

Ultrathin cryo-sectioning and immuno - gold labeling

A practical introduction



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List of abbreviations:

APD: Average Particle diameter
BSA: Bovine serum Albumin
CV: Coefficient of variation
EM: Electron microscope
FA: Formaldehyde
FCA: Foetal Calf Serum
GA: Glutaraldehyde
IgG: immuno-globulin G
MC: Methyl cellulose
PAG: Protein a-gold
PB: Phosphate buffer
PBS: phosphate buffered saline
PVA: polyvinyl alcohol
TA: Tannic acid
UA: Uranyl acetate

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1. General Introduction.

Immuno-cytochemical approaches are used to add detail at the molecular level to morphological studies. Specific immunoglobulins, usually of the G type are bound in situ to antigenic macromolecules, to which they are directed (the antigens). A marker attached to a specific IgG can be observed by EM, and indicates where the antigen is located. There are many different ways to carry out immuno-cytochemical localizations, all of which operate within technical limitations. Restrictions are set on the one hand by the need for preservation of the ultrastructure and on the other hand by immediate and complete immobilization of the antigen molecules. These requirements can be met by chemical fixation of cells or tissues using reagents like glutaraldehyde and formaldehyde. On the other hand, the denaturing effect of these fixatives on the antigens can reduce, or obliterate their binding with the specific IgG, resulting in a severe decrease of the immuno-labeling. In addition, cross-links between protein molecules that are induced by the fixative hamper the accessibility of the tissue (cellular) elements for the specific IgG and immuno-markers. In other words, good morphology and faultless localization require a tissue treatment that can destroy a significant part of the immuno-reaction. Compromises in this conflicting field are achieved in different ways. At the Cell Microscopy Center of the Utrecht Cell Biology Department we specialized in the approach of ultracryotomy. With that method we think that accessibility of antigens is optimal, since the immuno-reaction is done on ultra-thin sections of non-embedded materials. Damage during tissue processing is minimal. The technique is compatible with very mild fixation, and other denaturing steps, like dehydration in organic solvents and embedding in resins are not involved. The preservation and visualization of ultrastructural details are very good. This combination of conditions means that cryosections in general offer probably the best chances for high quality of the immuno-reaction and the ultrastructural image.

After Tokuyasu explored the use of thawed cryosections for immuno-EM studies in the early seventies and gave the principals of how to apply and where to go for further development of this technique, we bought in 1977 our first ultracryotome. From then on we have used ultracryotomy on a regular base in many EM-localization studies. In parallel, we worked on further developments of the technique, often in close contact with Tokuyasu and the EMBL group in Heidelberg, Germany (Griffiths) where such a facility was established a few years later. In many details the technique has improved considerably since these early years. First of all, the ultra(cryo)microtome went through an impressive series of changes which ultimately resulted in the newest instruments that make ultracryotomy a much easier job, in particular with the current use of diamond knives. Then the change to new immuno-markers. Inspired by the beautiful results of Jürgen Roth and colleagues (1978) on resin embedded sections, we (Geuze et al., 1981) started to use gold particles instead of ferritin, which was the first particulate marker applied in immuno-EM (Singer, 1959). This change yielded in the first place a routine double labeling procedure which is of paramount importance for localization studies. Furthermore the high electron density of the gold markers, in comparison to ferritin, brought Gareth Griffiths (Griffiths et al., 1984) to his modification of Tokoyasu's adsorption staining method that is still routinely in use. An other major improvement came up more recently, when Willisa Liou in our lab started to look critically into the damaging effect of the sucrose solution that had always been used for retrieving and thawing the cryo-section. The outcome of that study, in which the entire EM group participated, was a relatively simple modification in the method of retrieving cryosections, but it had a staggering effect on the preservation of the ultrastructure in the sections (Liou et al., 1996).

These are a few high lights from the many improvements that were introduced during the last 20 years and that made the cryo-sectioning for immuno-EM the reliable and powerful localization technique which it is now. This manual is an attempt to give an impression of how the technique is routinely done in our laboratory. It is used during practical courses, but it does not pretend to describe all possible alternatives found in other laboratories and neither did we try to give a complete overview of the literature. More information in these respects can be found, in the first place in the extensive book on immuno-cytochemistry by Griffiths (1993) and second in some recent reviews by Tokuyasu (1997) and Raposo and colleagues (1997).

Many laboratories expressed their interest in the skill that has been acquired in our group around ultracryotomy and immuno-gold labeling. In 1998 we decided to start annual courses on these issues in collaboration with LEICA microsystems (Vienna). Small groups of students receive a very practical training during a 10 days program in our laboratory in Utrecht. The first was successfully held in the fall of 1999. In 2003 we also organized a shorter course of 3 days just covering the Tokuyasu technique and immunolabeling. In the past more than 150 people have joined our course with great enthusiasm. We hope to extend this series in the future as a mean to communicate further developments in this field to our colleagues.

Utrecht, 1-7-2006

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2. Fixation

The aim of fixation is to (1) maintain the structural integrity of tissues and cells so that they can be studied in the electron microscope. Immuno-electron microscopy adds some additional demands: (2) The substances under investigation (antigens) must remain recognizable for an antibody. (3) The antigens should be arrested and immobilized at their natural site. (4) The antigens should stay accessible for the immuno-reagents used. Often these demands are in conflict with each other. Structure preservation and maintenance of natural distribution of the antigens need a decent and quick fixation but the same fixation may mutilate the antigenic site so that the antibody cannot recognize it. Furthermore it may intensify the matrix compactness due to cross-linking of cellular components and will hamper penetration of immuno-reagents.

In general, antigens attached to cellular components like membranes or the cytoskeleton will not easily be extracted from cryosections. When working with these naturally fixed antigens, mild fixation may be an advantage since cytoplasmic components are extracted from the sections and the accessibility for immuno-reagents is increased. When quantitative retention of antigens (especially soluble) is required a thorough fixation is essential.

The most frequently used fixation agents for immuno-cytochemistry are the hereafter mentioned aldehydes. One can find a useful survey over what is known about their chemistry in Griffiths' book (1993). These compounds are toxic. They are very reactive and volatile, in particular acrolein. Therefore it is important to handle them only in a fume-hood. They are neutralized by amine groups as in glycine. Therefore it may be a good idea to store them in the presence of some glycine powder.

All fixatives are designed to kill cells, so one should always take the necessary precautions and work in a hood.

2.1. Glutaraldehyde (GA) or pentaandial (O=CH-(CH₂)₃-CH=O), Mwt 100.

GA possesses two reactive aldehyde moieties, which mainly react with lysine residues of proteins. Proteins can be cross-linked internally or to each other. The fixation is irreversible and its penetration rate is relatively slow. The morphology is very well preserved. GA is considered a strong fixative in immuno-cytochemistry. The ultrastructure is stably preserved, but antigen denaturation often occurs.

2.2. Formaldehyde (FA) or methanal (O=CH₂), Mwt 30.

FA has only one aldehyde group that can react with amino acids and cross linking occurs in time due to the formation of methylene bridges. The formation of methylene bridges between molecules is enhanced at higher pH (8 - 8.2) and by using higher FA concentrations (> 5%) (Fox et al., 1985). When higher concentrations are used directly on cells or tissue, swelling may occur. When this appears to be the case the initial fixation can be done at low concentrations (2%) of FA, followed by increasing concentrations. Due to its size the penetration into tissues is very fast. It provides a good morphology especially after prolonged fixation. (8 - 72-hour). However this fixation process is partly reversible and prolonged storage of tissue in buffers without fixative may result in a loss of structural integrity. FA is considered a mild fixative. Due to that, it used to be a problem to preserve the ultrastructure in FA-fixed cryosections, but the introduction of new pick up procedures (see section 5.3) has improved that substantially. For good immuno-reactions FA is in general a better fixative than GA. Extraction of materials and less cross-linking favor penetration of immuno-reagents and the antigens themselves are less denaturated.

GA and FA can be used in various combinations. For instance our standard fixative is 2% formaldehyde + 0.2% glutaraldehyde. The effectivity of such mixtures is not an addition of reactivity of both components. Both aldehydes will compete for reactive groups. Therefore the mixture may result in stronger fixation than from 2% FA, but cross-linking may be less than when 0.2% GA is used without adding FA.

2.3. Acrolein, (propenal), O=CH-CH=CH₂, Mwt 56.

Acrolein has one reactive aldehyde group that mainly reacts with lysine. Furthermore the double bond between the carbon atoms may play a role in cross linking. Its fixation is irreversible and its penetration into tissues and cells is fast. Acrolein is mostly used in mixtures with GA or FA.

2.4. Other conditions affecting fixation quality.

2.4.1. pH.

The pH should be maintained within a physiological range during the fixation process. It is known that the aldehyde fixatives affect the pH when they react. Therefore the buffering capacity of the fixative solution should be strong. It is better to do the fixation in the presence of 0.1M phosphate buffer than in PBS. Some commonly used buffers like TRIS may be less suitable during fixation. Our concern is that the amino groups will quench the aldehyde action. Furthermore substances can be added to the buffers, that favor the preservation of specific cellular components like cytoskeleton elements (see cytoskeleton stabilizing buffers).

Suitable buffers are phosphate buffer 0.1M, pH 7.4, HEPES (0.1M, pH 7.4) or PIPES (0.1M, pH 7.4).

2.4.2. Temperature.

The initial fixation should be performed at room temperature or even at 37°C. At those temperatures the structural integrity of the cytoskeleton is better preserved. Furthermore the diffusion of the fixative into the tissue or cells is faster and the actual cross-linking will be more effective. After the initial fixation the specimens can be stored for longer periods of time at 4 °C .

2.4.3. Osmolarity.

A physiological osmolarity for a fixative would be 360 mOsm. Some fixatives like FA can increase the osmolarity to a great extend. For example 8% FA in a 0,1M phosphate buffer has

an osmolarity of 1300 mOsm. However the effect on cells is not as profound as these figures may suggest. First, the aldehydes are presumably uncharged when added to the cells and therefore they can pass freely through biological membranes. Second they start to react rather quick with amino moieties and therefore do not contribute as much as anticipated based on the molarity to the final osmolarity. However when rather slow reacting substances are used like FA, osmolarity might be taken into account (see also 2.3).

2.4.4. Duration of fixation.

The time during which specimens should be fixed depends on the nature of the antigen and the used fixative, its concentration and the temperature. Usually, 2 hr at room temperature is sufficient for fixation for GA containing fixatives. FA (without GA or acrolein) fixation is usually performed overnight. Prolonged fixation does not necessarily improve the ultrastructural preservation, but may affect the antigenicity of antigens under investigation. Furthermore prolonged fixation may harden the specimen and affect the sectioning properties.

2.4.5. Fixation procedures.

It is important to disturb the material to be fixed as little as possible prior to fixation. For instance do not wash cells when it is not necessary. Particularly ice cold media or even PBS will influence the cell's morphology. Rather leave them in the growing conditions and add fixative (see below). Also the fixative should be at temperatures of 20 -37 °C, at least at the beginning. Before perfusion fixation of tissues remove the blood with solutions at 37 °C and follow with the fixative, at room temperature, as soon as possible

2.4.6. Commonly used fixatives in immuno- EM.

The most widely use fixatives are:

1. 4% FA in 0.1 M PB, 2-96 hr.
2. 2% FA + 0.2% GA in 0.1 M PB, 1-2 hr

Further more many other combinations have been used in the past like:

1. 2% FA in 0.1 M PB, 2-96 hr.
2. 2>4(>6>8)% FA (10 minutes each step) in 0.1 M PB, 1-96 hr
3. 1% Acrolein + 0.1% (or 0.5%) GA in 0.1 M PB, 1 hr.
4. 2% FA + 1% Acrolein in 0.1 M PB, 1-48 hr.
5. 2% FA + 0.5% GA in 0.1 M PB, 1-2 hr.
6. 2% GA in 0.1 M PB, 2 hr.

PB = sodium phosphate buffer, pH 7.4. Evidently PB can be replaced by 0.1M PIPES, HEPES or PHEM buffer.

2.5. Handling of specimens

2.5.1. Cell suspensions.

Suspensions of bacteria, yeast and non-sedentary cell lines are preferably mixed gently with identical volumes of double concentrated fixative at culture temperature (normal strength buffer). Let the cells sediment, if necessary helped by gentle centrifugation (< 200 g). Remove the supernatant and resuspend the pellet in 1-1.5 ml fresh single concentration fixative and transfer to an Eppendorf tube for further handling. Usually, a minimum of 500.000 cells (bacteria 10 times more) is required to get a clearly visible pellet in an Eppendorf vial.

2.5.2. Sedentary cells.

Cells are grown in 6 cm Petri dishes. Replace the culture medium gently by 3 ml fresh medium the day before fixation. To start the fixation add 3 ml double strength fixative (normal strength buffer) at ambient temperature and mix gently, but quick. After one or two minutes, refresh with single strength fixative. After fixation, for harvesting, the cells are washed with PBS and PBS/gly and covered with 1-1.5 ml of 1% gelatine in PBS at 37 °C or room temperature, scraped from the dish with a rubber policeman or cell-scraper and transferred to an Eppendorf tube for further handling.

2.5.3. Tissues.

The main problem when fixing tissues is speed. Each delay for the fixative to reach the cells in the tissue of interest may hamper the ultrastructural preservation and induce serious relocation of antigens.

When possible, a "whole body perfusion" is performed. After sedation with a barbiturate (for example Nembutal, 20-30 mg/kg body weight) the abdominal cavity is opened and a needle is inserted in the abdominal aorta. An incision is made in the inferior vena cava close to the liver and the perfusion pump is started. First, to remove most of the blood, the vascular bed is flushed with PBS to which an anticoagulant can be added to prevent clotting. Then the fixative follows. During the flush ~1.5 times the blood volume (10ml for a 200g rat) is passed through the circulation in 0.5-1min. Fixative follows with the same flow for 5-10 min.

Another possibility is perfusion via the left ventricle. In that case the chest is opened, which stops the breathing and a needle is inserted into the left ventricle. The next steps are identical to the previous mentioned procedure. In this procedure the breathing stops as soon as the chest is opened, which urges the operator even more to handle the subsequent steps as quick as possible. On the other hand inserting the needle in the ventricle is easier than in the aorta, especially in small animals.

Tissue with poorly developed vascular beds should be quickly excised and be fixed by immersion. Similarly, human biopsies should follow the quickest route possible to the fixative and cut into small fragments as soon as being immersed in it.

