed leaving a perfectly rectangular, 50–100 μm high mesh.

20| Replace the trimming glass knife by a new glass knife or a cryodiamond knife. (Set the knife angle at 6°.) Make sure specimen and knife are tightened securely and set to the desired temperature, usually ~−120 °C. At this temperature, most of the 2.3 M sucrose-infiltrated specimens will give good results using a diamond knife and cutting 45–70 nm thin sections. One can try to vary temperatures depending on the material and the desired section thickness. When the specimens are very compact and/or fixed thoroughly, slightly higher temperatures are advisable. Thinner sections in general need lower temperature.

21| Align the knife edge with the block face and approach carefully with 0.1–0.5 μm steps using backlight illumination. When the backlight shadow disappears, set the microtome on automatic. As soon as the knife hits the block face, set the section thickness between 45 and 70 nm. GA-fixed specimens are usually cut at the lower side and FA-fixed ones at the upper side of that range. Set the cutting speed at ~1 mm s^{-1}.
Note: The color of the sections is a good indication of their thickness: silver indicating thinner and light-yellow a bit thicker sections.

Cut ultrathin sections form a ribbon that shifts from the edge, down over the knife. They should look shiny. If they look 'dull', there is probably too much compression (try lower temperature or a sharper (cleaner) knife) or chatter (make sure specimen and knife are tightened, knife angel is correct, etc.). The ribbon usually has a uniform appearance, but the thickness may vary often due to an older type of, or poorly maintained, cryoultramicrotome. This can also occur if something is wrong with the specimen or the knife. Some variation can be appreciated during the microscopy, as some cell structures are seen easier in either thinner or thicker sections.

22| Use the eyelash to keep the ribbon straight during sectioning and to cut it in length so that it fits onto a grid (take into account that the compression, which will largely be restored when the ribbons are picked up (in Step 23), is usually ~70%). Move the ribbon down the knife for the pick up.
? TROUBLESHOOTING

23| Switch off the ionizer, if in use, before picking up the sections. Pick up with 2.3 M sucrose can be done as described for semi-thin sections (Step 16). Since ultrathins are more vulnerable to overstretching by the surface tension after thawing on sucrose drops; it is advisable, in general, to use the sucrose/methyl cellulose mixture for section retrieval. The freezing of that mixture can be followed through the binocular. It starts from the periphery of the drop. The sections should be picked up at that moment, before the entire drop becomes frozen. Evaporated water from the drop will cause frost on the knife at the pick-up spot. So it is important to park the sections before being picked up on nonmetallic places that are at distance from the knife edge, that is, lower parts of the knife, the glue or a specially designed stage (Element Six, Diatome).

24| Withdraw the wire loop with sections from the cryochamber and let it thaw at room temperature. Do not enhance the thawing by breathing on the frozen drop. Thawing occurs within a few seconds.

25| After retrieval and thawing, place the sections on carbon-coated Formvar films on copper (or nickel) grids that remain affixed to the stickered surface of the microscope slide on which they are prepared. Copper grids should be completely covered with Formvar film, so that the copper is not accessible to phosphate-containing buffers like PBS, during the immunolabeling procedures (Step 44).

26| Perform a quick quality check of the sections by first detaching a grid carefully from the stickered slide and then floating it, section down, on distilled water (three times for 2 min) to remove the pick-up solution. Air-dry it for EM inspection. For more detailed structural observation at this stage, pick the sections up by the methyl cellulose/UA mixture and dry them on the grid in a thin film of this mixture (Steps 64–66).
■ **PAUSE POINT** Extra grids with sections covered by the drop of pick-up solution and still attached to the microscope slide can be stored indefinitely in slide boxes at 4 °C (ref. 18).

This makes it possible to cut many sections at one time, which can be used over a long period in different labeling experiments.

