

Embedding Small Specimens for TEM Examination

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When preparing specimens for TEM analysis, it is important that specimens routinely be large enough to manipulate with forceps and cohesive enough to hold together as a single entity. However, anyone working in EM will have some specimens that are too small to see or centrifuged pellets that easily fall apart during processing. These specimens can easily be lost during a routine dehydration and embedding protocol and therefore cannot be sectioned. If the specimen to be processed is too small to handle or will fall apart, then precautions can be taken to ensure that it can be safely embedded for sectioning. Although this article is written with the main focus on biomedical electron microscopy, these handling protocols can be applied to any small specimens that have to be thin sectioned. Embedding can be performed in resin or any other support medium.

Embedding in agarose

Many small specimens, and even fragile tissues and materials can be embedded in a 2% solution of low-melting-point agarose. Biological materials are chemically crosslinked in buffered aldehyde and then washed in buffer containing a small amount of glycine to neutralize the aldehyde. The small specimens are then immersed in a warm, and thus liquid, solution of agarose. When cooled, the block can be suitably trimmed and processed using routine dehydration and resin embedding protocols.

This approach has worked well for embedding embryos, cells and cell fragments in resin and for cryosectioning (Bierkamp *et al.*, 1999; Hook & Svalander 1991; Pedersen *et al.*, 2000). The method does have disadvantages. Even low melting point agarose exposes specimens to elevated temperatures, which may affect antigenicity, or if labile materials are being examined, the specimen may melt. Also many specimens are either too small, or the agarose is too opaque, for easy visualization and trimming. For these instances, other methods can be used.

Increasing the volume of the pellet

Another method for handling very small specimens is to bulk up the volume of the specimen with something that can be easily recognized in the section and ignored. For biological specimens, a small drop of red blood cells can be added to cells or cell fragments. The mixture is then centrifuged down into a tight pellet for subsequent handling. Very small amounts of the added material have to be used or the specimen can be lost in the extra mass. Alternatively, red cell ghosts, or even small amounts of keratin (from skin flakes) can be used to bulk up the pellet.

An advanced version of this pelleting method uses agarose or sepharose beads to bulk up the pellet. If affinity markers (antibodies or other molecules with specific binding abilities) are attached to the surface of the beads, then cells or cell fragments can be affinity purified and detected on the bead surface. Immobilized structures on the bead surface can subsequently be labeled with other affinity markers using pre-embedding labeling protocols.

Another approach to increasing the pellet volume, which also incorporates a cross-linking step is to embed the specimen in a fibrin clot (Raska *et al.*, 1998). The specimen is mixed with a small amount of fibrin and then reacted with fibrinogen to form a clot around the specimen. Specimens can be centrifuged down into a pellet before clotting or if they are too fragile, they can be left suspended in the clot.

Embedding on a solid substrate

Another, specialized way of handling small pellets and even single cells, is to attach the specimen onto a glass or plastic coverslip. Although regular coverslips can be used, it is easier to locate small structures if the coverslip has a grid etched into its surface. Coat the grid with something that will stick the specimen down (e.g. poly-L-lysine or alcian blue) and let the specimen settle onto the surface (material scientists may find other substances are more suitable for attaching small fragments onto glass). The specimen should be attached to the same side as the etched grid. Fix, dehydrate and embed the region where the specimen is attached in resin. The polymerized resin block (with, or without the coverslip, as the grid pattern is transferred to the resin) can be examined by light microscopy and specific regions can be identified and sectioned. It is possible to centrifuge specimens down onto the glass surface using a cytospin centrifuge. However, unfixed specimens should not be allowed to dry out. We have successfully used this approach to examine protozoans (Gardiner *et al.*, 1986)