2.5.4. Storage of fixed specimens.

After fixation with FA, FA/GA or FA/acrolein mixtures, 1.0% FA is usually added to the storage buffer. This is after a suggestion by Tokuyasu, who found the FA reaction with tissue constituents is partly reversible. After fixation with GA specimens should be OK in buffer for quite long periods, but even then, for reasons of uniformity and as a preservative, we usually add 1.0 % FA. Storage over longer periods of time in FA seems to harden the tissue which may improve the cutting conditions. It is possible to store tissue in FA for years, without any noticeable changes in morphology and antigenicity. Jan Slot once said: "Fixed cells never die".

Specimens can also be stored for a prolonged period of time in 2.3 M sucrose in a buffer at 4 °C (see section 3.2). This may affect the sectioning properties of the specimens. Some experiences indicated that the tissue becomes more brittle. Tissue can be stored indefinitely in 2.3 M sucrose while frozen in LN₂.

2.5.5. Shipment of fixed specimens.

During long distance shipment by plane, the fixed sample may become frozen and the ice crystals may cause substantial damage to the ultrastructure, in particular when only formaldehyde has been used for fixation. Therefore it is advisable to ship them in >1.8M sucrose, to which 1% FA can be added. Sometimes the tissues are sent together with immuno-reagents. Make sure that they are well separated from each other, for example in different Falcon tubes.

3. Support and cryoprotection

3.1. Extracellular support.

It has great advantages during sectioning if void spaces are filled with a substance which more or less has the same consistency as the cells or tissue. Therefore cells and tissues are prior to cryoprotection embedded in gels or cross linked meshwork's of macromolecules such as agarose, albumin, fibrinogen or gelatine. This serves several purposes:

(1). Cell suspensions are well concentrated and the blocks are easy to prepare and handle.

(2) The embedment improves the conditions for sectioning substantially, probably by equalizing the intra- and extra cellular spaces with regard to the presence of macromolecules.

(3) In sections of cell suspensions and to lesser extent in loose tissues, without embedding cell profiles float around individually when thawed on the pick up solution. When embedded in a meshwork they behave more coherently, which is particularly important for serial sectioning or correlative light and immuno-electron microscopy.

In general we use gelatine as supporting matrix. However low melting point agarose also is suitable especially when part of the specimens have to be osmium fixed and embedded in a resin.

3.1.1. Gelatine .

Gelatine comes in many "flavours". The source (pig-, cow-, fish- etc. skin) and the way it was treated and purified determine the stiffness of the final gel. Usually this is expressed as Bloom figure: the higher the Bloom figure the stiffer the gel will be (when the same percentage is used). The relatively high mol weight of the denaturised collagen prevents it from entering fixed cells. We use food quality gelatine, which is compatible with all the buffer systems we have tested till now.

3.1.2. Gelatine embedding of tissue

After fixation small blocks (< 1 mm³) or thin sheets (thickness ~ 1 mm) are prepared using a sharp razorblade. The blocks or sheets are rinsed in PBS/gly for 10 min. Infiltration with 10% gelatine in a buffer is done at 37 °C during 15-30 min while gently swirling the specimens. For good penetration of the gelatine the infiltration is often done in three steps, in 2, 5 and 10% gelatine successively. Each step 10-30 min at 37 °C. Then the gelatine is allowed to solidify at 4 °C. In case of loose tissues, like lung, the solidifying may be done under slight pressure, for instance by covering a small drop of gelatine containing some tissue fragments with a cover slip before lowering the temperature. Capillary forces are sufficient to pull away most gelatine so that tissue elements become more densely packed in the block (see fig 13.5 A).

3.1.3. Gelatine embedding of cell suspensions.

After fixation the cells are pelleted in an Eppendorf centrifuge. The fixative is removed and the cells are rinsed twice with PBS and 3X with PBS/Gly. The supernatant is removed and the pellet is resuspended in 1 ml 12% gelatine in buffer. After 10 min at 37 °C the cells are pelleted by centrifugation and the gelatine can be solidified on ice. Then, the tip of the tube that contains the pellet, is cut off with a stainless steel Teflon coated razor blade. Some 2.3M sucrose is convenient for lubrication when separating the gelatine embedded pellet from the tip of the tube. Keep the temperature strictly at 4 °C and prepare small blocks from the pellet. The centrifugation results in a

high density of cells in the final specimens. If the cells originally had a discoid shape, the cells are stacked perpendicular to the g forces (see fig 13.5 A)

3.1.4. Cell monolayers on filters.

After fixation in situ the filters are removed from their holder and cut into small square pieces (<1x1 mm). Due to the different sectioning properties of cells and filter the cells are often broken away from the filter during sectioning. This problem can partially be overcome by using a PVP/sucrose infiltration (8 to 18 hr at 4 °C). After infiltration small squares (<1x1 mm) are put upright (one single, or a few together) on a specimen holder and are frozen immediately in liquid nitrogen. Alternately, the filter fragments can be embedded in 10% gelatine, like in section 3.11 and then cryoprotected as in section 3.2.

3.2. Cryoprotection.

It is important to prevent ice crystal formation in the specimens during freezing. First of all that would damage the ultrastructure, but besides that, good cryosections can only be cut from vitreous frozen blocks. This makes freezing of fresh biological material a real problem. Ice crystal formation can only be prevented when freezing occurs within milliseconds. Heat conductance becomes the limiting factor with the result that in practice vitreous freezing is restricted to the very surface layer of, at best, ~10µm. This can be extended to a few hundred microns under high pressure conditions.

Working with chemically fixed tissue, as we do in the Tokuyasu technique, these freezing problems of ice crystal formation are simply circumvented by infusing sucrose into the tissue. Sucrose is a rather inert, hydrophilic compound that easily diffuses through the cellular membranes after fixation. It does not seem to affect the fixed tissue, even not at the highest possible concentration of 2.6M. Tokuyasu introduced the sucrose infusion and later it was shown by Griffiths et al. (1984; McDowall et al., 1983) that sucrose solutions of 1.8M or higher vitrify when they freeze, no matter how slow the freezing takes place. In sucrose infused tissue however, the cellular constituents and the extra cellular gelatine probably also contribute to the vitrification so that lower sucrose concentrations may be sufficient for proper cryoprotection. This can explain why Tokuyasu had good results with regard to cryoprotection when he used sucrose concentrations below 1.8M. He recommended initially 0.6 - 1.5M for different tissues, depending upon their protein density.

However, sucrose serves an other goal as well. Around 1980 we found that infusion of the tissue blocks with 2.3M sucrose improved the sectioning characteristics substantially (Geuze et al., 1980) for any tissue and cell preparation that we worked with. This high concentration of sucrose renders a plasticity to the frozen blocks that, when the sectioning temperature is dropped a little further, allows cutting of very thin sections that are still flat and glossy. Since then our lab uses 2.3M sucrose routinely for cryoprotection, usually buffered with 0.1M PB, but the buffering is not very critical at this stage. Alternately, also to improve sectioning, it is suggested to mix sucrose with polyvinylpyrrolidone (PVP). However, in our hands PVP appeared not to bring much improvement (with some exceptions, see 3.1.3). This is understandable since PVP is added with the idea to improve sectioning conditions mainly in the extracellular spaces (Tokuyasu, 1998). For that purpose we applied a gelatine meshwork (see 3.1), and it is doubtful if the long PVP molecules can readily penetrate into these gelatine filled spaces.

3.2.1. Sucrose infusion.

Small blocks of desirable shape and size are trimmed with a razor blade from the specimens. Since these are usually embedded in non-fixed 10% gelatine we have to do the shaping in the cold room or on ice. Cubic blocks with edges up to 0.5 mm are fine for thin sectioning. When specific tissue elements must be found, like glomeruli in kidney cortex or special airway elements in lung, one might start with substantial larger blocks and trim it down to the wanted elements after screening the tissue by LM in semi-thin sections (section 5.1). After shaping, the blocks are immersed with 2.3M sucrose in little vials (~1 ml) and incubated at 4 °C for

at least 4 hrs on a rotating table. Rotating speed, rotation angle and the air volume above the sucrose are best chosen so that the air is not fixed in the top part of the tube when rotating. That might cause specimens being trapped against the wall or the cover of the tube, in the air compartment where they may dry out to some extent. Try to make the air bubble small enough so that it moves from top to bottom during rotation which helps to agitate the sucrose solution. We leave them there usually overnight.

2.3M sucrose infusion in tissue that is not embedded in gelatine can be done at room temperature and takes at least 30min. This should be longer if PVP is added to the sucrose. It may be better to do the incubation overnight in that case.

4. Mounting and freezing.

4.1. Specimen holders

The copper or aluminium specimen holders should fit perfectly in the microtome otherwise the sectioning will be very irregular. Usually they are provided with the microtome by the manufacturer, however ordinary rivets seem to perform just as well and are much cheaper (see also remarks in section 4.3). The top of the specimen holder should be roughened, with a sharp metal point. This improves the grip of the specimen to the surface. Thereafter we sonicate them in acetone or 100% alcohol to remove any greasy material and metal remnants. With a binocular we check whether the specimen holders are perfectly clean and if no metal remnants are left behind. These remnants may ruin a precious diamond knife. The specimen holders can be reused after thorough rinsing in distilled water.

4.2. Mounting.

The specimen blocks are removed from the 2.3M sucrose by means of forceps or a tiny wire loop. Then they are placed on the table of the clean specimen holders. Be careful not to touch the table surface with anything except the specimen, in particular not with your fingers. 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. Sometimes the specimen has to be sectioned in a specific orientation, for instance cross sections of muscle fibres or kidney medullar tubules. It may be advisable in that case to cut the blocks in such a way that orientation still can be recognized, for instance elongated in the direction, perpendicular to the wanted sectioning direction. The final trimming of the blocks can then be done right before the mounting, so that you still remember which side of the block has to be the cutting face. The mounting procedure should be done as quickly as possible. Water evaporates from the specimen during air exposure, and then sucrose concentration becomes so high that sucrose crystals will be formed. For that reason we usually do this part, like the previous steps, under a dissection microscope in the cold room. If you do not have a cold room available, use ice and freeze your blocks one by one.

4.3. Freezing and storage of frozen specimens.

The mounted blocks, glued by the remaining sucrose to the specimen holder, are then put in LN₂ or in the cryochamber. We use Biozym cryotubes for storage, its caps fitted with two holes. The holes in the top of the cans are to prevent internal pressure and form also a grip for some firm tweezers during the opening or closing of the cans. An average LN₂ container can store 6-10 canisters, each of which can hold 6 of these aluminium cans. Each can contains an aluminium block, which prevents them from floating. Seven 8 mm holes, drilled in the metal block form seven numbered compartments, each of which can contain at least 8 pins with specimen blocks, we often use colour coding for the pins as well. In this way ~3000 specimen blocks can be stored in one LN₂ container.

When the specimens are relatively big, copper or, to a lesser extent, aluminum specimen holders cool down much quicker than the specimens and crevasses may emerge between the holder and the specimen. The same holds true if the top of the holder has not been cleaned properly (grease). During sectioning chattering, irregular sectioning will occur, in the worst case the specimen may break away from the holder. When this occurs it is possible to remount the specimen, since freezing is no longer a crucial step. Remove the specimen from the holder, put it back in 2.3 M sucrose (4 °C), leave it there for 30-60 min and remount. When cracks between specimen and holder remain a problem, it might help to freeze the specimen more slowly, for instance by not using LN₂, but the cold (-120 °C) environment in the cryo-chamber for freezing.

The sucrose needs to remain clear after freezing. If it turns milky the sucrose concentration is too low and water crystals have formed. This will affect the sectioning properties of your specimen. If the crystals have not damaged your specimen, renewed infiltration with "real" 2.3 M for 4 hr will solve this problem.

Frozen specimens (on specimen holders) can be stored in liquid nitrogen for months or even years. Prolonged storage in liquid nitrogen may freeze-dry the specimen surface to a certain extent. If this renders sectioning more difficult, remounting the specimen solves this potential problem.

5. Sectioning.

5.1. Initial trimming and sectioning of semi thin sections.

Follow the instructions in the microtome manual to install the knife and cool down the microtome to the desired temperature. When the microtome is stably adjusted to the chosen temperature, the pin with the frozen specimen is transferred to the cryo-chamber in the LN₂ filled aluminium can and fixed properly in the microtome arm. Semi-thin sections can be cut successfully from relatively large, rectangular block faces. Approach the front of the specimen carefully with a glass knife, align the long edge of the block face perpendicular to the knife edge and flatten the front by sectioning at relative high temperature (knife, specimen and chamber temp set at -90 to -100 °C) at a speed of 100 mm/s. Semi-thin sections of about 0.15 - 0.2 µm are cut using a glass knife or trimming diamond. Thicker sections may cause irreversible damage to the specimen. Chips may break away from the specimen or even the entire block can come off from the specimen holder especially if the specimen is surrounded by a lot of gelatine. The first sections will have a 'snowy' appearance and mostly consist of plain sucrose. When shiny coloured sections begin to appear, the actual tissue is being sectioned. Do not allow any snowy spots in the semi-thin section. Once the face of the block is nicely trimmed, retrieve a section from the knife with a 2.3 M sucrose droplet in a stainless steel loop (diameter 3 mm, steel wire diameter 0.3 mm mounted on a 15 cm long bamboo stick (see 13.5) or use the "perfect loop" from Diatome). When the droplet enters the cold nitrogen atmosphere it will start 'smoking'. When the smoking has stopped, push the almost frozen sucrose droplet against the section. This will stick to the droplet and stretch. Remove the droplet from the nitrogen atmosphere and melt it. Next touch the droplet with the section faced downwards on top of a microscope slide. Then retract the wire loop. The section will stay on the slide and can be stained for light microscopical inspection with for example 1% toluidin blue in distilled water. Do not use alcohol or other solvents in the staining solution, because the sections will be removed from the slide.

For routine inspection the shape of the block face is not very critical. Toluidin blue stained sections will give information as to whether one can go on to thin sectioning.

5.2. Mounting of LM-sections.

When the semi-thin sections are used for immuno-fluorescence (IF) studies it is good to trim the block edges, for instance by using a small piece of razor blade, held in an arterial

clamp. Less fast but more precise is mesa trimming either on the corner of the glass knife or a (diamond) trimming blade. Sharp even edges of the block face make it much easier to cut the sections in ribbons.