Immunofluorescence labeling

27| Take the silan-coated glasses that carry the semi-thin sections. Cover the wax-outlined areas around one scratch by the solutions described in the following steps. Wash by gentle pumping with a Pasteur pipette. Take care not to contaminate a marked area with antibodies from neighboring areas. Protect against drying, in particular during the longer incubation steps. For instance, place the slide on a moistened filter paper in a closed pair of Petri dishes. Drops of ~30 µl should be enough for the incubations with the immunoreagents. Never put these drops directly on dry sections. Except for Step 28, the procedure is done at room temperature and, after Step 37, protected from light if practically possible.

28| Wash with PBS (4 × 5 min). Wash for the first three times at 37 °C to remove the embedding gelatin and remnants of the methyl cellulose. Then return to room temperature for the final wash.

29| Wash with freshly made 0.1% wt/vol sodium borohydride in PBS (5 min).
! CAUTION Borohydride is very unstable. Use the solution immediately after making. Rinse well.

30| Wash with PBS (5 × 2 min).

31| Wash with PBS + 0.1% wt/vol Gly (2 × 5 min).

32| Wash with PBS + 1% wt/vol BSA (1 × 5 min).

33| Incubate with specific antibody diluted in PBS + 1% wt/vol BSA for >45 min.
▲ CRITICAL STEP The dilution of the antibody varies for each individual antiserum. In general, purified IgGs are used at concentrations ~1 µg ml^{-1}, but it can easily be a factor 10 up or down. Even harder to predict is the dilution at which antisera are used. One has to compare different dilutions for specific and nonspecific signal and look for the optimum. It is worth trying to prolong the incubation, for example, overnight (in the refrigerator). It may allow a further dilution by a factor of 5 to 10.

34| Wash with PBS + 0.1% wt/vol BSA (5 × 2 min).

35| Incubate with bridging antibody diluted in PBS + 1% wt/vol BSA (20 min).
Note: This is only required if the primary antibodies used in Step 33 do not bind with the fluorescent probe in Step 37. A bridging antibody binds specifically to the primary antibody, and is a specific target for the fluorescent probe.

36| Wash with PBS + 0.1% wt/vol BSA (5 × 2 min).

37| Incubate for 45 min with fluorochrome-coupled antibody (e.g., goat antirabbit IgG/Alexa 488, or goat antimouse IgG/Alexa 555) diluted in PBS + 1% wt/vol BSA.

38| Wash with PBS (5 × 3 min).

39| If desired, nuclear stain with DAPI for 5 min.
Note: Step 39 is optional. It may help to recognize the tissue elements without interfering with the immunoreaction.

40| Wash with PBS (5 × 3 min).

41| Wash with distilled water (5 × 3 min).

42| Embed the sections in a tiny drop of Mowiol and carefully place a cover slip without inclusion of air bubbles.

43| Leave the glass slide overnight at room temperature. Then seal the cover slips with nail polish.

44| FLM observation. During microscopy, use interference (Nomarsky) contrast to focus, first on the scratches, then find the sections and switch to fluorescence illumination.
■ PAUSE POINT The preparations can be stored for months at 4 °C in a dark place.

Immunogold labeling of ultrathin sections

45| Prepare 2% wt/vol gelatin plates by pouring a few millimeter layer of 2% wt/vol gelatine in PB in 3 cm Petri dishes (add 0.02% wt/vol sodium azide to the gelatine). Let them solidify at 4 °C and place the plates in sealed boxes in the refrigerator, where they can be stored for weeks.

46| Detach the grids with sections (freshly cut or after storage) from the slides and place them on the 2% wt/vol gelatine plates at 4 °C, the sections facing the gelatine. They can be kept there for a few hours, or overnight.

47| First melt the gelatine plate plus grids under a lamp (~40 °C) until fluid, and then place it in a 37 °C stove for another 20 min. This step removes sucrose, methyl cellulose and remnants of the embedding gelatine.

