Biological Sample Preparation for SEM Imaging of Porcine Retina

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Introduction

Sample preparation is a critical step in scanning electron microscopy (SEM) imaging. This is especially true for biological samples because of charge build-up and sensitivity to vacuum and electron beam damage. In terms of ultrastructure imaging, a variety of advancements in detectors and approaches have improved biological imaging such that fewer steps are required for sample preparation. However, the conventional approach incorporating osmium tetroxide fixing, ethanol dehydrating, critical-point drying, and coating still finds useful application. This paper evaluates three biological sample-preparation methodologies for imaging the ultrastructure of immature porcine retina. The three preparation methods examined are critical-point drying (CPD), hexamethyldisilazane (HMDS) dehydration, and direct imaging by environmental scanning electron microscopy (ESEM). Preparation methodologies were evaluated based on resulting image quality and reduced potential for artifacts.

Methods and Materials

Specimen en bloc dissection. Porcine eyes were fixed for 5 days in a 10% formalin solution and then transferred to phosphate buffered saline (PBS). Extraocular tissue was dissected away, and globes were hemisected anteroposteriorly along the sagittal plane with a scalpel. A core through the retinal layers, choroid, and sclera was made with a 4 mm diameter trephine and placed on an aluminum stub using carbon dots or an aluminum crucible (for direct examination in ESEM).

Critical-Point Drying (CPD). The first step in the CPD process was to dehydrate the specimen with ethanol. In general, more gradual dehydration minimizes surface tension effects, but there is some ambiguity as to the duration and incrementation of this important step for fragile biological tissues. We selected a dehydration procedure that is standard at our facility for liver and kidney specimens: samples were dried in 70% ethanol for 12 hours and increased to 95% ethanol for two changes lasting one hour each. To ensure complete ethanol saturation, the dehydration solution was increased to 100% ethanol for three changes lasting one hour each. All samples were critical-point dried using a PELCO CPD2 Critical Point Dryer (Ted Pella Inc.). Temperature and pressure were closely monitored to ensure the samples were not prematurely dried or thermally damaged. Six samples were prepared with 2% osmium tetroxide, and 6 samples were prepared without osmium tetroxide to determine if post-fixation improved imaging and minimized charging artifacts and thermal damage. Additionally, all samples were sputter-coated with ~10 nm of gold-palladium and imaged using a Helix detector in low vacuum on the FEI NovaNano 630. The Helix detector is an FEI NovaNano detector that allows imaging of non-conductive samples in low-vacuum mode. Pressures were varied from 0.25–0.4 Torr, but the accelerating voltage remained at 7 kV.

Hexamethyldisilazane (HMDS). Ethanol dehydration was implemented, as described above, followed by three changes of 100% HMDS for 30-minute durations. After the third change, specimens remained in HMDS until all of the solution evaporated. Samples were sputter-coated with gold-palladium and imaged on an FEI Quanta 600 FEG in high vacuum with an Everhart-Thornley detector and also an FEI NovaNano 630 in low vacuum with a Helix detector.

Environmental Scanning Electron Microscopy (ESEM). ESEM imaging captures specimens in their natural hydrated state and can augment information obtained in other SEMs using extensive sample preparation. Unfortunately, examining the ultrastructure of the retina in its natural state is hindered by the vitreous of the eye. Vitreous is a viscous substance (composed of 99% water by volume) that sits atop the retina surface. Therefore, retina was dissected as described previously, but the specimens were dehydrated slightly in 70% ethanol for 1 hour, and vitreous was physically removed by gently suctioning with a medicine dropper. ESEM was performed on an FEI Quanta 600 FEG with a Peltier stage and gaseous secondary electron detector.

Results

All SEM imaging methods, except the ESEM, allowed resolution of the filament-like collagen matrix. Initially, the CPD and HMDS samples were found to have peculiar spherical artifacts in the collagen matrix (Figure 1). By adjusting the accelerating voltage, chamber pressure, and ethanol procedure individually (not shown), we determined that these artifacts were the result of the ethanol dehydration protocol. Accordingly, a modified dehydration protocol was implemented to incorporate more gradual ethanol increases. The ethanol concentration in the dehydrating solution was increased from 30% to 50% and incrementally increased by 10% up to 100%. The duration of each iteration was 10 minutes. Two additional increments at 100% for 30 minutes ensured complete ethanol saturation throughout the tissue. By modifying the ethanol dehydration protocol to slow the dehydrating process, the artifacts were significantly decreased (Figure 2). Subtle changes were made to chamber pressure and working distance to analyze the resulting images qualitatively.