For IF, sections are placed on 3-aminopropyltriethoxysilan coated microscope slides. With a diamond pen we scratch lines of ~1.5 cm perpendicular to the length axis of the slide. One slide can carry 3 scratches at ~2cm distance from each other. After the scratching, make sure that all glass splinters are removed with a paint brush or compressed air. 3 or 4 pick-up drops with semi-thin sections can be placed alongside a scratch (scratch and sections at the same side of the slide). Two different specimens needing identical immuno-staining can be placed on each side of the scratch. For different labeling procedures sections have to be placed along different scratches. A fine wax pen is used to demarcate the area with sections around a scratch. The wax line prevents the incubation drops from pulling away from the scratch area. The sections with sucrose can be stored in a cool place for several days prior to immuno-labeling (see section 6.13).

5.3. Fine trimming.

After LM-inspection, the block face has to be trimmed carefully to a smaller size for ultrathin sectioning. The smaller the block the easier the ultrathin sectioning will be. In contrast to plastic-embedded blocks, which are usually pyramid-like with a trapezoid face, we prefer for cryosectioning a rectangular block face, trimmed perpendicular at the sides. The edges should be very straight, without irregularities and with opposite sides perfectly parallel. Normally, block faces of 0.6-0.4 x 0.3-0.4 mm are suitable for thin sectioning. The trimming of the sides needs to be done more precisely than for semi-thin sectioning. Experienced people with steady hands may still make use of the razor blades and do it manually. However for those who feel less confident about their skill the so-called mesa trimming is recommended. In that method the trimming is done with the microtome. A 45° glass knife with a right angle corner is used to cut away the specimen material at one side of the planned block face, just like the first face trimming was done: ~ 0.2 µm thick sections are cut with the instrument at very high cutting speed (100 mm/s). When the sectioning is deep enough (50-100 µm) the specimen is turned 90° and the procedure is repeated until at all sides of the block are trimmed. This method is more time consuming than the first one but it requires less experience and yields a perfect rectangle.

The trimming can also be performed using diamond trimming tools. They allow thin sectioning and the sides are specially designed for trimming specimens. The trimming blades with a 90° angle have the advantage that the block edge stays the block edge and the size of the block stays the same. However the knife is not sharpened on the sides, so it does not cut. There are also knives available with a 20° or a 45° side that are sharpened and do cut, so the sides will be like the block face. With these blades a pyramid shaped block is created.

The material that is trimmed off may be a troublesome pollution on knife and specimen. It can be removed carefully blowing it away with nitrogen. Care should be taken not to introduce frost in the system, which will happen when the warm air from outside the cryobox is used, since it contains water vapour. Alternatively LN2 can be poured on the knife and specimen, but that destabilizes the temperature and one may have to wait for it to equilibrate again. Also sucrose drops in the wire loop can be used for cleaning.

5.4. Ultrathin sectioning.

Replace the trimming knife with a perfect glass knife or a diamond knife (see section 8). Make sure that the specimen and knife are tightly secured and set the temperature of specimen, knife and chamber at -120 °C. At this temperature most of 2.3 M sucrose infiltrated specimens will give good results using a diamond knife and cutting 50 nm thin sections. However, sectioning is possible from -85 to -170 °C and some variations should be tried depending on the material one is cutting and the section thickness one is aiming at. When the specimens are very

compact and/or fixed thoroughly slightly higher temperatures than - 120 °C are advisable. With regard to section thickness, thinner sections in general need lower temperature.

Align the knife edge and the block face and approach the knife edge carefully with 0.1 to 0.5 µm steps using backlight illumination. With a perfect specimen and knife, sections ranging from 20 to 100 nm can be cut. Usually the feed is set between 45 and 70 nm: GA fixed specimens usually at the lower and FA fixed specimens at the upper side of that range. The colour of the sections is indicative of the section thickness, just like in plastic sections. They should be shiny as well, if they look “dull” there is probably much compression or chatter. When the sections are regular in thickness over a long period then the actual thickness may not deviate significantly from the setting. However it is very difficult to section without compression. Depending on the geometry of the knife the compression varies usual from 30 to 60%. When the thickness changes in time, (which happened often in the older cryotome types) this indicates that there is something is wrong with the specimen, the knife, or the instrument’s set up or condition. In this case section thickness is only to guess. In particular when the knife is not perfect, the thin ones are often compressed and disregarded with the result that people please themselves with the “good” ones that are actually too thick.

One of the problems is static electricity. Due to the extremely dry atmosphere in the cold nitrogen atmosphere, the knives will be charged during sectioning. As a result the sections may stick to the knife surface and will not stretch properly. On the other hand, when they are touched with an eyelash they may escape into the air. Especially with diamond knives, this can be a problem. To overcome this difficulty a regulated ionizer (Diatome, Bienne, Switzerland) can be mounted in the cryochamber. By careful ‘tuning’ the electrostatic forces the sections will smoothly slide over the knife surface and are easy to handle with an eyelash. The use of the ionizer is not always the solution to the sticking (and thus compression) problem. The knives should be perfectly clean (see addendum). Also, diamond knives from different manufacturers cut differently, and it is advisable to experiment with the ionizer strength and knife clearance angles. Element 6 (previously Drukker International, Cuyck, Netherlands) claims that its knives have more hydrophilic surfaces. This would benefit the sliding of the sections over the diamond surface and make the cutting of flat sections easier.

To be able to make the perfect section, a perfect clean knife is an absolute requirement. A perfect glass knife is rare, but will cut just as well as a diamond knife at cryo-temperatures. However, it takes much experience and time to prepare a perfect glass knife, and therefore we usually prefer a diamond knife, it lasts about one year and saves a lot of time.

5.5. Section retrieval (Pick-up).

Once the ultracryomicrotome produces relatively flat, minimally compressed sections, they have to be retrieved from the knife. Under the right conditions modern ultracryomicrotomes, like the Leica Ultracut S or T/FCS and more recently the FC6/UC6, regularly produce ribbons of sections. Usually the sections are ‘guided’ with an eyelash on top of a wooden stick away from the knife edge. The ribbons are detached from the knife edge and moved aside, somewhere down on the diamond knife or on the glue between knife holder and diamond knife. The size of the ribbons should not exceed about half the diameter of the wire loop used to retrieve the sections (see below), so that the sections have space to spread out over the pick-up drop during thawing. Usually no more than 4 or 5 sections should be included. When enough sections are collected, stop the sectioning and pick them up. The ionizer should be turned off during section retrieval. As described in section 5.1 a wooden stick with a stainless steel loop (loop diameter of 2.5 mm, wire diameter 0.3 mm) is dipped into a pick-up solution. Retracting the wire loop fast out of the pick-up solution, in a direction perpendicular to the plane of the loop, will result in a large drop. Slow withdrawal, sideways, will decrease the drop size. The size of the drop is important since a larger drop will take longer to freeze. We have currently two different pick-up solutions in our laboratory.

The first solution is 2.3 M sucrose in 0.1M PB. The wire loop filled with a sucrose droplet is introduced into the cryochamber. While looking through the binocular, the droplet is brought to a position near, but not yet interacting with the sections. Usually some “smoke” is emerging from the droplet. Once it stops ‘smoking’ press the droplet gently on top of the gathered sections. Do not try to pick up too many sections otherwise the surface of the droplet becomes ‘overcrowded’. Looking through the droplet the sections are visible, and sometimes it is possible to see the sections stretch. Soon after that the sucrose solution is frozen. Remove the droplet from the cryochamber and let the sucrose drop with sections thaw. Most people help the thawing by gently breathing on the drop. The effect of that is questionable and when uranyl or fixatives are added to the retrieval solution (see below) it may be a risky habit. After thawing, press the droplet with the section side down on a Formvar carbon-coated grid. To check the quality of the sections, the sucrose is removed by floating one grid on distilled water. After approximately 5 minutes the grid is removed from the water, air dried and viewed in the electron microscope. If the sections are satisfactory, the other grids can be stored as described in section 5.6

The second solution we use is a 1:1 mixture of 2.3 M sucrose in PB and 2% methyl cellulose in distilled water which is prepared just before use. Prepare this mixture fresh from cold solutions (4 °C) and mix the constituents gently. Mixing at room temperature will give white deposits, which is probably due to withdrawal of water from the methyl cellulose solution by the sucrose before the mixing is completed. We started using this second pick-up solution in 1996 after we found a vastly improved ultrastructure in the sections picked up this way (Liou et al., 1996). Since then this is the standard way to pick up sections in our lab. The viscosity of this solution is much lower and it freezes much quicker. The freezing is obviously crystalline. It is easy to follow under the binocular how it starts freezing at the edge (white rim) of the drop in the loop. Right at that moment the section should be picked up. Otherwise the drop is completely frozen and the sections can not be retrieved. The sections should be parked somewhere away from the knife edge since water vapour is emitted from this drop causing ice deposits on the knife. Picking up of the sections should be done from a non-metallic surface like the diamond itself, a special designed stage or the glue around the diamond knife, but not the knife holder. With this solution the sections do not stretch on the droplet, so what you see is what you get!! This implies that only the shiny flat sections give optimal results. It needs some experience to use this pickup method successfully, but it pays off at the end. The fine structure is much better as compared with sections retrieved with 2.3 M sucrose.

In most cases 2.3 M sucrose or sucrose/methylcellulose are suitable pick-up solutions. However when delicate lipid rich structures are involved these pick-up solutions are not sufficient to retain lipids in a cryosection. An additional “on-section” fixation is necessary to preserve lipid-rich structures and the lipids in membranes. Willis Liou used for these purposes a mixture with final concentrations of 2% methyl cellulose and 2% uranyl acetate in distilled water (Liou et al., 1997). The sections must be of outstanding quality in terms of thickness (50 nm) and flatness. She also had some success by thawing cryosection of fresh, cryo-immobilized material in pick-up solutions containing mixtures of uranyl and aldehyde fixatives. In other words she did the complete chemical fixation on the sections (Liou et al., 1996).

5.6. Storage of thawed cryosections.

After the sections have been put on top of the grid, they can be stored in two ways. First, the grids can be transferred to 2% solid gelatine plates (sections facing the gelatine). The plates are prepared by pouring the gelatine, while fluid into 3.5 cm Petri dishes until they are about half full. For buffering of the gelatine solution see 10.6. To prevent drying out the 3.5 cm plates are stored in Petri dishes at 4 °C in a closed container. Keep the gelatine cool during sectioning. These gelatine plates are convenient for short storage of the grids, up till 24 hrs at 4 °C. If you want to store grids longer the gelatine plates must be kept in a humid environment, furthermore the grids should be made out of nickel, copper grids will turn black due to corrosion.

Recently we introduced storage of sections while still covered by the pick-up drop of sucrose or sucrose/methyl cellulose (Griffith and Posthuma,2002). The grids remain attached to the stickered microscope slide on which they are prepared (see 8.1). In that position the grids with sections are stored in a closed microscope slide storage box at 4 °C. It was found that drying under these conditions did not affect the ultrastructure or immunoreaction.

6. Immuno-labeling.

During the immuno-labeling procedure the sections are incubated over a series of different drops by floating the grids on top of the drops, sections facing the drop. For most rinsing steps drops of 100-200 µl are used which can carry up to 5 grids at once. For antibody and immuno-gold solutions we use 5-10 µl for each grid. The drops are placed on a clean and flat surface. This is easily achieved by using a parafilm sheet that is adhered to a glass plate by some distilled water. The cover sheet of the Parafilm is removed while the incubation proceeds, so that the surface is kept clean. The grids can be transferred by wire loops or by forceps. Using the latter, less of the incubation fluid is transferred to the next drop, making washing more efficient and the adhering fluid may decrease the concentration of the immuno-reagent which are usually in very small drops (5-10 µl). To transfer a minimum of excess fluid, pull the grids sideways and gently from the drops. Excess adhering fluid can be removed with filter paper, but be careful: Never let part of the section side of the grid become dry. Drying can damage the ultrastructure and a dry grid surface adsorbs dirt when put on the next drop. Also, when that drop contains immuno-globulins 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 stay dry throughout the procedure. If a grid accidentally sinks and one wants to save it, wash it in distilled water. Then dry the back side by carefully wiping with filter paper and let it float, first on clean distilled water before entering the incubation procedure again.

For a typical immuno-labeling of the sections, transfer the grids over the incubation media in section 6.1 through 6.11 successively. Routinely all media are buffered by PBS until the washing steps in distilled water. PBS is probably not critical and when needed, other buffers can be used as well. Be aware that grids may corrode when very long incubation procedures are followed (see also section 3.1.2). The incubations are at ambient temperature, unless indicated differently. A short schedule is given in the addendum.

6.1. 2% gelatine/PB at 30-37 °C for 15-20 min.

If sections are stored on gelatine plates warm the gelatine to 40°C and keep them on the melted gelatine for 10 min. The grids, stored on the glass slides with Suc/MC must be transferred to a cold 2% gelatine plate before melting it. Then warm it to 40 °C and keep on the fluid gelatine for at least 20 min, in order to remove the dried mixture of sucrose and methyl cellulose.

6.2. 10-20mM (~0.15%) glycine for 5 min.

6.3. 1% BSA/PBS for 2 min.

Steps 6.1-6.3 are meant to prepare the sections for the immuno-labeling. They quench free aldehyde groups (glycine) and to block other sites where the immuno-reagents may stick non-specifically (gelatine and BSA). Other proteins that may be used as well are: Cold Fish skin gelatine, ovalbumin, 1-5% (fetal) calf serum, diluted goat serum, or 0.1-1% acetylated BSA (Aurion).

An other step we sometimes use is a solution of NaBH₄. However, be careful because there are reports of bad effects on the ultrastructure. Concentrations of 0.1-1% have been used, but the effect of the borohydride step has not been well established in our lab. This may be partly because the compound is very unstable (it is more stable at higher pH, see Tokuyasu, 1997) which could explain the rather inconsistent results. It should be used immediately after being prepared, or even refreshed once during an incubation period which is usually 10-15min. The idea is that it may quench free aldehyde groups, breaks double bonds in molecules induced by (GA) fixation and thus restores antigenicity. Furthermore the tiny hydrogen bubbles may render the sections more open which results in an improved penetration of the immuno-reagents during the incubations. In case of immuno-fluorescence, NaBH₄ treatment can take away auto-fluorescence induced by GA.

