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The Good, the Bad and the Ugly: Task-Specific Fixation for Connective Tissues

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Tissues with high water content, mediated by highly concentrated proteoglycans, are exceptionally resistant to ultrastructural preservation. Due to their net negative charge, proteoglycans allow the retention of substantial water and also interact strongly with cells, collagens and other molecules to form a hydrated, integrated, and resilient matrix. Proteoglycan content varies within specific tissue compartments, but is ubiquitous to all connective tissue matrices. A significant challenge presented to the Microscopist is in the stabilization of this fragile and extensive proteoglycan network, whose structure is dependent on retained water which itself must be removed to be compatible with most embedding media. Furthermore, and regrettably, proteoglycans are largely (70%) extracted during conventional glutaraldehyde/Osmium fixation. The remaining structures (cells and collagen fibrils) are separated by large open spaces previously occupied by the extensive proteoglycan matrix and water. Cryo-stabilization (high pressure freezing followed by freeze-substitution) is unique in its ability to stabilize the intimate proteoglycan/collagen/cell associations; however particularly in skin, many landmark structural entities are masked by the retained proteoglycans and cannot be visualized. HPF/FS is triumphant in revealing the ultrastructure of smaller organisms; however the successes in cooling exceedingly hydrated tissue ice-crystal free are so rare that biopsies must not be sacrificed to this procedure. Certainly, the few images of well cryo-preserved connective tissue need consideration when deliberating spatial relationships in this difficult tissue (1).

Certain cationic dyes such as ruthenium hexamine trichloride may be added to the fixation cocktail to retain proteoglycan (2, 3). Improvement in tissue structure is obvious even in the light microscope, however the resulting PG matrix is not space filling, rather it precipitates at the sites of interaction with other tissue components (collagen fibrils and cells).

Much of the work in our laboratory is in the ultrastructural localization of various tissue components, requiring an entirely different approach to tissue preservation. For immuno-EM, we do not include steps to preserve the proteoglycan network. Without retained PG, the remaining cellular and structural entities contrast well in the resulting in open space. Often we evaluate anchoring fibrils, composed of type VII collagen, which are entirely masked in well frozen skin. Anchoring fibrils anchor into the sub-basal densa of the epithelium, then extend into the papillary dermis where they form loops which entrap banded collagen fibrils (think "Velcro"). Effectively, they "staple" the epithelium onto the underlying dermis. A defect in collagen VII results in the severe blistering disease Epidermolysis Bullosa. We are currently evaluating several Clinical therapies designed to ameliorate this disease by introducing WT collagen VII, which incorporates into the basement membrane to form functional anchoring fibrils. Skin biopsies are often sent to us from the far side of the world and must be received with little degradation, from which we can proceed with immunocytochemical protocols. Most of the antigens of interest are not viable following fixation; therefore exposure to aldehyde at the point of origin is not an option. Instead, we direct our collaborators to immerse tissues in Dulbecco's serum-free culture media, which allows cellular viability even after several days. Upon receipt, we typically diffuse primary antibody and secondary antibody-1.4nm gold conjugates diluted with media into the tissue, with diffusion accelerated by microwave energy, so that tissues are immunolabeled and ready for chemical fixation within 2 hours. Fixation, dehydration and embedding are also accelerated by microwave energy, which not only reduces working time but also tissue extraction.

On some occasions a region of interest cannot be exposed sufficiently to the diffusion of antibody, necessitating that immunolabeling be carried out on the surface of sections cut from embedded tissue. Here it is necessary to expose the tissue to some degree of chemical fixation so that it may withstand the rigors of dehydration and embedding. Although some antigens may be localized following preparation for best ultrastructure (3% glut, 1% OsO4, epon embedding), our "go to" fixative is ice-cold 4%paraformaldehyde with 0.1% glutaraldehyde for 30", PLT (progressively lower temperature) dehydration to 90% EtOH, and embedding in LRWhite. With the addition

of triethanolamine this method will also retain GFP and YFP fluorescence, allowing correlative light and electron microscopy (CLEM) (4). With added triethanolamine the media also will polymerize at 37 C. The degree of ultrastructural preservation is not satisfying, but may be "good enough" to allow definitive localization.

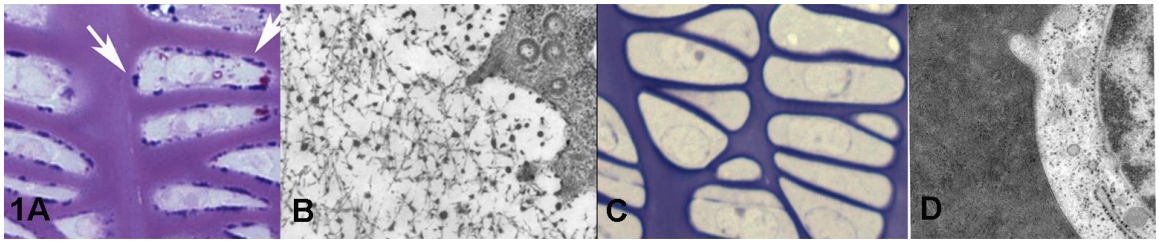

Figure 1. Fixation in glutaraldehyde allows shrinkage of chondrocytes (A) and extraction of matrix (B). Ruthenium Hexamine Trichloride added to glutaraldehyde and Osmium retains much of the proteoglycan within the matrix, resulting in far less shrinkage of chondrocytes and a dense matrix (C). Preserved by high pressure freezing, the matrix and chondrocytes are optimally preserved (D).

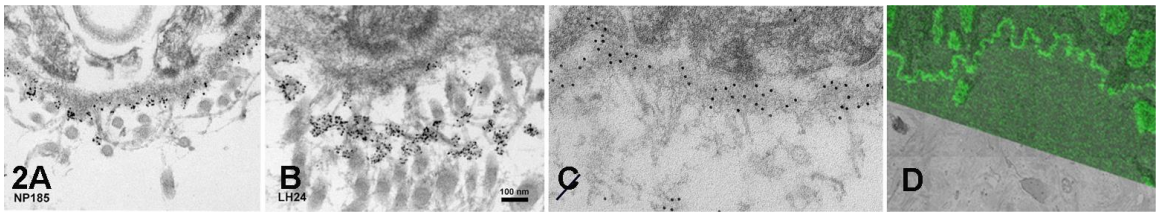

Figure 2. When unfixed human skin is incubated in dilute antibody to collagen VII followed by secondary immuno-gold, antibody to the N-terminus (A) and C-terminus (B) are resolved at distinct regions of the anchoring fibril. Labeling the surface of sectioned skin with a polyclonal type VII antibody succeeds only if the skin is lightly fixed and embedded in LRWhite, resulting in less definition of tissue ultrastructure (C). The secondary antibody complex in (C) is a complex of 1.4nm gold with a Alexa 488, allowing correlative LM localization to the epithelial basement membrane (D).

References

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