48| For immunolabeling, incubate the sections in a series of solutions as mentioned below in Steps 49–61. Perform these incubations by floating the grids on top of drops of various solutions, sections facing the drop. For rinsing, use drops of 100–200 µl each of which can carry several grids at once. For antibody and immunogold incubation, individual grids should be placed on drops of ~5 µl. The drops are placed on a clean and flat surface of a parafilm sheet that is adhered to a glass plate by a thin film of distilled water. Put a drop of water on a glass plate, cover it with a parafilm sheet and squeeze out excess water by sliding a clean rod over the cover of the parafilm sheet. The water layer should be really thin so that it does not creep between the parafilm and its cover. The cover sheet of the parafilm is removed only from the immediate working area, so that the remaining surface is kept clean.

Transfer the grids by wire loops or by tweezers. Handling with loops is easier. However, with tweezers less of the incubation fluid is transferred to the next drop, which makes washing more efficient. Since the fluid carried with the grid dilutes immunoreagents in very small drops, it is important to transfer a minimum amount of fluid: when a loop is used, pull the grids sideway and slowly from the drops. If tweezers are used, excess fluid can be removed with filter paper; but take care not to dry the section side of the grid. Drying can damage the ultrastructure, and a dry grid surface adsorbs dirt when put on the next drop. When that drop contains immunoglobulins or gold particles, these will be adsorbed to the dry grid as well, resulting in high background labeling. On the other hand, the backside of the grid should remain dry throughout the procedure. If a grid accidentally sinks and one wants to save it, transfer it into distilled water. Then dry the backside by carefully wiping it with filter paper and let it float, the section side down, first on clean distilled water, before returning it back to the incubation procedure. All incubations are done at room temperature. Cover the incubating grids with a Petri dish against dust and evaporation, especially during the relatively long incubations on small drops of immunoreagents.

49| Wash with PBS + 0.1% wt/vol Gly (5 × 2 min).

50| Wash with PBS + 0.1% wt/vol BSA for 2 min.

51| Incubate with specific antibody diluted in PBS + 1% wt/vol BSA for >20 min. Use the dilution used for immunofluorescence (see Step 33).

52| Wash with PBS + 1% wt/vol BSA (4 × 2 min).

53| Incubate with bridging antibody (e.g., rabbit antigoat, sheep or mouse, or swine antirabbit IgG) diluted in PBS + 1% wt/vol BSA for 20 min.
Note: Step 53 is only required if specific antibodies with weak binding capacity for PA are used in Step 51, like sheep and goat IgG, or mouse monoclonals. Alternatively, Step 53 may be used to enhance the gold signal. However, the bridging step decreases the resolution, increases the background[19] and brings about complications that make double-labeling more difficult.

54| Wash with PBS + 1% wt/vol BSA (4 × 2 min).

55| Incubate with PAG, freshly diluted in PBS + 1% wt/vol BSA for 20 min.
Note: IgG-gold can be used instead of PAG, which may be advantageous, in particular when the specific antibody (Step 51) has a weak affinity for PA. However, the same disadvantages hold as mentioned for the bridging antibody (Step 53).

56| Rinse with PBS + 0.1% wt/vol BSA (2 × 2 min).

57| Rinse with plain PBS (4 × 2 min).

58| Stabilize the reaction by incubating with 1% wt/vol GA in PBS for 5 min.
! CAUTION This step should be done in the hood.

59| Rinse with PBS (2 × 5 min).

60| For double-labeling (**Figs. 1,3**) and triple-labeling (**Fig. 2**), repeat Steps 49–58 with different antibody and PAG combinations.

61| Wash with distilled water (fresh, not from plastic bench-bottles) (~6 × 1 min).

Contrast and drying

62| Float the grids for 5 min on 2% wt/vol uranyl oxalate/acetate, pH 7(refs. 8,16).
Note: The importance of this step is controversial. Tokuyasu introduced it to stabilize membrane lipids. Some say it enhances the final contrast.

63| Pass the grid once briefly over water.