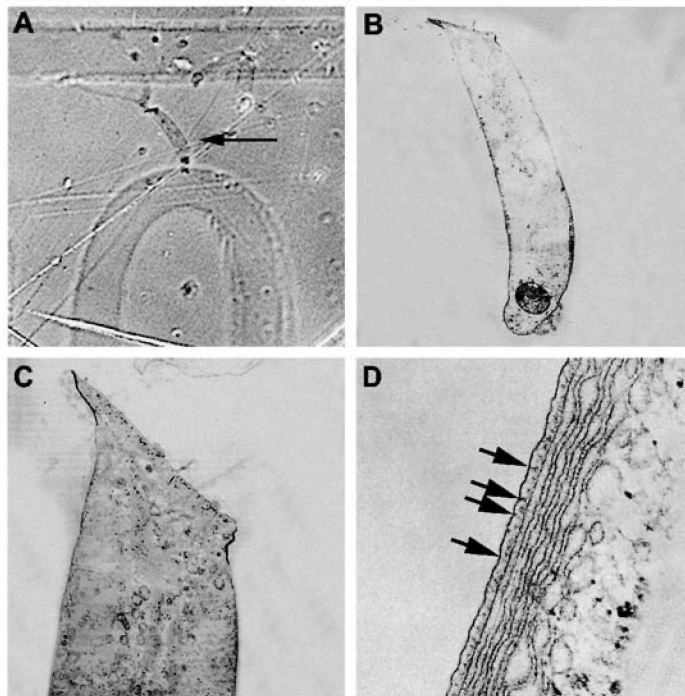
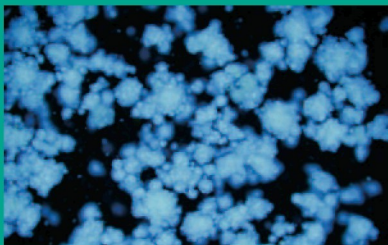
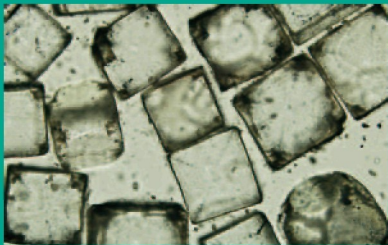


Figure 1. Electron microscopy of a single cell. This is an example of how a single outer hair cell can be embedded in resin, identified by light microscopy and sectioned for examination in the transmission electron microscope. Outer hair cells were isolated from the organ of Corti by physical dissection and enzyme treatment. The separated cells were left to settle on a glass coverslip on which had been etched a locator grid. The glass coverslip was then immersed in 2.5% buffered glutaraldehyde, washed, exposed to 1% osmium tetroxide, dehydrated in ethanol and propylene oxide and finally embedded in epoxy resin. The glass was removed from the polymerized resin block by immersion in liquid nitrogen, the exposed surface cleaned with 70% ethanol and the block examined by phase contrast light microscopy. Figure 1A. The image observed by phase contrast light microscopy. An individual outer hair cell can be observed in the resin (arrow). Marks on the resin surface and the locator grid mark (in this instance the cell was located in square O5, part of the letter "O" is visible in the image). Figures 1B – D. The outer hair cell located in Figure 1A was identified and the resin around it trimmed away. The block was glued onto a firm support (in this instance an empty resin block was used) and placed into an ultramicrotome. Thin sections approximately 70nm thick were produced, collected on metal specimen grids, contrasted and examined in a transmission electron microscope. The arrows in Figure 1D highlight small bridges on the cell periphery that appear to connect the outer cell membrane with inner membranes that are presumed to be rough endoplasmic reticulum. It is clear that single cells can be identified in resin blocks and sectioned for electron microscopy. Subcellular detail is only affected by the mechanical damage that occurs before the cells are chemically cross-linked.