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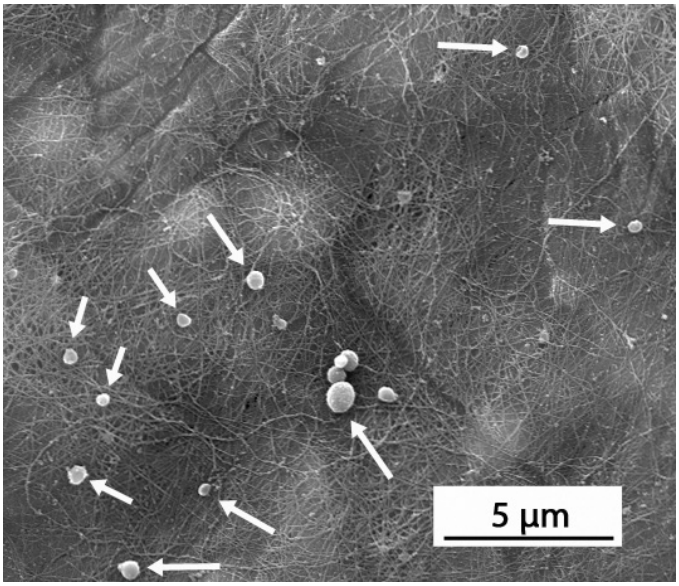

Figure 1: Spherical artifacts (arrows) found in the collagen matrix of the porcine retina. The dehydration procedure was thought to be the cause of the artifact and was changed for subsequent specimens. Imaged using the Everhart-Thornley detector in high vacuum with a magnification of 8000× and 20 kV accelerating voltage.

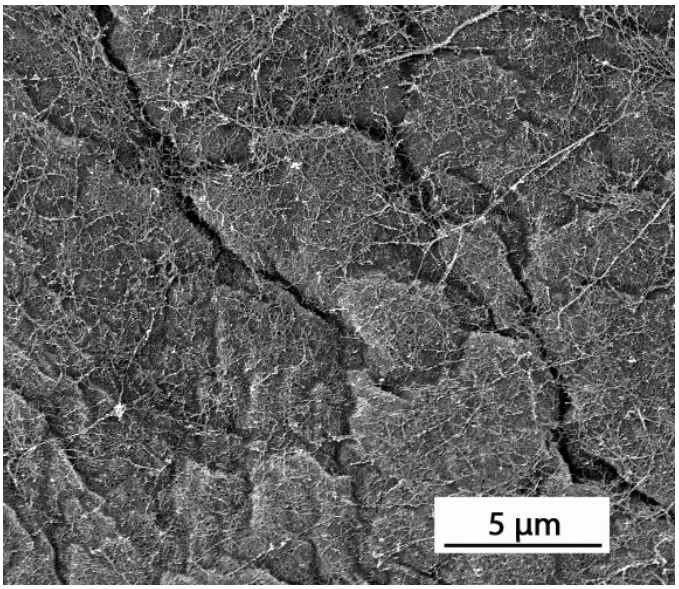

Figure 2: Retina sample that was critical-point dried using a more gradual dehydration protocol to mitigate the spherical artifacts of Figure 1. Imaged using Helix (SE) detector in low vacuum with a magnification of 8000×, 0.298 Torr chamber pressure, and accelerating voltage of 7 kV.

Samples without osmium tetroxide from the CPD preparation yielded crisp images of the collagen matrix on the retina surface using the Helix detector at low vacuum (Figure 3). Specimens subsequently treated with osmium tetroxide, also imaged at low vacuum with a Helix detector, yielded indistinguishable results (Figure 4).

A comparison of the HMDS image (Figure 5) with the CPD images (Figures 3, 4) showed no distinct difference between the two preparation methods. No charging or drying artifacts were observed.