64| Pass the grids for a few seconds over two drops of UA/MC and leave them for 5–10 min on a third drop of that mixture. Use the same two UA/MC rinse drops for all the grids being contrasted at that moment. All the drops should be on ice. Cover the drops carefully.
Note: At low temperature, methyl cellulose is less viscous and penetrates better into the sections, so that the sections become integrated in the dried methyl cellulose film. When this part is not done properly, the methyl cellulose forms a sheet over the sections, which often tears off under the electron beam.

65| Pick up the grid in a wire loop of an inner diameter between 3.5 and 4 mm, by sliding the loop underneath the grid on the UA/MC drop and lifting it from the drop. Now the grid is in the center of the loop with a small volume of UA/MC hanging underneath. Tilt the loop with grid to an angle of 45–60° (grid on top), touch the side of the loop to well-absorbing filter paper and slowly drag along the filter paper until no more UA/MC comes off into the filter paper. A thin even film of UA/MC will be left in the loop, covering the sections on the grid at the center of the loop. The thickness of the remaining film on the grid is dependent on how fast the absorption of excess UA/MC occurs: the slower the absorption, the thinner the remaining film will be. If the film breaks during the sliding on the filter paper, one may return the still moist grid to the UA/MC drop and repeat the process. In most cases though, the absorption is about finished at the moment it breaks. The grid remains adhered to loop at one side and is allowed to dry there.

66| Leave the grids to dry at room temperature for at least 10 min. They can be removed from the loop with fine tweezers.
! CAUTION Be careful with the heavy metal uranium in this part of the procedure. Discard all remnants properly (also see TROUBLESHOOTING at Step 3), including the filter paper containing absorbed UA/MC. It is advised to use a disposable substrate like parafilm sheets.
■ **PAUSE POINT** Store the dried sections in grid boxes at room temperature. They can be kept in good condition for indefinite periods.

● TIMING

Provided that all solutions have been made, the entire procedure takes approximately three to four working days. Of course, this depends very much on the number of preparations to be cut and whether single- or double-labeling is required. An example of a schedule is the following:
Day 1 (Steps 1–3): fixation, 2 h overnight
Day 2 (Steps 4–8): rinsing, scraping of the cells, pelleting and cutting of small blocks, sucrose infusion overnight
Day 3 (Steps 9–26): mounting of blocks on pins, cooling the microtome and knive, cleaning knive, block trimming, cutting and checking of semi-thin cryosections, ultrathin cryosectioning for 1 antibody labeling
Day 4 (Steps 27–66): single- or double-labeling, contrasting

? TROUBLESHOOTING

Step 3

If there is reason to believe that structural artifacts, or faulty molecular rearrangements occur during the fixation at ambient temperature, one might consider freezing before fixation. Freezing should be done under stringent conditions to prevent serious damage of the cells by ice crystals. The most advanced procedure at the moment is HPF. HPF equipment is available from several providers (Leica Microsystems, Vienna; Baltzers, Liechtenstein). Two ways have been described to fix the frozen samples chemically. One approach, section fixation, is also mentioned in the '*Note*' of Step 3. In the other approach, the rehydration method, the frozen samples are dehydrated at –90 °C during cryosubstitution in acetone. Chemical fixation occurs then at low temperature in acetone. After fixation, the samples are rehydrated at 4 °C and further treated as after Step 4. This method is rather new. Although the ultrastructure, in particular of the cellular membranes, tends to suffer from the dehydration step, some protocols are reported, which yielded good results[1,20].

Step 6A(iii)

If for some reason gelatin cannot be used in the procedure, one can try low melting point agarose, but that has to be infiltrated at higher temperature and it is not so easy to wash away after sectioning (Steps 28 and 47). A second alternative is to skip the gelatin embedding and replace the 2.3 M sucrose by a mixture of PVP and sucrose[9,10]. PVP also remains in extracellular spaces, but does not form gels, and is less helpful in making sample blocks.