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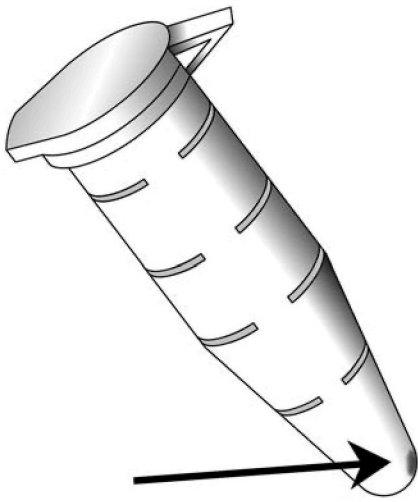


Figure 2. Centrifugation of small specimens. If the hinge of an Eppendorf tube is placed uppermost in a 45° fixed angle rotor, the material in the tube will always be centrifuged to the same spot below the hinge.

and are continuing its application to the study of single, isolated mammalian cells. This method is limited to examining specimens that are visible by light microscopy, and the method may not be useful for larger pellets of cells or cell fragment (which may fall off the glass coverslip).

Gelatin embedding

Gelatin embedding offers a good approach for embedding small specimens for TEM examination. There are many advantages to gelatin

embedding that make it useful for even novice users. A 10% solution of gelatin will be liquid at 37°C so specimens to be embedded are never exposed to high temperatures (as may be the case with agarose embedding). If it is determined that specimens are incorrectly embedded after the gelatin has solidified, then the solution only needs to be re-warmed to 37°C for it to become liquid again. Gelatin can easily be removed from embedded specimens, or from sections, if required, by washing with warm aqueous medium.

Unlike agarose, which encases specimens, gelatin tends to stick to biological specimens and gives extra support. This is especially important if the specimen has to be cut into smaller pieces after being encased. Cells and tissues will fall out of agarose when it is handled too much, but gelatin remains attached to, and between, the specimen.

Really small specimens are chemically crosslinked with buffered aldehyde, treated with glycine and placed in a thin polypropylene centrifuge tube (about 0.5 ml total volume). The specimens are gently centrifuged to the bottom of the tube, re-suspended in a small volume of warm 10% gelatin and centrifuged again to form a tight pellet (Amigorena *et al.*, 1994). It may still be possible to obtain centrifuge tubes with sharp pointy ends on the inside surface. These work best because the pellet fragments are deposited in the very tip of the tube when centrifuged using a horizontal rotor.

Acknowledgements:

I would like to thank Ms. S. Wu and Mr. A Webster for their help with preparing the images used in this article.

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After the gelatin had gelled, cut off the tip of the tube, which is easy if the tube is made of polypropylene, and embed using a routine protocol. For cryosectioning, we soak the gelatin block in 2.3M sucrose and freeze by immersion in liquid nitrogen. For embedding biological specimens in epoxy resin we fix the gelatin in 2% buffered glutaraldehyde, post fix with osmium tetroxide and dehydrate in ethanol. The gelatin turns dark and the tip remains visible making orientation of the block a simple procedure.

An alternative centrifugation step of small specimens or cell fragments is to centrifuge them down, in

gelatin, to a known location in an Eppendorf tube. When Eppendorf tubes are used in a fixed-angle rotor, pellets always deposit at the same place - on the tube surface furthest away from the center of the rotor. If the lid hinge is placed outward, the pellet at the bottom of the tube will always be found under the hinge. Small, and even invisible pellets that have been embedded in gelatin can be easily found in this region. A few fine hairs or dust particles in the specimen make detection easier under a dissecting microscope.

Finally, a pelleted gelatin-embedded specimen can be easily manipulated if it is removed from the bottom of the centrifuge tube and placed onto a glass slide covered with Parafilm. Warm gelatin can be poured over the pellet and a second Parafilm-coated slide pressed over the first. Spaces placed between the two slides (folded strips of Parafilm work well for this) create a space where the specimen, embedded in a thin film of gelatin, can be found. Small fragments or tiny cell pellets can be easily seen in the gelatin film and cut out in small blocks if the gelatin is kept cool.

