Dehydration and Rehydration Issues in Biological Tissue Processing for Electron Microscopy

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Electron microscopy (EM) is an indispensable tool for the study of ultrastructures of biological specimens. Every electron microscopist would like to process biological specimens for either scanning electron microscopy (SEM) or transmission electron microscopy (TEM) in a way that the specimens viewed under the electron microscope resemble those seen *in vivo* or *in vitro* under the light microscope. This is, however, often easier said than done because biological tissue processing for EM requires careful attention of the investigator with regard to the numerous processing steps involved in specimen preparation, such as fixation, dehydration, infiltration, embedding, and sectioning. Each of these steps can easily distort the fine structures of the specimen by introducing mechanical, chemical, or thermal damage. In this article, I will focus on two of those distorting effects which I found were especially critical in the multi-step tissue processing of biological specimens. These effects are caused by dehydration and rehydration.

Dehydration of biological specimens is an important step in tissue processing for both SEM and TEM. The purpose of dehydration is to remove water from the biological specimens so that they can be further processed for EM. This is important for SEM specimens because it facilitates replacing water with acetone, which then can be exchanged with liquid carbon dioxide or other transitional fluids for subsequent critical point drying. Dehydration is important for TEM specimens as it facilitates the replacement of the specimen's water content with ethanol and then with propylene oxide, a standard transitional solvent that further dehydrates the specimen and is highly miscible with the most widely used plastic embedding media. Incomplete dehydration results in inadequate infiltration and polymerization of the embedding medium. Furthermore, e.m. specimens must be free of water when exposed to the high vacuum ambient of an electron microscope.

The typical dehydration protocol includes the gradual replacement of water within a biological specimen by using a graded series of dehydration agents. I found the following protocol to be the most reliable: 50% of ethanol or acetone for 5 minutes, 70% for 10 minutes, 80% for 10 minutes, 90% for 15 minutes, and 100%, two times, for 20 minutes. All dehydration steps are to be performed at room temperature. It is important that the volume of the dehydration solution is much larger than that of the specimen (at least 10 times as large). Furthermore, it is important that the specimen is not removed from bath to bath during the dehydration process to avoid mechanical damage to the specimen (*e.g.*, caused by forceps) and to avoid exposing the specimen to ambient air. Instead, it is recommended that the liquid is poured out of the vial being used, allowing the specimen to remain behind undamaged and unrehydrated.

Ethanol and acetone are the most common solvents used for dehydration. Other substances include propylene oxide, dimethoxypropane, and methanol. Unfortunately, acetone, ethanol, and other dehydration agents tend to shrink biological specimens and can extract tissue components such as lipids and proteins. Ethanol is perhaps the most widely used dehydration agent because it is less toxic, less volatile, does not extract bound lipids as much as acetone does, and causes less swelling at the beginning of the dehydration process and less shrinkage at the end. Acetone, on the other hand, is more flammable, extracts tissue components, but causes less tissue shrinkage. Finally, propylene oxide is an irritant, extremely volatile, a potentially carcinogenic substance, and extracts both fixed and unfixed lipids. Swelling occurs in most tissues when dehydration is initiated and it is offset somewhat by shrinkage as soon as the higher concentrations