Step 22

During this process static electricity of the knife can be a serious problem, causing the ribbons to detach from the knife and fly away or, contrarily, they may adhere too strongly to the knife surface and not stretch properly. Some characteristics of the diamond knives may be an important factor in this phenomenon. One manufacturer (Element Six) claims that their knives have a more hydrophilic surface, which counteracts electrostatic interactions between knife and sections. Another provider (Diatome)

designed a regulated ionizer that can be mounted in the cryochamber for tuning the electrostatic forces. Both systems can contribute to a smooth sliding of the sections over the knife surface and substantially facilitate the picking up of stretched sections.

ANTICIPATED RESULTS

For optimal results it is advisable to try different types of fixation, and test concentrations of antibodies and gold probes. FA fixation is a good choice for the localization of membrane proteins. Probably because some extraction of cytosolic proteins occurs during the mild fixation with FA, membranes often stand out in beautiful contrast. Acrolein is the smallest monoaldehyde and rapidly penetrates cells and tissue. It also renders extra contrast to membranes. GA is a strong crosslinker, and is preferred in cases of the detection of soluble proteins. GA fixation results in an overall dense morphology. To achieve optimal contrast, microscopy is best done at a voltage of 80 kV and with a smaller aperture (diameter 20 μm) than commonly used for ultrathin Epon sections.

An important factor influencing the quality of morphology in cryosections is the thickness of the embedding medium, methylcellulose. This varies between experiments, grids and grid meshes. In general, cryosections from cells fixed in PF only are more fragile and need a thicker supporting layer. Furthermore, the thinnest cryosections are not necessarily the best. In cases that sections have spread too much, it is worthwhile to look between section folds where the morphology is often better. Thus it is important to inspect a grid for the most favorable morphology.

Ultrathin cryosections can also be used for other than IgG-mediated specific localizations like mRNA[21], lectins and toxins (see **Fig. 3**).The figures illustrate examples of cryosections from cells and tissue that were double- or triple-immunolabeled with differently sized gold particles.

The components localized and gold particles sizes in nanometers are indicated on the figures.

ACKNOWLEDGMENTS We are very grateful for the long and excellent collaboration with Elly van Donselaar, Janice Griffith, Viola Oorschot and George Posthuma. Their technical skills and devotion to deliver optimal results were essential to set the standards of the above protocols. From outside, our group we like to acknowledge Dr. Gareth Griffiths with whom we had fruitful discussions all over the years. In particular, we thank Dr. Kyoteru Tokuyasu, our master.