6.4. The specific antibody solution in 1% BSA/PBS for >20 min. 5 µl drops.

Anti-sera and IgG preparations are usually diluted in 1% BSA/PBS, but other blocking proteins or buffers can be used as well. The dilution for anti-sera has to be worked out in each different case. Affinity pure IgG preparations are used in concentrations of 2-20 µg/ml. Incubations at 37 °C can be considered. The grids are on 5 µl drops which may dry out easily, in particular when the incubation time is prolonged or when the incubation is at 37 °C. Therefore it is important to cover the drops, for instance by a small petri dish.

In general, the duration of the immuno-incubation is not very critical, the minimum time we use is 20 min at room temperature. One should realize that antigens might be extracted from the thawed sections. That may happen to molecules that do not react with aldehydes, like membrane lipids, but also soluble proteins may escape easily when the fixation is weak (Posthuma et al., 1987). In such cases it is better to keep the incubation short. On the other hand, proteins that are integrated in membranes or bound to the cyto-skeleton will not easily escape and labeling of these may be favoured by longer incubation periods, during which all sorts of soluble compounds may be extracted, so that penetration into the section may improve. When the incubation is extended to the next day we usually keep the grids overnight at 4 °C in a moisture atmosphere. In that case the antibody may be further diluted (~10 times) when non-specific binding becomes a problem. .

6.5. Rinse with 0.1%BSA/PBS; 4 x 2 min.

6.6. Bridging antibody in 1% BSA/PBS; >20 min.

This incubation step is used when: (1) The specific antibody in step 6.4 does not or weakly react with protein A in step 6.8. Many monoclonals do not react with protein A. In most cases mouse monoclonal specific antibodies are followed by a rabbit anti mouse IgG preparation. (2) A signal amplification is wanted. Be aware of the fact that background may increase more than the specific labeling and that the resolution is badly affected by the bridging step (Slot et al., 1989). For amplification of reactions with rabbit specific antibodies we use swine anti-rabbit IgG (Nordic, Tilburg, Netherlands) in dilutions of 1 : 1000-2000 (depending on the batch).

6.7. Rinse with 0.1% BSA/PBS; 4 x 2 min.

6.8. Protein A-Gold (PAG) in 1% BSA/PBS; >20 min.

Like in steps 6.4 and 6.6, the duration of this incubation is not very critical, but some say that after 30 min background levels may go up. PAG is used well diluted in order to suppress the background. According to the size of gold particle used, PAG is diluted so that the optical density, measured over 1 cm at 520 nm, is:

PAG 5 - 8 nm:	OD 0.1
PAG 9 - 12 nm:	OD 0.2
PAG13 - 20 nm:	OD 0.3

At these dilutions the actual protein A concentration is below 1 µg/ml.

An alternative to PAG is IgG-Gold (IGG), in which for instance goat IgG directed to rabbit, mouse, or any other specific IgG is complexed to gold particles.

Reasons why we prefer PAG over IGG:

- Advantage for preparation. Protein A is a well defined molecule. Every preparation gives with PAG reproducible results. PAG tends to form less clusters than IGG.
- In general background levels are lower in PAG labeled sections, except in some specific cases (see below).
- PAG localizations have better resolution than IGG.
- PAG recognizes specific IgG from various species.
- In sequential double label procedures with PAG we found less interaction between the different reactions (co-labeling).

Disadvantages of PAG are:

- Protein A binds less stable to colloidal gold particles. For that reason we make fresh PAG probes every 4 months. Another consequence of the relatively weak binding of protein A to gold is that a very weak gold binding protein is needed as background blocker in the PAG solution. BSA is perfect for that purpose. Do not dilute PAG in gelatine, since gelatine binds strongly to gold and you may end up with a gelatine-Gold instead of the PAG complex.
- There is a chance that PAG binds to endogenous IgGs present in the interstitium, depending on what species the tissues are from. If stronger fixation is not an option and one cannot ignore the extracellular labeling, the use of IGG is recommended unless the specific antibody is from the same species as the tissue, which would make things worse. This is usually the case when monoclonals have to be used in mouse tissues.

For more information on Gold probes, see section 9.

6.9. Rinse in plain PBS 4 x 2 min.

6.10. 1% Glutaraldehyde in PB or PBS; 5 min.

This step should be done in the fume-hood. The aim is an extra fixation of the immunoreagents on the sections before the rinsing and contrast-staining part of the procedure. We noticed occasionally some loss of labeling at this stage, after which GA fixation was introduced as a routine step. However, in many cases of single labeling the GA incubation can probably be omitted as well.

In a double labelling procedure GA fixation is required in between the two labeling sequences. GA inactivates free IgG binding sites for protein A that are introduced during the first round of labelling. GA fixation is less successful in denaturing IgG molecules when a bridging IgG or IgG-gold is used.

The GA treatment can be a problem when the second reaction is GA sensitive. Alternatively FA can be used, However we noticed that this on-section treatment is often not as bad as one would expect for antigens that are killed when GA is used for the initial cell fixation.

There are two possible explanations for that. First, GA sensitivity is often a matter of penetration barriers that are created by the cross-linking effect of GA. It may well be that these barriers can not be formed anymore in a weakly fixed section that has gone through a series of incubations. During these incubations many molecules have been extracted, that could be part of these barriers. Second, if GA can not be used for initial fixation, one usually fixes with FA. Since both aldehydes react primarily with the same groups, largely amines of protein molecules, it may well be that the GA reaction is rather mild with proteins in sections that are prefixed with FA

6.11. Rinse with distilled water; >6 x 1 min.

It is important to remove the phosphate or other ions thoroughly from the sections in order to prevent uranyl precipitates during the final staining and embedding in section 7. Use fresh and good quality distilled water.

6.12. Contrast enhancement

Before drying, the sections have to be stained for contrast and supported by polymers in order to prevent drying artefacts. In our lab staining and support are routinely achieved by final incubation and drying of the grids in a uranyl acetate (UA) / methyl cellulose (MC) mixture. Both ingredients of this, what he called, adsorption staining were suggested by Tokuyasu. The actual procedure was later worked out by Griffiths (1984). For alternative staining/embedding methods see Tokuyasu 1998. The standard procedure goes as follows:

6.12.1. 2% uranyl oxalate/acetate, pH 7; 5 min.

Grids are floated on this solution for 5-10 min. Be careful not to introduce any salts in this or following solutions, this will inevitably yield precipitates.

The purpose of this step is not very clear. Some say it enhances contrast but in case of doubt one might consider to omit it from the procedure. Tokuyasu originally introduced it to stabilize membrane lipids (1978).

6.12.2. Rinse with water 2X

briefly

6.12.3. UA staining and MC embedding.

Go quickly (seconds) over 2 drops of UA/MC, pH 4, and leave the section floating for 5-10 min on a 3rd drop of that mixture. All UA/MC drops should be on ice. MC solutions are less viscous at lower temperature, so that they can penetrate better into the sections. Cover the drops with a Petri dish to prevent drying.

The standard mixture is made by mixing 4% UA :2% MC = 1 : 9. But other concentrations can be tried as well. Higher concentrations of UA result in better negative staining of membranes but also cause a general increase in electron density, which makes the gold particles more difficult to see.

Instead of MC a mix of 4% PVA (mwt 10,000) and 4% UA (9:1) can be used.

6.13. Drying.

Pick up the grid in a wire loop with an inner diameter between 3.5 and 4 mm. Push the loop in the UA/MC drop at some distance from the grid, bring it underneath and lift the grid from the UA/MC drop. Now the grid is in the center of the loop with excess UA/MC hanging underneath.

Tilt the loop and grid to an angle of 45-60° (the excess UA/MC downwards) and touch the side of the loop to well absorbing filter paper (for instance Whatman no 50) with the sections facing the filter paper. The amount of excess UA/MC should be enough to make contact and disappear into the filter paper, leaving behind a thin even film on the surface of the grid, which remains in the loop centre. When too much UA/MC comes with the grid when picked up, the contact and absorption to the filter paper will be too vigorous and the grid may float away. The amount of excess is easily controlled by picking up the grid more or less sideways and gently from the UA/MC drop. The thickness of the remaining film on the grid is depending on how fast the absorption of excess UA/MC occurs, i.e. the more the absorptive capacity of the filter paper, the thicker the remaining film. When the drain of UA/MC into the filter paper stops (meanwhile, the loop can be moved sideways over the paper), the grids are allowed to dry at room temperature for at least 20 min. They can be removed from the loop with fine tweezers.

Be careful with the heavy metal uranium in this part of the procedure. Discard all remnants properly. Think of the pieces of filter paper with absorbed UA/MC.

6.14. Double labeling.

For double labeling, after the GA step in section 6.10, rinse the sections with PBS (2x 1 min) and 0.1% glycine/PBS; 5 x 2 min. Then repeat the steps described in section 6.3 through 6.10 for labeling a second antigen (section 6.4) with a different size PAG (section 6.8). As mentioned in section 6.10, it is important that bridging antibodies used for this stage of the double labeling procedure are not reactive to the IgG of species used in the first labeling. Therefore it is often not possible to use 2 monoclonal antibodies in this double labelling procedure. Only mouse IgGs of type 2a, which can be labeled directly with PAG without using the bridging antibody are suitable for double labeling in this sequential procedure.

Even within the limitations set by obvious cross reactions between the 2 antibodies one occasionally encounters less understood interactions between the first and second labeling. Sometimes the second gold sticks to the first, even though no bridging antibody is used. Most likely even after the GA treatment, protein A still has some affinity to particular subclasses of immuno-globulins. We also encountered cases where the first gold sticks to some extent to the second antigen. This can only be explained if (part of) the first immuno-complex dissociates from its initial site and binds again at some stage during the second labeling procedure via the second specific antibody. Furthermore, it is a not uncommon phenomenon that the labeling intensity for one or both of the antigens is lower in the double labeling than in single labelled sections. Specific characteristics of each different antibody preparation make that these problems are not always as manifest. To deal with them, it is important to always reverse sequences of labeling for the two antigens and also to take into account observations in parallel, single-labeled sections when you evaluate double-labeling patterns.

A very good alternative for the sequential procedure is based on IGG labeling and can be applied when both specific antibodies are generated in 2 different species. For instance: anti-A from mouse and anti-B from rabbit. They are labeled by 2 different IGG probes: goat anti-mouse IgG-Gold 5 nm and goat anti-rabbit IgG-Gold 10 nm respectively. This double labeling can be performed conveniently in one labeling round: In section 6.4 the grids are incubated in a mixture of anti-A and anti-B and in section 6.8 one uses a mixture of the 5 and 10 nm IGG probes.

6.15. Immuno-fluorescence labeling.

Semi-thin sections on glass-slides (section 5.1) are labeled for IF by essentially the same sequence of incubations as described for EM in section 6.1 through 6.9 and finally 6.12, except that PAG is replaced by a fluorochrome coupled to a secondary IgG (e.g. Alexa 488-goat

anti-rabbit IgG from Jackson Labs. is used for all our polyclonals). When the specimen is fixed with GA, the sections are treated with 0.1% borohydride (1 mg/ml) in PBS for ~10 min.

For IF staining we cover the wax outlined areas (section 5.2) with incubation medium in the labeling steps (section 6.4, eventually 6.6 and 6.8). ~20 µl should be enough. The washes in between and after can be facilitated gently by Pasteur pipette. Be careful not to flush antibodies over neighbouring areas with sections that are labeled for different antigens.

After the final wash with distilled water, make the surface nearly dry, place tiny drops of Mowiol (or Vectrashield) in each area with sections and place a coverslip over the sections. Carefully avoid air bubbles and too much Mowiol. Let the Mowiol spread out for an hour or so and then seal the rims with nail polish. The slides can be stored in the dark at 4 °C for months.

6.16. Controls.

For checking non-specific labeling induced by the bridging antibody, or the PAG or IGG probes, sections done in parallel should be treated in identical incubation procedures as the experimental grids, replacing the specific antibody (section 6.4) by an antibody known to be non-reactive to the specimen.

Checking for non-specific labeling induced by the specific antibody can best be done by processing and labeling tissue or cells that are similar to the specimen, but are lacking the antigen. This is a feasible control in all studies on transfected gene products or when “knock out’s” are available. The second best control is probably to adsorb the reactive antibodies to pure antigen preparations. However there are some pitfalls here. If the antigenic sequence, or part of it occurs in other proteins, these spurious reactions will be quenched as well in the adsorption test. So, it is important to accompany these tests by electrophoresis and immuno-blotting. This does not always work, antigen molecules in solution may have different three dimensional properties than the molecules in the sections. Therefore, it may be that some sub-populations of the antibodies do not bind to the free antigen and still give some reaction on the sections, in which case one might confuse the specific reaction for a spurious one. If you incubate sections with pre-absorbed AB solutions we often found huge clusters on our sections.

For checking non-specific labeling induced by the bridging antibody, or the PAG or IGG probes parallel sections should be treated in identical incubation procedures as the experimental grids, replacing the specific antibody (section 6.4) by an antibody of the same species known to be non-reactive to the specimen.

7. Knives.

Cryosectioning demands a perfect knife, which can be either a diamond or a glass knife. Nowadays cryo-diamond knives are of excellent quality. Although rather expensive they save money at the same time. The sectioning properties of a diamond knife are outstanding and remain good for as long as a year. Even facing and shaping the specimen can be done with ‘blunt,’ or ‘old’ diamond knives (thus giving them a second life as trimming knife), with semithin diamond knives or with specially designed diamond trimming knives.

However, ultrathin sectioning and semithin sectioning can also be performed with a glass knife. The quality of a well made glass knife is just as good as a diamond knife, but its sharpness does not last very long.

7.1. The art of making glass knives.

The preparation of a glass knife starts by selecting good glass rods from which they are made. The glass should be tough and preferably without consistency differences in the glass (Pittsburgh glass). When a new batch of glass rods arrives, the dimensions and quality should be checked. The width of the rods must be constant. Moreover in the lateral sides of the rods distortions cannot be tolerated. If the rods do not meet your requirements send them back to the manufacturer.