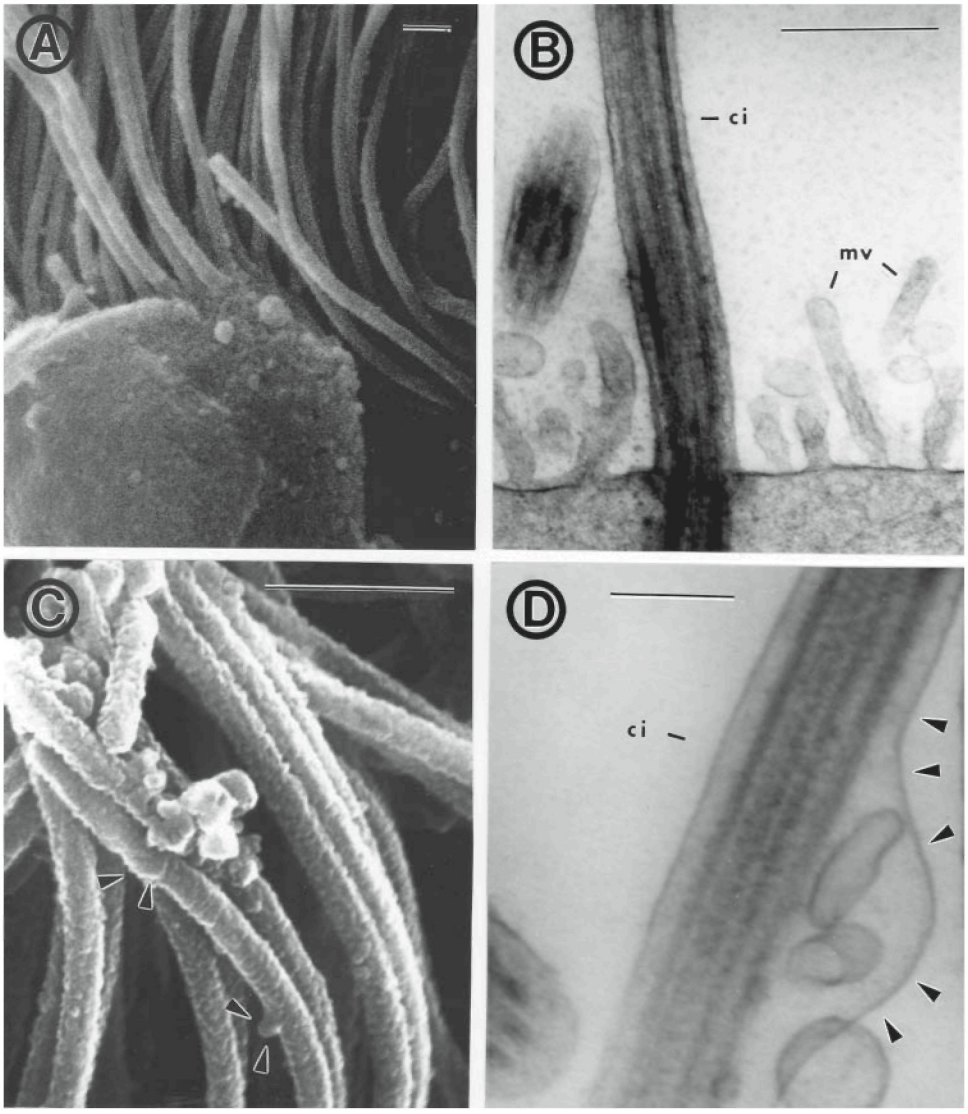

Figure 1. Scanning electron micrographs (A and C) and transmission electron micrographs (B and D) of ciliated tracheal epithelial cells. A successful dehydration procedure of these tissues results in good preservation of cilia (ci) and microvilli (mv). In contrast, specimens exposed to air experience rehydration resulting in significant swelling of cilia and a bleb-like appearance of the membrane that covers the cilia (arrowheads). Magnification bars, 1 μm (A), 0.5 μm (B), 0.75 μm (C), 0.2 μm (D).

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of solvents are approached. In order to minimize the side effects of dehydration agents, it is recommended that the total time used for performing the entire dehydration procedure should be kept as short as possible. For example, the total time required for the dehydration protocol I suggested above is approximately 1 hour and 20 minutes. This relatively quick processing time significantly limits damage resulting from excessive changes in solvent concentrations, reduces swelling and shrinkage, keeps extraction of tissue components at a minimum and, at the same time, allows for the complete removal of water from the specimen. The successful dehydration of ciliated tracheal epithelial cells as viewed by SEM and TEM is shown in Figures 1A and B.

Rehydration is another important factor which deserves discussion in regard to biological tissue processing. Rehydration is the reversed process of dehydration. Rehydration means that a specimen takes up water from the environment. This can happen when a specimen is accidentally exposed to ambient air for a certain period of time. Rehydration of biological specimens during processing is almost always accidental, but critical in EM, because it can lead to the description of artifacts. Figures 1C and D shows ciliated tracheal epithelial cells which were exposed to air causing swelling of tissue components. Rehydration of biological tissues can be avoided by either conducting additional processing steps immediately or by placing the partially processed specimens in a desiccator until they are needed.

Rehydration can also pose a significant problem in dehydration agents. Dehydrants are hygroscopic, which means, they readily absorb atmospheric moisture. For example, a bottle of absolute ethanol or acetone left open for a short time will readily absorb water from the air to a degree that this chemical solvent will lose its capability of eliminating all of the water from tissue during the dehydration procedure. Since rehydration can become a big problem in tissue processing, it is recommended to keep bottles of dehydrants tightly sealed. Bottles of absolute ethanol that have been opened often, and were stored on the laboratory shelves for months or even years, must be suspected of having absorbed significant volumes of water rendering them useless for the dehydration steps at higher concentrations (*i.e.*, 80% through 100%). However, they may be used to prepare the lower percentage dehydrants (*e.g.*, 50% and 70%) used in the first two steps of the suggested dehydration protocol. To avoid water absorption in the vials and the escape of volatile solvents during the dehydration experiment, I recommend using vials with caps. Furthermore, I found that it is best to store stock solutions of acetone and absolute alcohol over drying agents such as anhydrous calcium chloride in tightly sealed bottles. Immediately before use, adequate volumes of these substances can be filtered through fresh anhydrous calcium chloride to provide highly active dehydrants for the protocol. Molecular sieves can also be used to desiccate dehydration agents.

In summary, there are many possible ways to successfully perform dehydration in biological tissue processing, and to avoid the risks of rehydration. Although I described a suitable and reliable protocol of dehydration for ciliated tracheal epithelial cells, the correct protocol depends on various factors, including the type of specimen to be studied, the time allotted to the investigation (*e.g.*, clinical or research setting), prior experience with dehydration procedures, and the chemicals or financial resources available. ■

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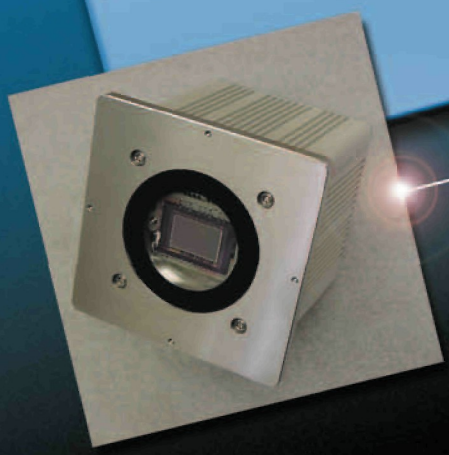

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