Published online at http://www.natureprotocols.com
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. van Donselaar, E., Posthuma, G., Zeuschner, D., Humbel, B.M. & Slot, J.W. Immunogold labeling of cryosections from high-pressure frozen cells. *Traffic* **8**, 471–485 (2007).
2. Roth, J., Bendayan, M. & Orci, L. Ultrastructural localization of intracellular antigens by the use of protein A-gold complex. *J. Histochem. Cytochem.* **26**, 1074–1081 (1978).
3. Geuze, H.J., Slot, J.W., van der Ley, P.A. & Scheffer, R.C. Use of colloidal gold particles in double-labeling immunoelectron microscopy of ultrathin frozen tissue sections. *J. Cell Biol.* **89**, 653–665 (1981).
4. Slot, J.W. & Geuze, H.J. A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* **38**, 87–93 (1985).
5. Slot, J.W. & Geuze, H.J. Sizing of protein A-colloidal gold probes for immunoelectron microscopy. *J. Cell Biol.* **90**, 533–536 (1981).
6. Tokuyasu, K.T. Immunocytochemistry of ultrathin frozen sections. *Histochem. J.* **12**, 381–403 (1980).
7. Tokuyasu, K.T. A technique for ultracryotomy of cell suspensions and tissues. *J. Cell Biol.* **57**, 551–565 (1973).
8. Tokuyasu, K.T. Membranes as observed in frozen sections. *J. Ultrastruct. Res.* **55**, 281–287 (1976).
9. Tokuyasu, K.T. Use of poly(vinylpyrrolidone) and poly(vinyl alcohol) for cryoultramicrotomy. *Histochem. J.* **21**, 163–171 (1989).
10. Liou, W., Geuze, H.J., Geelen, M.J. & Slot, J.W. The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. *J. Cell Biol.* **136**, 61–70 (1997).
11. Griffiths, G. Fixation for structure preservation and immunocytochemistry. in *Fine Structure Immuno-cytochemistry* Chapter 3 26–89 (Springer-Verlag Berlin Heidelberg, Germany, 1993).
12. Tokuyasu, K. & Okamura, S. A new method for making glass knives for thin sectioning. *J. Biophys. Biochem. Cytol.* **6**, 305–308 (1959).
13. Johnson, T.J.A. Aldehyde fixatives: quantification of acid-producing reactions. *J. Electron Microsc.* **2**, 129–138 (1985).
14. Liou, W., Geuze, H.J. & Slot, J.W. Improving structural integrity of cryosections for immunogold labeling. *Histochem. Cell Biol.* **106**, 41–58 (1996).
15. Oorschot, V., de Wit, H., Annaert, W.G. & Klumperman, J. A novel flat-embedding method to prepare ultrathin cryosections from cultured cells in their *in situ* orientation. *J. Histochem. Cytochem.* **50**, 1067–1080 (2002).
16. Griffiths, G., McDowall, A., Back, R. & Dubochet, J. On the preparation of cryosections for immunocytochemistry. *J. Ultrastruct. Res.* **89**, 65–78 (1984).
17. Geuze, H.J. & Slot, J.W. Disproportional immunostaining patterns of two secretory proteins in guinea pig and rat exocrine pancreas cells. An immunoferritin and fluorescence study. *Eur. J. Cell Biol.* **21**, 93–100 (1980).
18. Griffith, J.M. & Posthuma, G. A reliable and convenient method to store ultrathin thawed cryosections prior to immunolabeling. *J. Histochem. Cytochem.* **50**, 57–62 (2002).
19. Slot, J.W., Posthuma, G., Chang, L.Y., Crapo, J.D. & Geuze, H.J. Quantitative aspects of immunogold labeling in embedded and in nonembedded sections. *Am. J. Anat.* **185**, 271–281 (1989).
20. Zeuschner, D. *et al.* Immuno-electron tomography of ER exit sites reveals the existence of free COPII-coated transport carriers. *Nat. Cell Biol.* **8**, 377–383 (2006).
21. Herpers, B. & Rabouille, C. mRNA localization and ER-based protein sorting mechanisms dictate the use of transitional endoplasmic reticulum-golgi units involved in gurken transport in *Drosophila* oocytes. *Mol. Biol. Cell* **15**, 5306–5317 (2004).
22. Möbius, W. *et al.* Immunoelectron microscopic localization of cholesterol using biotinylated and non-cytolytic perfringolysin O. *J. Histochem. Cytochem.* **50**, 43–55 (2002).
23. Möbius, W. *et al.* Recycling compartments and the internal vesicles of multivesicular bodies harbor most of the cholesterol found in the endocytic pathway. *Traffic* **4**, 222–231 (2003).
24. Frens, G. Controlled nucleation for the regulation of the particle size in monodisperse gold suspension. *Nature (Phys. Sci.)* **241**, 20–22 (1973).
25. Zsigmondy, R. & Thiessen, P.A. *Das Kolloidale Gold* (Akademische Verlagsgesellschaft M.B.H., Leipzig, Germany, 1925).
26. Horisberger, M. & Rosset, J. Colloidal gold, a useful marker for transmission and scanning electron microscopy. *J. Histochem. Cytochem.* **25**, 295–305 (1977).
27. De Roe, C., Courtoy, P.J. & Baudhuin, P. A model of protein-colloidal gold interactions. *J. Histochem. Cytochem.* **35**, 1191–1198 (1987).
28. Horisberger, M. & Clerc, M.F. Labelling of colloidal gold with protein A. A quantitative study. *Histochemistry* **82**, 219–223 (1985).