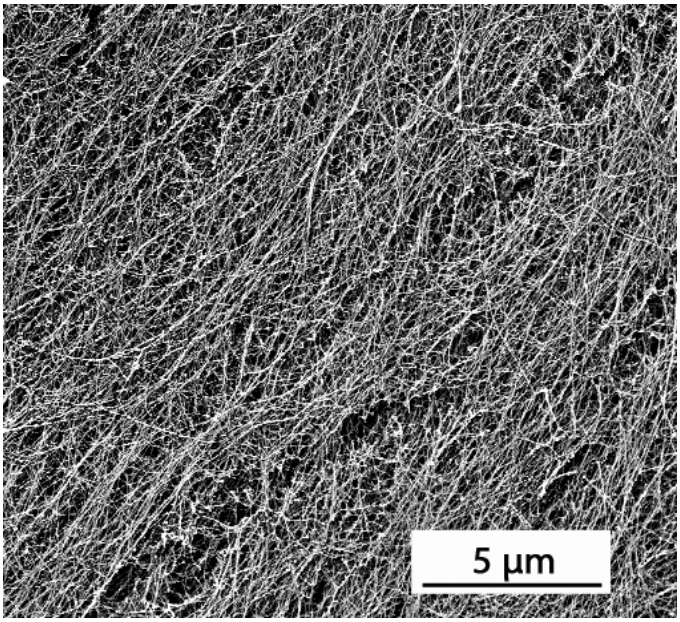

Figure 3: Retina sample critical-point dried without osmium fixation using Helix detector in low vacuum. Image taken at a magnification of 8000×, 0.261 Torr chamber pressure, and accelerating voltage of 7 kV.

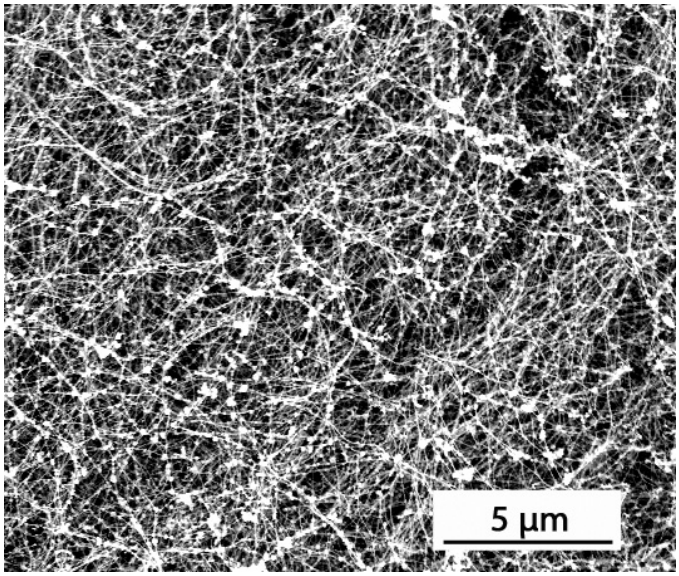

Figure 4: Retina sample critical-point dried with osmium fixation using Helix detector in low vacuum. Image taken at 8000× magnification, 0.376 Torr chamber pressure, and 7 kV accelerating voltage.

The ESEM was unable to resolve collagen fibers on the retinal surface (Figure 6). Multiple attempts with varying temperature and pressure parameters yielded no progress in the imaging results. Furthermore, air drying during imaging posed a significant problem because of the curling of the thin layers of the retina.

Discussion

In our study, CPD and HMDS preparation methods both provided acceptable image quality and minimal artifacts. Although CPD is the most common preparation method, HMDS requires no specialized equipment nor precise

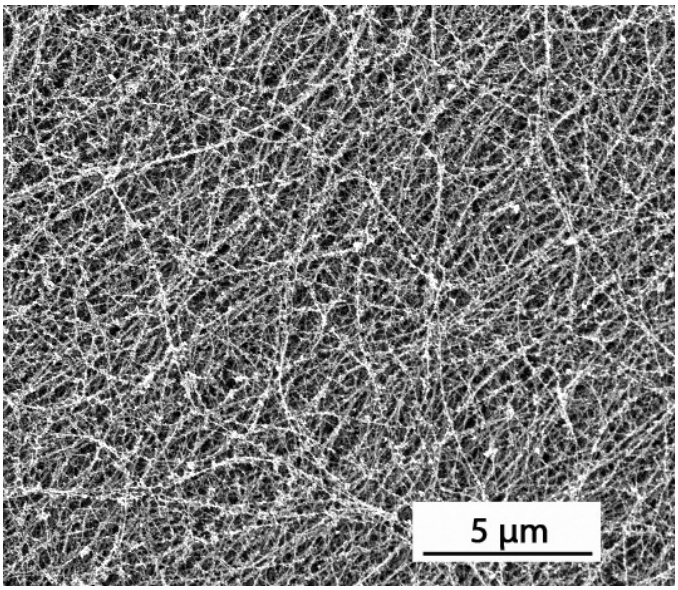

Figure 5: Retina sample prepared with HMDS dehydration and imaged using Helix detector in low vacuum. Magnification of 8000× was used with a 7 kV accelerating voltage and 0.301 Torr chamber pressure.