The best way to make knives is according to Tokuyasu's balanced break method (Tokuyasu and Ukamara, 1959) using a Leica knife maker. In theory a perfect knife is made by breaking a glass rod in two equal parts, next each part is broken in two equal parts etc., till squares of 2.5 x 2.5 cm are produced (Fig. 7. 1.A). This balanced breaking method yields squares, with freshly broken surfaces perpendicular to the upper and lower surface of the square because equal forces have been applied to the glass during the breaking. However, in practice it is virtually impossible to produce squares using this method due to imperfections in glass dimensions and tolerances in the knife maker. Therefore the method has been adapted by Griffiths. He first makes double squares in a row from a glass rod (fig. 7.1.B) The freshly broken surfaces will not be perfect, but when a double block is broken into two squares the forces applied to each side will be identical thus ensuring at least one side of the square has a surface perpendicular to the upper surface. Two glass rods are broken into double blocks using the short scoring line and placed on a shelf in the same order as they were made. Next the twin squares are broken into squares. All right-side and left-side squares are collected separately in the order in which they are made. First all the right-hand squares are broken into triangles using the same length of the scoring device. The sharpest knives will be obtained when the breaking occurs exactly on the diagonal of the square. Furthermore the crack should develop slowly using moderate forces and should be perpendicular to the upper surface of the square. To meet all these requirements the midpoints of the upper pins of the knife maker must necessarily be positioned at equal distances from the scoring line. The support pins at the lower surface of the glass should be positioned exactly beneath the scoring line (figure 7.1 C). These are factory settings, and should be checked if you suspect the pins to be ill positioned. (This can be checked by breaking several twin blocks. If the breaking plane shows the same aberration repeatedly, the positioning of the pins is wrong). To be able to fracture the squares along the diagonal the position of the square is carefully examined after each fracture and if necessary adjusted. If the counter piece is hardly visible and the sharp edge is made out of the freshly broken plane, the knife is probably quite good (figure 7.1. D, E, F). Since positioning of the square may need some trials, first use all the right-hand squares (which are more identical to each other than mixture of right and left). When that is finished the same procedure is followed for the series of left-hand squares. Knives with a convex edge (figure 8.1. G) or with elevated stress lines (fig 7.1. H) should not be used.

Preparing knives in the above-mentioned way seems laborious, but the invested time is not idly spent, since the ideal knife will save much time and annoyance during sectioning.

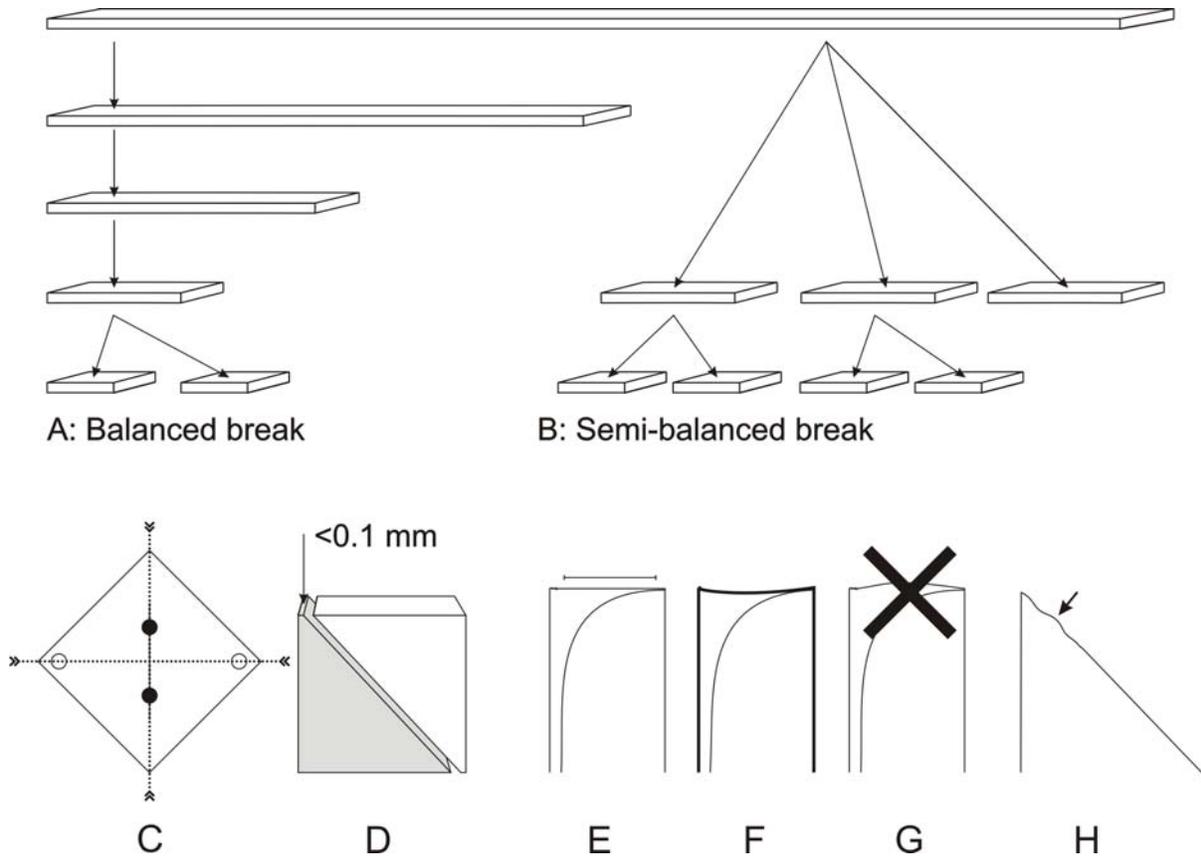


Figure 7.1, Preparation of glass knives

7.2. Diamond knives.

The cutting angle and the surface of a diamond knife determine its sectioning properties. The cutting angle is composed out of three angles the “inbuilt” clearance angle, the clearance angle (usually 6°) and the knife angle. It is easily to understand that the smaller the sum of all the angles is, the less resistance a section has during sectioning. Smaller knife angles result in less compression of the sections. Knives with a cutting angle of even 15° have been used for this purpose. However such a knife is very vulnerable. For normal use knives with a knife angle of 35° or 45° are used.

The surface of the diamond knife is carefully designed by its manufacturer and determines whether it is hydrophobic or hydrophilic and to what extent charges build up during the sectioning. To be sure that the surface you start with is as good as new, any knife should be thoroughly cleaned (see 13.2).

The charging is very important for the sectioning characteristics of the knife. During sectioning the friction between the section and the knife generates a static charge (diamond is a very good insulator). In the dry nitrogen atmosphere of the cryochamber the charge remains on the diamond knife and the sections either stick to the knife or are pushed away from the knife.

Sectioning with a diamond knife is very difficult if the charge on the knife cannot be regulated. The ionizer provides a way to regulate the charge which remains on the knife by creating positive and negative charges in the cryochamber. The position of the ionizer tip and the power of the ionizer together determine which charge remains on the diamond surface. When used correctly, the sections “float” on the charges on the knife surface and compression and other sectioning artefacts are minimal.

8. Formvar carbon coated grids.

We use carbon-coated Formvar films that are spread over hexagonal copper grids. Nickel grids can be used as well and have the advantage of being more inert to chemicals, like PB or PBS, but their magnetism can be a nuisance. When the Formvar film is without holes the interaction between buffers and the copper grid can adequately be avoided. Only in case of long storage on PB or PBS this may be a problem. Sometimes the Formvar is damaged along the grid-bar edges. Then incubation media can penetrate to the backside of the grid, dry out and cause typical precipitation zones along the bars. It has been found that this phenomenon occurs in particular with certain brands of grids. Maybe the sharpness of the bar edges varies.

8.1. Formvar film.

First clean the Erlenmeyer flask and its stopper thoroughly with acetone and chloroform (analytical grade). Dry the flask and put 1.1 g Formvar (Merck) and a cleaned magnetic stirring rod in the flask. Place it on a magnetic stirrer and add 100 ml chloroform (analytical grade) while stirring. After 10 -20 min the solution is ready.

Next clean hexagonal 100 mesh copper grids. Take no more than 200 grids in a 15 ml glass tube. Add 1 ml distilled water and 200 μ l NH_4OH (25%) and rinse the grids with a Vortex until the solution becomes lightly blue. Discard the ammonia solution and wash and stir several (up to 10) times with distilled water so that no traces of ammonia are left, which may cause brown spots on the grids. Finally, wash twice in acetone and dry the grids on filter paper in a 37 °C stove. This grid cleaning method is risky as too long an exposure to the ammonia causes crystals to appear on the copper which are shed on the Formvar film when it is made. Alternately we use 1% H_2SO_4 in distilled water for 10 seconds (vortex), immediately followed by a copious rinse with water. If they are left in the H_2SO_4 any longer the grids will dissolve. For Nickel grids 2 rinses with acetone are sufficient to clean them. Let them dry in a 37 °C stove.

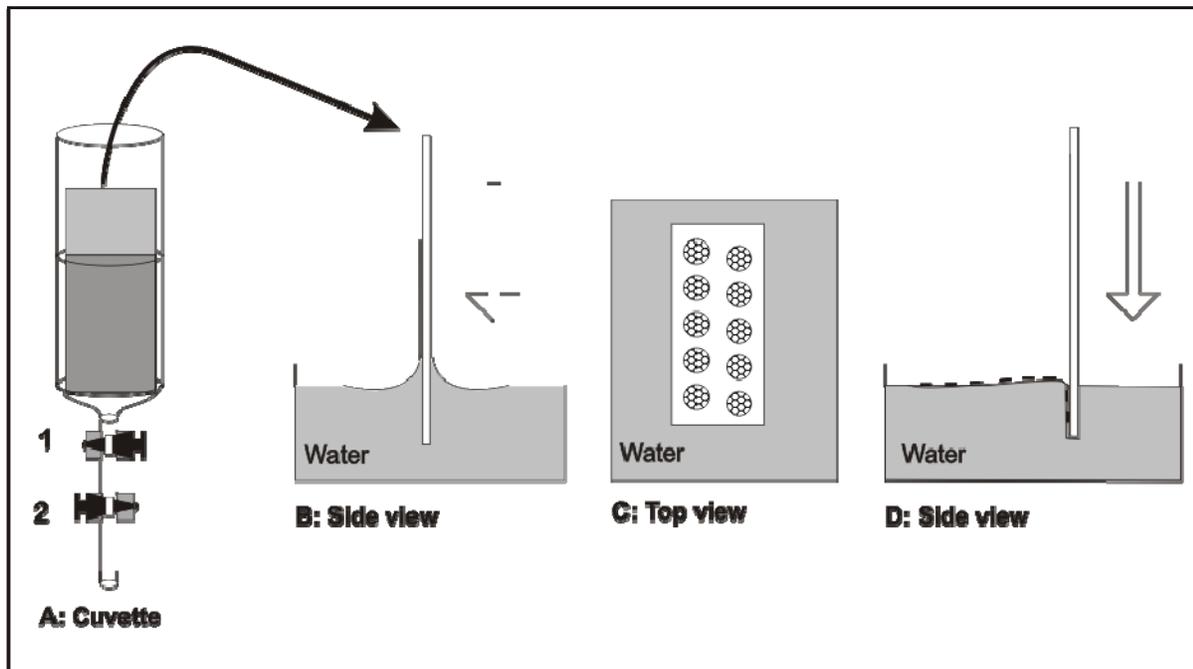


Figure 8.1. Production of Formvar coated grids

The film is made on a microscope slide. The surface of the slides should be without glass crystals. In the Formvar film these particles will produce holes. The surface is cleaned by wiping the slide with a chamois and next with lens paper. Then use a dust remover to blow away the last dust particles. If the slide is too clean, the film can not be removed from the slide. The Formvar solution is poured 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, the second one to switch on or off. The size of the funnel is chosen so that it can hold a microscope slide in upright position (Fig. 8.1 A). The level of the Formvar solution in the funnel is about 2/3 of the length of the slide. The slide is carefully placed in the funnel with a pair of tweezers and the top of the funnel is covered to reduce evaporation of the chloroform. Then the lower valve is opened with one turn. The upper valve is set in such a way that the Formvar/chloroform solution flows out of the funnel in 14 seconds. Remove the slide from the funnel after 30 seconds and 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, or 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 (Fig 8.1.B) The thickness of the film should be about 50 nm, which will give it a silver-white appearance on the water surface (use a lamp). The cleaned grids can be placed on the film (shiny side up, Fig 8.1.C), avoiding irregularities in the film. Once the film is covered with grids, a microscope slide provided with a sticker is gently pressed on the film into the water. The film with grids will attach to the sticker. Be sure to submerge the slide far enough into the water so that the film is totally submerged before the slide can be removed from the water and dried (figure 8.1 D).

To check whether the film is of good quality, the grid is observed in the electron microscope at 20,000 x for holes and dirt. Furthermore the strength of the film is tested by removing the objective aperture and focusing the beam. The film should not break.

8.2. Carbon coating of Formvar films.

The Formvar film on the grids is coated with carbon to render it hydrophilic and better electrostatic properties. At 0.01 Pa (10^{-4} Torr) a very thin layer of carbon is evaporated onto the film using standard techniques. A good indication of the thickness can be obtained by placing a small piece of adhesive tape on the sticker with grids, which is removed after carbon evaporation. The border line between non coated and carbon-coated surface should be just visible.

9. Immuno-gold markers.

Spherical gold particles can be prepared in colloidal sols. The gold particles stay 'in solution' due to their charges. Addition of electrolytes renders the sol instable. However, when colloids like proteins are added under proper conditions, these bind spontaneously to the gold spheres. After binding of sufficient protein a gold sol becomes stable in the presence of electrolytes. The binding of proteins to gold is fairly strong, when optimal binding conditions are chosen (De Mey et al., 1981; Horisberger and Clerc, 1985), but variable for different proteins (de Roe et al., 1987). The proteins normally maintain their biological activities, but too strong a binding may affect it (Horisberger and Clerc, 1985). The high electron density makes stabilized gold particles convenient tools for affinity cytochemistry in electron microscopy. Thus, immunoglobulins, lectins and specific ligands for several receptor systems, bound to colloidal gold particles, are popular markers for delineating intracellular locations and pathways of endogenous and exogenous substances. Gold markers were first used for immuno-cytochemistry by Faulk and Taylor (1971). They attached gold particles directly to the specific IgG molecules, before these were allowed to react with the antigens in the tissue. This direct method was later largely replaced by two indirect methods.

In indirect methods the gold is coupled to IgG molecules that recognize specific IgG molecules, that in their turn bind to the antigen in the section. The other common indirect method is the PAG method, in which staphylococcal protein A is used to recognize specific IgG's. This method was introduced by Romano and Romano (1977) for whole mount labeling and later, for labeling on sections, by Roth and colleagues (1978).