For all embedding methods where specimens have been chemically cross linked with buffered aldehyde, make sure the chemical fixative has been neutralized or washed away with a primary amine solution, and when embedding with gelatin make sure the gelatin has gelled before manipulating it. Keeping gelatin cold will make sure it stays solid. ■

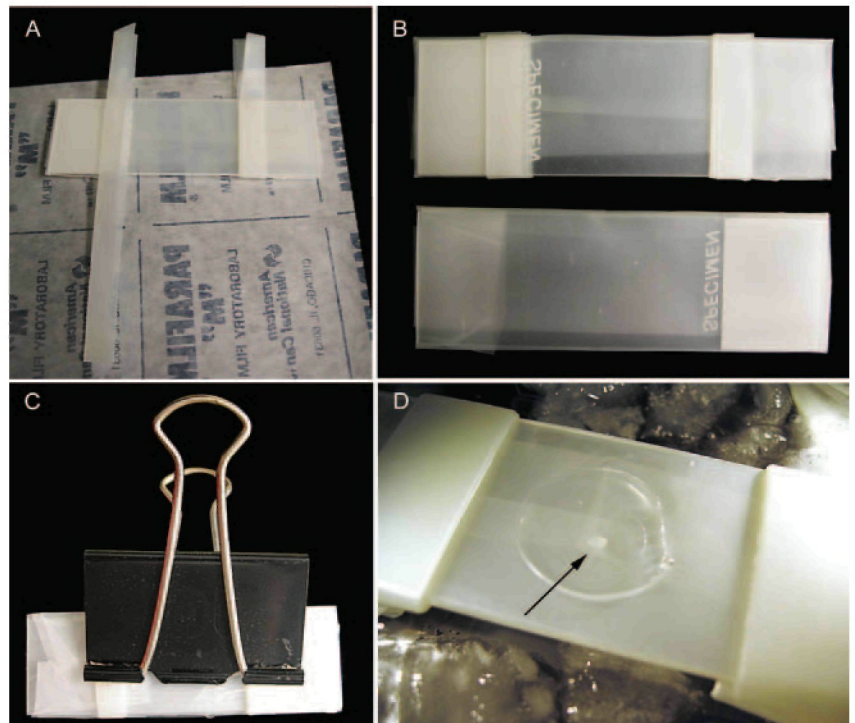


Figure 3. Embedding in gelatin. Small specimens can be placed between two ParafilmM[®] covered glass slides. The slides are completely wrapped in sheets of ParafilmM[®]. One of the glass slides is then wrapped again with folded strips of ParafilmM[®] to act as spacers (FIGs 3A and B). The small specimen is then placed on the slide with spaces, covered with warm 10% gelatin and covered with the second slide. The two slides are held in place with a clamp (FIG 3C) and left at 4°C until the gelatin has set. When the two slides are separated (FIG 3D), the specimen (indicated in FIG 3D with an arrow) will be embedded in a thin film of solidified gelatin. If the gelatin is kept on ice it will remain solid and the specimen can be easily cut out of the gelatin sheet for further processing. (Many thanks to Dr Eija Jokitalo of the EM Unit at the University of Helsinki for introducing me to this method of embedding cells).

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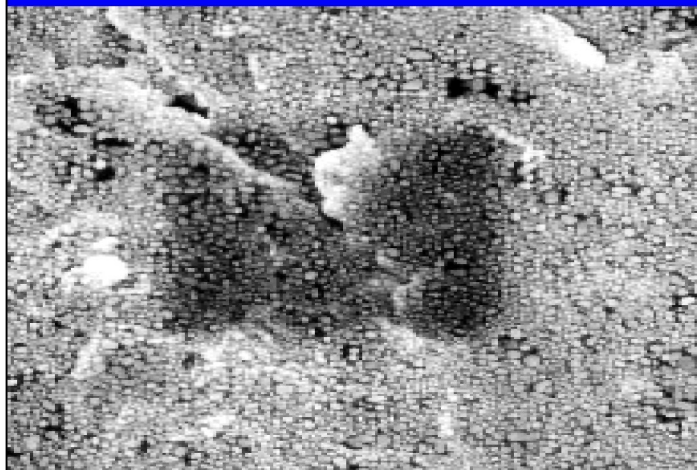
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