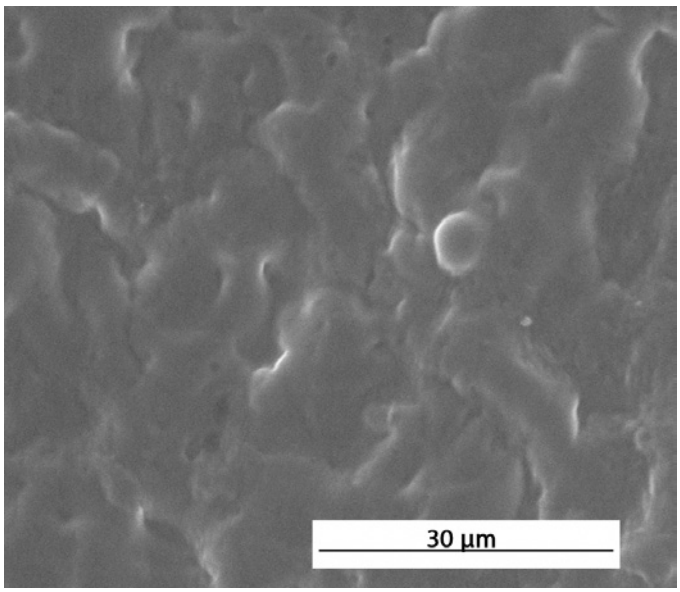

Figure 6: ESEM image taken of retinal surface at 6.499 Torr of water vapor with a magnification of 4000× and 7 kV accelerating voltage. The presence of vitreous (99% water) impedes visualization of the collagen matrix on the retina. Attempts to minimize vitreous were unsuccessful, and specimen became thermally damaged (i.e., retina layers curling) within 20 minutes of application of the beam.

monitoring of the samples, resulting in lower time and cost commitments than CPD. For immature porcine retina, we found that the HMDS images were indistinguishable from CPD images and therefore conclude that HMDS is suitable for delicate tissues as long as imaging is conducted in low vacuum. This agrees with other studies that have shown the efficacy of HMDS [1, 2] on non-retina animal tissues. However, CPD still appears to be the preferable method for plant specimens [2].

The ESEM approach was by far the least time-consuming of all the methods tested, and the costs were minimal. Unfortunately, imaging the retina surface using this technique proved difficult because of the thickness of the vitreous fluid layer, the poorer image resolution, and the finite duration of the specimen in the ESEM chamber before it became thermally damaged by the electron beam. ESEM has been used to resolve features on the nanometer scale, but this can be difficult with a wet sample [3]. Collagen fibers on the retinal surface are on the order of 10 nm in diameter. The small size of the collagen matrix and the presence of vitreous on the retina surface make ESEM imaging a poor choice for investigating retina ultrastructure.

Another challenge with ESEM imaging is the limited time for imaging of the biological specimen inside the chamber. Despite having some control over pressure and temperature, biological specimens are very susceptible to beam damage and deterioration, and samples may only be imaged once. Typically, biological specimens can be imaged for 30–60 minutes before significant drying artifacts damage the sample [4]. In our study, the retina lasted 20 minutes, perhaps because of the thin (~200 µm) and multilayered structure.

Conclusion

SEM of biological specimens is such that subtle changes in sample preparation can alter image quality as well as introduce artifacts. From our investigation of preparation methodologies for a delicate biological tissue (that is, retina), we conclude that the CPD and HMDS preparation techniques both result in similar image quality, but HMDS clearly has the advantage of being less time-consuming and less costly. If specimens come from previously fixed tissue, additional fixation with osmium tetroxide is unnecessary when using a Helix detector in low vacuum. Regardless of preparation method, gradual ethanol gradient steps should be used to reduce the potential for drying artifacts. ESEM was not found to be useful for imaging collagen in retina samples given the resolution requirements, the natural presence of vitreous on the surface, and the thin multilayered structure that is extremely susceptible to thermal damage.

Acknowledgments

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