9.1. Preparation of gold sols.

Colloidal gold sols are prepared by condensation of metallic gold from a supersaturated solution created by the reduction of Au^{3+} , usually in the form of gold trichloride. The circumstances under which reduction and sol formation take place determine two important characteristics of the resulting gold particles: (a) their size, henceforth expressed as the average particle diameter (APD); and (b) their size variability, i.e. the coefficient of variation (CV) of the diameter of particles in a sol. When the CV exceeds 15% sols are considered to be heterodisperse, and when the CV is smaller than 15% the designation homodisperse is given to a sol. The size of the gold particles is critical. Large particles can be observed at low magnification and are suitable for scanning EM observations, whereas small particles, which favor the reaction sensitivity, are appropriate for high resolution transmission electron microscopy. Homodispersity of the markers is not only convenient for microscopical observation, but is also important for double labeling techniques (Horisberger and Rosset, 1977; Geuze et al., 1981, Slot et al., 1981, 1985 and 1991).

Many different methods have been used for the preparation of gold sols. The nature and concentration of the agents used for Au^{3+} reduction are crucial for determining the APD and CV of the sols, but factors like reaction temperature and pH are also important. We will describe two methods; both are relatively simple and together they allow gold sols with APD ranges from nearly 3 nm up to 150 nm to be reproducibly prepared. Still smaller gold markers can be prepared by other methods, down to <1nm particles (Hainfield, 1987; Baschong and Wrigley, 1989; Van der Plas and Leunissen, 1993). However, markers less than 3 nm in size are not easy to detect in cryosections stained with uranyl. Therefore these small particles have to be enlarged by silver

enhancement (Danscher and Norgaard, 1983; Holgate et al., 1983). Up to now the advantages of that technique (better penetration resulting in higher sensitivity) on cryo-sections are questionable and we restrict ourselves to the 3 - 150 nm range.

The solutions used in both procedures are freshly made except for the gold chloride solution. The latter is made in stock at 1% strength by dissolving the contents of a 1 gram capsule, commercially available from several suppliers, in 100 ml distilled water. Clean the capsule carefully (no grease!) before breaking it in the 100 ml water. The solution can be stored in the refrigerator for months. Be careful, the solution is very acidic and caustic.

The glass ware should be carefully cleaned, but no special coating is required.

9.1.1. Citrate gold procedure(Frens, 1973)

Gold sols with ADPs ranging from approximately 14 nm to nearly 150 nm can be prepared by using citrate for the reduction of Au^{3+} .

- Take 100 ml of a 0.01% HAuCl_4 solution in distilled water
- Heat until boiling
- Add a variable volume (4 - 0.32 ml) 1% trisodium citrate. $2\text{H}_2\text{O}$ and mix.

The mixture is boiled until the color turns bright orange-red where 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 less than 15 min. The APD of the resulting sols is approximately 14 nm when 4 ml of the citrate solution is used, and rises slowly when less citrate is added. A maximum APD of approximately 150 nm is described. Sols in the range of 15 - 25 nm are homodisperse, but when the APD increases further they become heterodisperse.

9.1.2. Tannic acid citrate gold procedure (Slot and Geuze, 1985).

Gold sols with APDs ranging from nearly 3nm to 15 nm can be prepared using mixtures of tannic acid and citrate (TA-C) for the reduction of Au^{3+} .

To make 100 ml of a TA-C sol, two solutions are made.

(A) The gold chloride solution (250 ml beaker):

- 1 ml 1% HAuCl_4 .
- 79 ml distilled water.
-

(B) The reducing mixture (50 ml beaker):

- 4 ml 1% tri-Na-citrate . $2\text{H}_2\text{O}$.
- A variable volume 1% TA (Aleppo tannin, Mallinckrodt, St. Louis, code 8835)
- 25 mM K_2CO_3 , to correct the pH of the reducing mixture. A volume is added equal to the volume of 1% TA used. When less than 1 ml of 1% TA is used, no carbonate needs to be added.
- Distilled water to make the volume of (B) up to 20 ml.

(A) and (B) are heated to 60 °C. Then add (B) quickly to (A) while stirring. Red sols are 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. After the sol formation is finished, evident from the red color, the sols are heated to boiling.

When in the procedure the volume of 1% TA added rises from 0.01 to 5 ml, the APD of the sols gradually falls from 15 nm to nearly 3 nm. For example, if 0.015, 0.1, 0.5, or 3 ml 1% TA is added to the reducing mixture, APDs of about 14, 9, 6, or 3.5 nm respectively can be expected.

Such sols are sufficiently homogenous so that they can be used as markers in multiple staining experiments.

The source of TA is critical. Only Aleppo tannin gives the reported results. Use well cleaned laboratory glassware.

9.2. Protein binding to gold.

Colloidal gold sols are unstable in the presence of electrolytes. When electrolytes are added the colour turns blue and finally the gold precipitates. When a protein is added in sufficient concentration, it is adsorbed to the gold particles, which makes the sol stable in the presence of electrolytes. The minimum amount that can stabilize 10 ml of a gold sol was originally called the gold number of a protein (Zsigmondy and Thiesen, 1925). The gold number, or stabilization concentration, is different for each protein and can be established as follows (Zsigmondy and Thiesen, 1925; Horisberger and Rosset, 1977). In small test tubes 250 μ l samples of gold sol are mixed with samples of a protein solution in a range of 0.25 - 2.50 μ g protein per tube. After one minute 25 μ l 10% NaCl is added. The lowest protein concentration that prevents the red to blue colour change from occurring (which can be judged visually) is taken as the stabilization concentration, and from that value the gold number can be calculated.

In TA-C sols the colour change upon the addition of salt is slow and masked by the brownish colour contributed to the sol by the tannic acid. This is particularly noticeable with the smallest gold particles, where relatively high concentrations of tannic acid have been used for their preparation. This may raise a problem when establishing the stabilization concentrations or performing whole mount Immunolabeling. It can be circumvented by adding low concentrations of H₂O₂ (<0.2%) to the sol.

Low electrolyte concentration in the protein solution used for binding the gold is usually recommended in order to prevent aggregation of gold particles upon addition of the protein. However, Lucocq and Baschong (1986) reported that protein preparations in various commonly used buffers could be complexed with gold without obvious aggregation.

The binding of protein to gold is pH-dependent. According to Geoghegan and Ackerman (1977), in general stable complexes can be achieved at a pH equal to, or slightly higher than, the iso-electric point of the protein involved (see also section 9.2.1).

9.2.1. Protein A-gold (PAG) preparation

Binding of staphylococcal protein A to gold particles had been studied by Horisberger and Clerc (1985). They found that a sol binds more protein A close to the pI (pH 5.1), but that the binding of protein A is more stable at higher pH. On the other hand, the tighter binding at higher pH (e.g. pH 7) may have a denaturing effect on the protein and make the probes less reactive. Therefore it is advised to do the binding at pH 6. Furthermore, the results of Horisberger and Clerc showed that gold sols can bind much more protein A than indicated by the gold number. However, this "excess" protein A is bound less tightly and may dissociate during the first weeks after the preparation. The dissociated protein A will compete for reactive sites with the PAG and lower the immuno-labeling intensity. In the past we had that experience, but since modifications in the binding procedures as described below (according to the findings of Horisberger and Clerc), our probes are at least stable for months. It is apparently important to do the binding around pH 6 and to add no more protein A to the sol than the stabilization concentration as determined by Zsigmondy's visual colour change test. A more sensitive test, based on EM observation of aggregate formation upon adding salt, was introduced by Lucocq and Baschong (1986). With that method they found much higher stabilization concentrations than with Zsigmondy's test. Apparently Zsigmondy's gold number indicates a rather arbitrary protein concentration, where the gold reaches some, but not complete stability in the presence of salt. Fortunately, this value seems to coincide with the concentration beyond which, at least in the case of protein A, protein is less stably bound to the gold. Therefore Zsigmondy's test is adequate for determining the concentration

of protein A that should be added to a gold sol, whereas the EM test proposed by Lucocq and Baschong is most probably not.

Even when these optimal binding conditions are taken into account, the binding strength of protein A to gold particles is of moderate strength (see also section 6.8; De Roe et al., 1987) and ultimately a preparation may lose reactivity. Therefore, it is better to renew the PAG probes a few times per year.

Protein A binding to gold

- Protein A (Pharmacia, Sigma or LKB) is dissolved in distilled water at 0,2 mg/ml. A gold sol is prepared as described in Section 8.1 and used the same or the next day
- The pH of the sol is adjusted 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 protein A (" 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. When the pH is set the preparation of the PAG has to be finished because the sol becomes instable.
- Determine the stabilization concentration (gold number) of protein A in small samples as described above (Zigmondy's test).
- The PAG probe can now be prepared by adding this concentration of protein A to the sol at pH6, while stirring gently.
- A few minutes after the addition of protein A the pH is brought to 7 - 7.5 by adding 0.1 N NaOH. Then add bovine serum albumin (BSA), at a final concentration of 0.1%, to be sure that the gold particles are stabilized maximally. For BSA we use Sigma fraction V. Make a 10% stock solution in distilled water, neutralize it with NaOH, and centrifuge at 100,000g for 1 hr. Store without azide in the freezer. We found BSA an excellent secondary stabilizer for PAG probes. Probably because its binding to gold is very weak.

Purification of the PAG complex

The most commonly used procedure to purify the pA-gold is centrifugation. We found the best result by gradient centrifugation as follows (Slot and Geuze, 1981):

- The preparation is concentrated by centrifugation: e.g. 5 nm gold, 45 min at 125,000 g^{av}; 10 nm gold, 45 min at 50,000 g^{av}. The resulting pellet is composed of a large loose part and a small tightly packed part. If the centrifugation force is too high the tight pellet becomes large and an increasing amount of aggregates of gold particles is introduced into the preparation.
- Remove the supernatant without disturbing the pellet and resuspend the loose part of the pellet in a small volume
- The concentrated probe is now purified by layering it over a 10 - 30% continuous glycerol (or sucrose) gradient in 0.1% BSA/PBS. The gradient is centrifuged for 45 min at 41 000 rpm (5 nm gold) or for 30 min at 18 000 rpm (10 nm gold) in a SW 41 rotor (Beckman Instruments) or at the appropriate speed in any other type of rotor. The dark red band is then collected. It contains essentially a monodisperse PAG preparation without free protein A.

Alternatively, purification can be carried out by column chromatography after concentration by osmosis, as suggested by Wang et al. (1985). We performed this procedure as follows:

- Take 20 ml of a crude pA-gold preparation prepared as above, except that now 0.01% BSA is added, and put it in a dialysis bag. The bag is immersed in 30% polyethylene glycol (PEG) 20 000 while agitating. The degree of concentration is followed by weighing, and after approximately 2 h the 10 - 20 x concentrated preparation is collected.
- For purification of this concentrate we use a 14 x 0.7 cm column (ACA 22 gel, LKB) which is equilibrated with PBS + 0.1% BSA. Samples of about 250 µl of the concentrate can be

applied to the column. When run at a flow rate of - 3 ml h⁻¹ the red gold complex comes off after about 1 h.

- Collect 250 µl-fractions. Pool the three of four most concentrated ones.

Purification on a column is a convenient alternative when adequate centrifugation equipment is not available. Free protein A is completely removed, but we found the separation of single markers from aggregates not as good as after gradient centrifugation. This is of less importance for PAG preparations, in which usually only a minor part of the gold particles occurs as aggregates. However, gold complexes with other proteins sometimes do contain significant amounts of aggregates. We observed this in certain IGG preparations. Then separation on a gradient may be preferable.

9.2.2. IgG-gold (IGG) preparation.

With the exception of monoclonals, IgG preparations are composed of many fractions with a wide range of pI values. De Mey and colleagues (1981) found it advisable to bind IgG to gold at pH 9, which is on the alkaline side of the pI range. To prevent aggregation of IgG and the subsequent occurrence of clumps in the IgG-gold preparation, special care has to be taken during the preparation of the IgG fraction from antisera. We prepare goat anti-rabbit IgG as follows (Slot and Geuze, 1984): 4 ml of the goat antiserum is adsorbed to 2 ml CNBr-activated Sepharose gel to which rabbit immuno-globulin is bound, for 2 h at room temperature. Then the gel is washed with PBS and anti-rabbit immuno-globulin is eluted with 4 ml 3 M KCNS and the solution is dialyzed at room temperature against 2 mM borax, pH 9. If some precipitation occurs one has to dilute the IgG solution prior to dialysis. Essentially the same procedure was followed to purify swine anti-rabbit IgG.

Except for pH differences, complexing of immuno-globulins to gold and subsequent purification of IGG fractions by gradient centrifugation or on columns are identical to the pA gold procedure. Therefore both the IGG preparation and the sol have to be adjusted to pH 9.

9.3. Storage of gold markers.

Gold markers can be stored for months in the refrigerator without noticeable loss of reactivity. Ultimately, however, the probes may lose activity due to the dissociation of the proteins from the gold particles (Horisberger and Clerc, 1985; De Roe et al., 1987).

9.4. Commercial protein A-gold

In order to keep our PAG probes fresh we renew them every 4 months. We noticed that they often lose reactivity after some months. This may explain why commercial probes, which are usually offered 2-6 months old, are not always satisfactory. Our routine preparations are 5, 10, 15 and 20 nm. They are suitable for triple labeling (see J. Cell Biol., 113, 123-135, 1991).

In the past, several labs have repeatedly asked for samples of our probes. That became a burden for us and in addition the periods between different mailings made us believe that they often worked with sub-optimal probes. Therefore, it was in their as well as our interest that we decided to commercialize our product to some extent. We now offer subscriptions for our protein-A/gold. A subscription form and prices can be found on our website [HTTP://www.cmc-utrecht.nl](http://www.cmc-utrecht.nl).

10. Recipes.

10.1. BSA 10%.

10 % of Bovine Serum Albumine (Sigma A-9647) in distilled water (Milli Q). The solution must be stirred slowly (to prevent foaming) over night in the cold room. The pH is set to 7.4 with 1 N NaOH and azide is added to a final concentration of 0.02%. Finally the solution must be centrifuged for 1 h at 100.000 G. The supernatant is stored in small aliquots in the refrigerator.

10.2. Formaldehyde 16%.

To prepare 16% FA in water, 16 g of paraformaldehyde powder (Polysciences, Inc.) is dissolved in 90 ml of distilled water and heated to 65 °C, at which temperature it is kept for 15-20 min. While stirring, 5N NaOH is added until the pH = 7.4 (pH paper). The pH adjustment is slowly, so give it some time. After cooling down to room temperature distilled water is added to a final volume of 100 ml. The solution is cooled and filtered. FA is stored in small aliquots at -20 °C.

Before use the fixative is heated till 65 °C and the solution is clear. Next the stock is diluted to the appropriate concentration in 0.1M phosphate buffer. A 0.1M PIPES or HEPES buffer can also be used if phosphate interferes with one of the reagents. The buffers used to prepare fixatives need to have a good buffer capacity to maintain a pH of about 7.4 during fixation.

10.3. FORMVAR for grid coating

Weigh 1.1 gram Formvar (Formvar 1595 E Merck 12164) in a 100 ml volumetric flask and add chloroform (Merck 2447) to 3/4 full, stir until dissolved then fill with chloroform to 100 ml and let rest overnight in a dark place at room temperature.

10.4. Gelatine.

When gelatine is solved in 0.1M PB gelatine of many brands (including all of the chemical companies that we tested) give some flocculent precipitate, which can introduce an annoying pollution on the sections. One can circumvent this by using different buffers. PBS already solves most of the problem. At least visually in many cases the gelatine solution is clear, but small nuclei of precipitate may still occur. TRIS buffer works even better resulting in clear gelatine solutions in most cases. However, in particular when gelatine is used for storage of grids chloride containing buffers, like PBS and TRIS-HCl, often cause corrosion of the copper grid. Indeed there should be a Formvar film as a barrier between the buffer and the copper, but that appears often leaky after longer incubation or storage. We skipped all of these problems by screening many gelatine brands on their resistance against phosphate precipitate. We found an ordinary local food brand that is completely compatible with PB, up to concentrations of 12% (W/V).

10.5. Gelatine 10%

Put 10 gram gelatine powder in 75 ml 0.1M phosphate buffer. After stirring for 10 min at room temp the solution is warmed till 60 °C (4-6 hrs). When the gelatine has dissolved the solution is cooled till 37 °C, and 200 µl of a 10% azide solution is added. Finally ad buffer till 100 ml. The homogenous 10% gelatine solution is poured into 5 ml vials and placed at 4°C until use.

10.6. Gelatine 2%

Put 2 gram gelatine powder in 75 ml 0.1M phosphate buffer. After stirring for 10 min at room temp the solution is warmed till 60 °C (4-6 hrs). When the gelatine has dissolved the solution is cooled till 37 °C, and 200 µl of a 10% azide solution. Finally add buffer till 100 ml. While still at 37°C the solution is poured into small Petri dishes (diameter of 3cm) and cooled in the refrigerator and next stored in tight fitting containers at 4°C.

10.7. Methyl cellulose 2%

For a final volume of 200 ml, 196 ml of distilled water is heated to a temperature of 90 °C, and 4 g of methyl cellulose (Sigma M-6385 Methyl Cellulose 25 centipoises) are added while stirring. The solution is rapidly cooled on ice while stirring, until the solution has reached a temperature of 10 °C, add water till 200 ml. Seal with Parafilm and stir overnight in the cold-room (4 °C). Let the solution "ripen" for 3 days (no stirring) at 4 °C and spin at 100,000G for 1.5 hr. The supernatant is poured in 10 ml vials with a screw cap and can be stored at 4 °C in the dark for about 3 months

10.8. Methyl cellulose - uranyl acetate pH4.

Add to 90 ml of the methyl cellulose solution 10 ml of 4% uranyl acetate and mix gently. Centrifuge the solution for 95 min at 29,000 rpm (4 °C). The supernatant is poured into 50 ml vials with screwcap and can be stored at 4 °C in the dark for about 3 months.

10.9. Mowiol.

Add together in 50 ml glass bottle with screw top: 6 gram glycerol (analytical grade) and 2.4 gram Mowiol 4-88 (Hoechst). Mix well, add 6 ml distilled water and leave for 2 hrs at room temperature. Add 12 ml of 0.2M TRIS-HCl, pH 8.5. (TRIS buffer: 2.42 gram of TRIS-hydroxy-amino-methane in 80 ml dist. water. Set pH to 8.5 with 1 N HCl and adjust to 100 ml with distilled water). Place this mixture for 2 hrs in 50 °C water bath while gently agitated. Centrifuge for 15 min at 5000 g. Collect the supernatant and store in small aliquots at -20 °C.

10.10. Phosphate buffered Saline (PBS) stock solution (10X)

80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄·2H₂O, 2.3 g NaH₂PO₄·H₂O, add water up to 1000 ml. Dilute 1:10 prior to use.

10.11. Phosphate buffer 0.2M, pH 7.4(stock solution)

Prepare separately a solution of 0.2M Na₂HPO₄ and a solution of 0.2M NaH₂PO₄. Prior to use the two solutions are mixed in a 81:19 ratio for a pH of 7..

10.12. PIPES 0.2M, pH 7.4 (stock solution)

Prepare the PIPES solution in distilled water. The pH is set to 7.4 with NaOH while dissolving the PIPES, otherwise it will not be completely solubilized.

10.13. PVP (Polyvinylpyrrolidone) - sucrose

This is an infusion material for very low fixed or highly hydrated specimens. The PVP seems to create in general more favourable cutting conditions. It renders plasticity to the blocks, which allows cutting of very thin and well stretched sections.

If made in A.D. and not in buffer, the pH is 10.

In a 100 ml beaker, make a mark for 20 ml.

Weigh: Na ₂ CO ₃	0.69 g
PVP mw 10,000	3 g
Sucrose	11.62 g

Mix powders then add A.D. (or buffer) to 20 ml and place on a magnetic stirring plate and stir for 3 hours (light sensitive, wrap in aluminium foil).

Of course you can also make this in the buffer of your choice.

10.14. Silan coated microscope slides.

Take clean microscope slides and place them during 1 min in a mixture of silan (3-aminopropyltriethoxysilan) and ethanol (100%) 1 : 19. Then transfer the glasses to in distilled water, 2x 1 min. Take them out and let the excess water absorb in filter paper. Then dry the glasses for 3 hrs at 57 °C.

10.15. Sucrose 2.3M :

Dissolve 78.73 g sucrose sucrose (Baker 0334) in in a final volume of 100 ml 0.1M phosphate buffer. Stir until the sucrose is completely dissolved and aliquot in 1 ml vials.

10.16. Uranyl oxelate pH 7.

- Make up 4% Uranyl acetate in distilled water
- Make up 0.3 M Oxalic acid.
- take 4% Uranyl acetate and 0.3 M oxalic acid and mix 1:1
This will give you 2% UA in 0.15 M Oxalic acid.
- add 25% Ammonium Hydroxide drop by drop to a pH of 7 or 8. Do this with constant stirring in the hood and check periodically with a pH indicator stick. If you beyond 7.5 or 8 then a precipitate forms and you can start all over again. Also, if the Ammonium is too old or has been opened too many times, there will be a substantial amount of CO₃²⁻ in the bottle and will give you a neutral UA with less contrasting ability and possible precipitates.

10.17. Uranyl acetate pH 4

A 4% uranyl acetate (SPI) solution is made in distilled water.

11. Suppliers

Decon: <http://www.decon.co.uk>

SPI: <http://www.2spi.com/>

Drukker: <http://www.e6.com/e6/page.jsp?pageid=1155>

Diatome: <http://www.diatome.ch/>

Leica: http://www.leica-microsystems.com/EM_Specimen_Prep

12. References.

Baschong W, Wrigley NG. Small colloidal gold conjugated to Fab fragments or to immunoglobulin G as high resolution labels for electron microscopy. A technical overview. *J. Electron Microsc. Tech.* 1989; 14:313-323.

Danscher G, Norgaard JOR. Light microscope visualization of colloidal gold on resin-embedded tissue. *J. Histochem. Cytochem.* 1983; 31:1394.

De Mey J, Moeremans M, Geuens G, Nuydens R, deBrabander M. High resolution light and Electronmicroscopic localization of tubulin with the IGS (immuno-gold staining) method. *Cell Biol. Int. Rep.* 1981; 5: 889-899.

De Roe C, Courtoy PJ, Baudhuin P. A model of protein-colloidal gold interactions. *J. Histochem. Cytochem.* 1987; 35: 1191-1198.

Faulk WP, Taylor GM. An immuno-colloid method for the electron microscope. *Immuno-chemistry.* 1971;8:1081-1083.

Fox CH, Johnson FB, Whiting J, Roller PP. Formaldehyde fixation. *J. Histochem. Cytochem.* 1985; 33:845-853.

Frens G. Controlled nucleation for the regulation of the particle size in monodisperse gold suspension. *Nature(Phys.Sci)* . 1973;241:20-22.

Geoghegan WD, Ackerman GA. Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold. *J. Histochem. Cytochem.* 1977; 25:1187-1200.

Geuze HJ, Slot JW, Scheffer RCT, van der Ley PA. Use of colloidal gold particles in double-labeling immuno-electron microscopy of ultrathin frozen tissue sections. *J. Cell Biol.* 1981; 89:653-665.

Geuze HJ, Slot JW. Disproportionate immuno-staining patterns of two secretory protein in guinea pig and rat exocrine pancreas cells. An immuno-ferritin and fluorescence study. *Eur. J. Cell Biol.* 1980; 21:91-100.

Griffith JM, Posthuma G. A reliable and convenient method to store ultrathin thawed cryosections prior to immunolabeling. *J. Histochem. Cytochem.* 2002 50: 57-62.

Griffiths G, McDowell A, Back R, Dubochet J. On the preparation of cryosections for immuno-cytochemistry. *J. Ultrastruct. Res.* 1984; 89:65-78.

Griffiths G. Fine structure immuno-cytochemistry. New York: Springer-Verlag, 1993; 459.

Hainfield JF, Furuya FR. A 1.4 gold cluster covalently attached to antibodies improves immuno-labeling. *J. Histochem. Cytochem* 1992; 40: 177

Holgate CS, Jackson P, Cowen PhN. Bird CC. Immuno-gold-silver staining: New method of immuno-staining with enhanced sensitivity. *J. Histochem. Cytochem.* 1983; 31:938.

Horisberger M, Rosset J. Colloidal gold, a useful marker for transmission and scanning electron microscopy. *J.Histochem.Cytochem.* 1977; 25:295-305.

Horisberger M, Clerc MF. (1985). Labelling of colloidal gold with protein A. A quantitative study. *Histochemistry.* 1985; 82:219-223.

Liou W, Geuze HJ, Geelen MJH, Slot JW. The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. *J Cell Biol.* 1997; 136: 61-70.

Liou W, Geuze HJ, Slot JW. Improving structural integrity of cryosections for immuno-gold labeling. *Histochem. Cell Biol.* 1996; 106: 41-58.

McDowall AW, Chang JJ, Freeman R, Lepault J, Walter CA, Dubochet J. Electron microscopy of frozen hydrated sections of vitreous ice and vitrified biological samples. *J. Microsc.* 1983; 131:1-9.

Mühlpfordt H. The preparation of colloidal gold particles using tannic acid as an additional reducing agent. *Experientia.* 1982; 38:1127-1128.

Posthuma G, Slot JW, Geuze HJ. The usefulness of the immuno-gold technique in quantitation of a soluble protein in ultrathin sections. *J. Histochem. Cytochem.* 1987; 35:405-410.

Romano EL, Romano M. Staphylococcal protein A bound to colloidal gold: a useful reagent to label antigen-antibody sites in electron microscopy. 1977; *Immuno-chemistry* 14: 711-715

Romano EL, Stolinski C, Hughes-Jones NC. An antiglobulin reagent labelled with colloidal gold for use in electron microscopy. *Immuno-chemistry* 1974; 11:521-522.

Roth J, Bendayan M, Orci L. Ultrastructural localization of intracellular antigens by the use of protein A-gold complexes. *J. Histochem. Cytochem.* 1978; 26:1074-1081.

Singer S.J. Preparation of an electron-dense antibodyconjugate. *Nature (London).* 1959; 183:1523-1524.

Slot JW, Geuze HJ. A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* 1985; 38:87-93.

Slot JW, Geuze HJ, Gigengack S, Lienhard GE, James DE. Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. *J. Cell Biol.* 1991; 113; 123-135

Slot JW, Geuze HJ, Weerkamp AJ. Localization of macromolecular components by application of the immuno-gold technique on cryosectioned bacteria. In: *Methods in Microbiology: Electron Microscopy in Microbiology* P.F. Mayer, editor, Academic Press Ltd. London. 1988; 20:211-236.

Slot JW, Geuze HJ. Gold markers for single and double immuno-labeling of ultrathin cryosections. In: J.M.Polak, I.M. Varndell (eds): *Immuno-labelling for electron microscopy.* Elsevier Science Publ. b.v., Amsterdam, 1984; 129- 142.

Slot JW, Geuze HJ. Sizing of protein A-colloidal gold probes for immuno-electron microscopy. *J.Cell Biol.* 1981; 90:533-536.

Slot JW, Posthuma G, Chang LY, Crapo JD, Geuze HJ. Quantitative aspects of immuno-gold labeling in embedded and nonembedded sections. *Am. J. Anat.* 1989; 185:271-281.

Tokuyasu KT, Immuno-cytochemistry on ultrathin cryosections. In: cells, a laboratory manual, Vol. 3 Subcellular localization of genes and their products. Eds. D.L. Spector, R.D. Goodman and L.A. Leinwand. Cold Spring Harbor Laboratory Press, 1997. pp. 131.1 - 131.27

Tokuyasu KT, Singer SJ. Improved procedures for immuno-ferritin labeling of ultrathin frozen sections. J. Cell Biol. 1976; 71:894-906.

Tokuyasu KT, Ukamara S. Glass knives for electron- microscopy. J. Biophys. Biochem. Cytol. 1959; 6:305.

Tokuyasu KT. A study of positive staining of ultrathin frozen sections. J. Ultrastruct. Res. 1978; 63:287-307.

Van der Plas P, Leunissen JL. Ultrasmall gold probes: characteristics and use in immuno- (cyto)chemical studies. Meth. Cell Biol. 1993; 37:241-257.

Wang BL, Scopsi L, Nielsen MH, Larsson LI. Simplified purification and testing of colloidal gold probes. Histochemistry. 1985; 83:109- 115.

Zsigmondy R, Thiessen PA. Das Kolloidale Gold. Akademische Verlagsgesellschaft M.B.H., Leipzig. 1925

13. Addendum

13.1. Protein A- G affinities

Affinity of Protein A, Protein G and Protein A/G, for various immunoglobulin's as reported in literature (source Internet)

	Protein A	Protein G	Protein A/G		Protein A	Protein G	Protein A/G
Human IgG	S	S	S	Cat IgG	S	w	S
Human IgG1	S	S	S	Chicken IgG	nb	nb	nb
Human IgG2	S	S	S	Cow IgG	w	S	S
Human IgG3	w	S	S	Dog IgG	S	w	S
Human IgG4	S	S	S	Goat IgG	w	S	S
Human IgM	w	nb	w	Guinea Pig IgG	S	w	S
Human IgA	w	nb	w	Horse IgG	w	S	S
Human IgA1	w	-	S	Horse IgG(c)	w	nb	w
Human IgA2	w	-	S	Horse IgG(T)	nb	nb	nb
Human IgD	nb	nb	w	Monkey IgG (Rhesus)	S	S	S
Mouse IgG	S	S	S	Pig IgG	S	w	S
Mouse IgG1	w	w/s	w/s	Rabbit IgG	S	S	S
Mouse IgG2a	S	S	S	Rat IgG	w	w/s	w/s
Mouse IgG2b	S	S	S	Sheep IgG	w	S	S
Mouse IgG3	S	S	S				
Mouse IgM	nb	nb	nb				
<i>Legend</i>							
w : Weak binding		w/s : Indifferent					
S : Strong binding		nb : No binding					

13.2. Diamond knife cleaning procedure

Materials

- Squeeze bottle with distilled water
- Squeeze bottle with ethanol 99.5%
- Razorblade cleaned with benzoin or acetone to remove the grease contamination
- 2% Decon 90 in distilled water, freshly made every week
- Firm Balsawood rod (see remarks) bevelled to an angle of 90°
- Firm polystyrene rod, bevelled to an angle of 90°
- Glass Petri dish (10 cm diameter)
- Glass Petri dish (depth \pm 4.5 cm) filled with distilled water
- Latex gloves
- Binocular
- Running tap water

Important remarks

- Do not use calcium rich “hard” tap water (use distilled water in this case)
- Test a brand new knife first without cleaning
- Instead of polystyrene you can use Balsawood for the “daily” maintenance. Boil Balsawood (10x10cm) for 10 min. in distilled water to remove minerals, rinse with distilled water, cut small rods parallel with the veins and leave the rods of balsawood overnight in 2% Decon, dry the rods and store them.



Boil Balsawood for 10 min. in distilled water

For daily use: take a rod from the storage, bevel it to an angle of 90° and leave the rod for a few minutes in the 2% Decon before you run it across the cutting edge. Avoid bringing the knife-edge in contact with the darker veins in the Balsawood as this can damage your knife.

- During cleaning with the rod (polystyrene or Balsawood), be sure your knife and rod are wet from the 2% Decon. If the rod is too dry you will contaminate the knife.
- Dry the knife always immediately with a photographers blower after rinsing with ethanol. Only until the knife-edge is dry. Remnants of evaporated ethanol on the knife-edge are “deadly” for sectioning.
- Rinse always in the direction from the cutting edge down to the bottom of the knife

Daily maintenance

Materials:



2% Decon 90 in distilled water in Petri dish and beaker
polystyrene rod bevelled to an angle of 90°
razorblade cleaned with benzoin or acetone

Before cutting:

1. Use the knife directly from the box and put it in the cryochamber or
2. Rinse the knife under running tap water for 10 sec.(see remarks)
3. Rinse the knife with distilled water



rinse the knife under running tap water



rinse the knife with distilled

water

4. Work under the binocular and put a few droplets of 2% Decon on the knife-edge



under the binocular:



put a few droplets of 2% Decon on the knife-edge

5. Dip the polystyrene rod (or Balsawood, see remarks) in the 2% Decon and run the wet rod 4 times across the cutting edge (avoid the dark veins and work under the binocular!!) Use each time a fresh place on the rod. If you see oil-like colours on the knife-edge, the cleaning will be bad. Prevent this by keeping the wood longer in Decon or keep the knife-edge wet by droplets of 2% Decon



under the binocular:

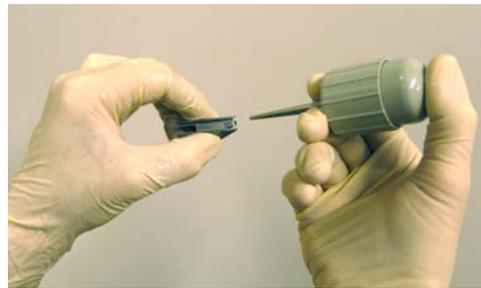


run the wet rod across the cutting edge

6. Rinse the knife under running tap water (see remarks)
7. Rinse the knife with distilled water
8. Rinse with ethanol 99.5%



rinse with ethanol 99.5%
photographer's blower



dry the knife-edge directly with a

9. Dry the knife-edge directly with a photographer's blower. Blow the droplets from the knife-edge down to the bottom. Only until the knife-edge is dry.
10. Put the knife again in the cryochamber (be sure the knife is really dry before you put it in the cryochamber)

After cutting:

1. Take the knife out of the cryochamber and let it thaw a bit
2. Repeat step 2 – 9 (before cutting)
3. Be sure the knife is really dry before you put it in the cryochamber again or put it in the box.

Contaminated knife

Materials:



2% Decon 90 in distilled water in Petri dish
Latex gloves
razorblade cleaned with benzoin or acetone
polystyrene rods bevelled to an angle of 90°
glass Petri dish (depth \pm 4.5 cm) filled with distilled water

1. Submerge the knife overnight in 2% Decon in a glass Petri dish (10 cm)
2. Rinse the knife under running tap water for 10 sec. (see daily maintenance)
3. Rinse the knife with distilled water (see daily maintenance)
4. Take the deep Petri dish with distilled water (under the binocular), put gloves on, hold the knife under the water surface and clean it with the bevelled polystyrene rod by gently running the rod across the cutting edge 6-8 times. Use each time a fresh place on the rod



under the binocular



under water: run the rod across the cutting edge

5. Repeat if necessary
6. Rinse the knife under running tap water (see daily maintenance)
7. Rinse the knife with distilled water (see daily maintenance)
8. Rinse with ethanol 99.5% (see daily maintenance)
9. Dry the knife-edge directly with a photographer's blower. Blow the droplets from the knife-edge down to the bottom (see daily maintenance)
10. Be sure the knife is really dry before you put it in the cryochamber or put it in the box.

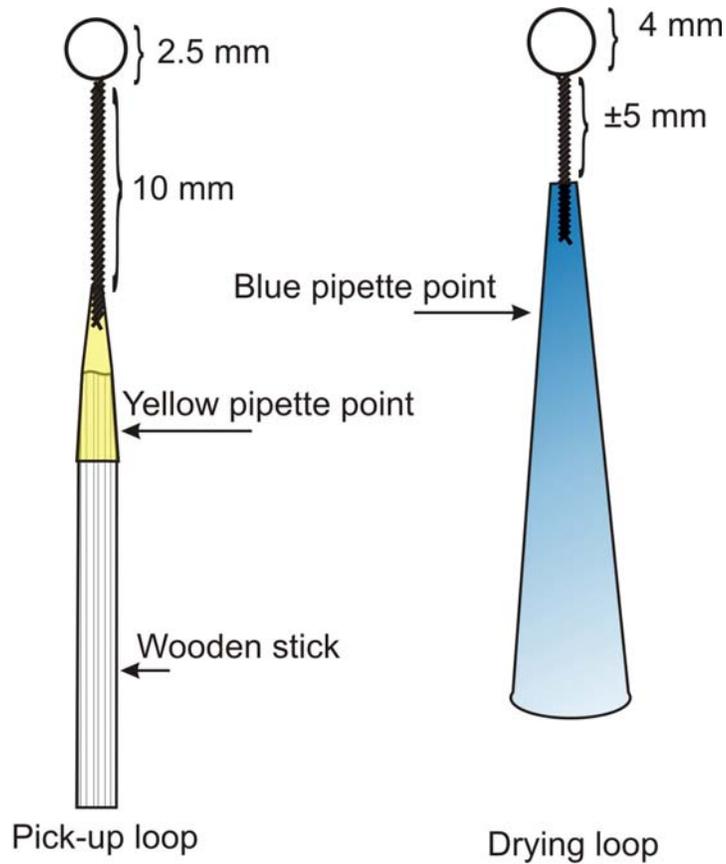
13.3. Short incubation schedule.

Labeling with protein A-gold, the mentioned times are just guidelines. Shorter incubation times may decrease the amount of labeling. The incubations are performed at room temperature. In our standard immunolabeling procedure, thawed cryosections on carbon-coated Formvar copper grids are floated sectionside down on drops of:

1. 2% gelatine in PB (phosphate buffer Ph 7.4 0.1M) (10 min)
2. 0.1% glycine in PBS (2 x 2 min)
3. 0.1% BSA in PBS (PBS/BSA 0.1%) (2 min)
4. Specific antibody diluted in PBS/BSA 1.0% . Drops of appr. 5 μ l containing 5 - 20 μ g/ml specific antibody are sufficient to incubate one grid..... (20 min)
5. Wash on PBS/BSA 0.1%..... (4 x 2 min)
6. Only if specific antibodies with weak binding capacity for protein A are used, like sheep and goat IgG and some monoclonals, or when an enhancement of the gold signal is desired: Bridging antibody (rabbit anti goat, sheep or mouse; swine anti rabbit) diluted in PBS/BSA 1.0% (20 min)
7. This is followed by washing like in step 5.
8. Wash on PBS/BSA 0.1%..... (4 x 2 min)
9. Protein A/gold, diluted as indicated for each batch in PBS/BSA 1.0%. Drops 5 - 10 μ l per grid. Gold probe dilutions are made fresh and used immediately.(20 min)
10. Rinse with PBS/BSA 0.1 % 2 x 2 min, followed by plain PBS (4 x2 min)
11. Stabilize the reaction on 1% glutaraldehyde in PBS (5 min)
12. Wash on PBS (2 x 5 min)
13. In case of double labeling ,repeat steps 3 - 9 with different antibody, protein A/gold combination after a 5 x 2 min rinse with PBS/glycine.
14. Distilled water (fresh, not from plastic bench-bottles)..... (> 6 x 1 min)
15. Uranyl pH 7 (5 min)
16. Distilled water briefly
17. Rinse with ice cold MC-UA
18. Ice cold MC-UA (5 -10 min)
19. Loop out the grids and reduce the MC-UA to an even thin film, and let it dry.

Never let the grid become wet on the back surface or dry out during the incubation procedure, this will generate many background gold particles.

13.4. Loops (drawing)

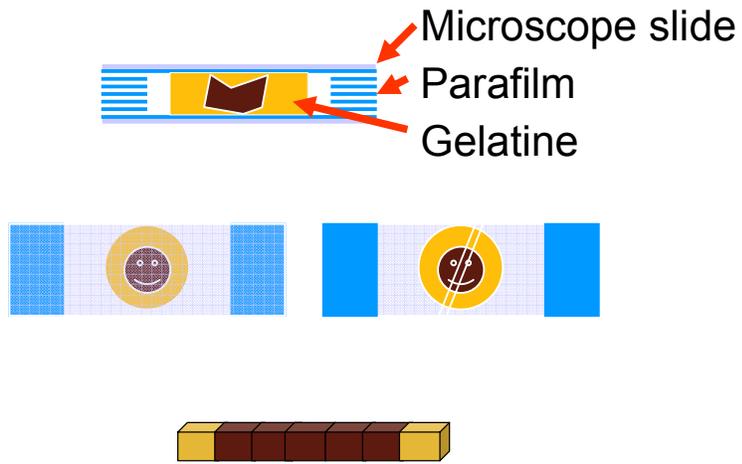


Wire : soft stainless steel diameter 0.3 mm
(used by orthodontists)

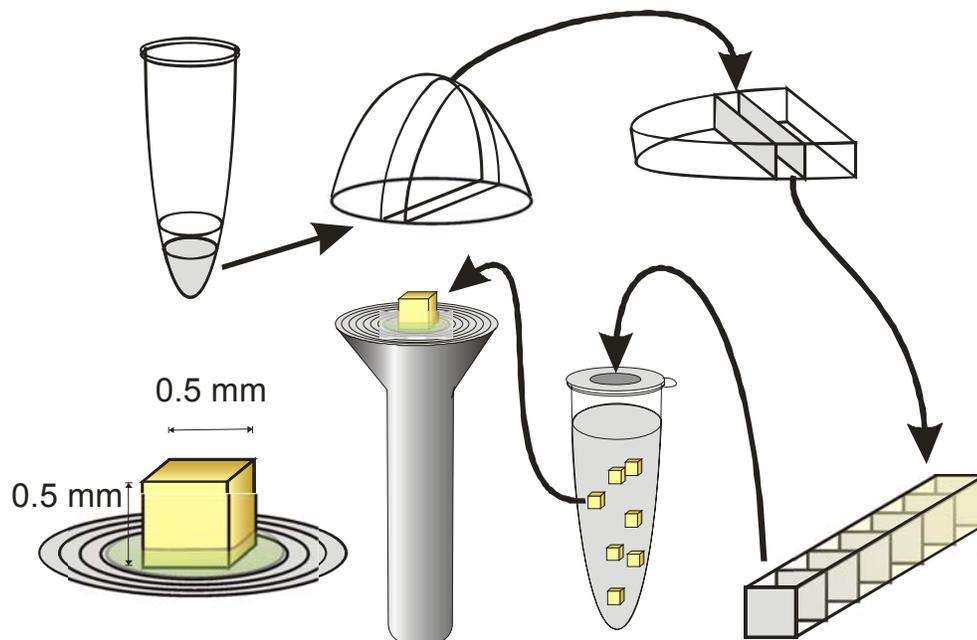
Pickup loop: make a 2.5 mm wire loop of 0.3 mm soft stainless steel, the length of the shaft should be about 1.5 cm long. Fill a yellow Eppendorf tube with approximately 100 microliter resin (EPON or comparable resin). Press the shaft of the wire loop in the opening of the tip for 0.5 cm and push a wooden stick into the other end. Let the EPON polymerise at 80 °C overnight.. (optional: remove the excess part of the Eppendorf tip).

Drying loop: Made in a similar way, only use 250 microliter of EPON solution and omit the wooden stick.

13.5. Embedding of tissue and cells in gelatine (drawing)



A: embedding tissue in gelatine and prepare blocks



B: embedding cells in gelatine and prepare blocks