

# Sample Preparation for Scanning Electron Microscopy: The Surprising Case of Freeze Drying from Tertiary Butanol

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## Introduction

For scanning electron microscopy, our ability to examine wet specimens continually improves thanks to advances in instrumentation; nevertheless, drying wet samples remains indispensable, particularly for high resolution or for a sample that needs to be imaged repeatedly. In many laboratories, the standard method for drying is critical-point drying. In this method, samples are dehydrated with ethanol and then infiltrated with cold liquid carbon dioxide, which is then heated up under pressure to pass through its critical point, forcing the carbon dioxide to undergo a transition from a super-critical liquid to gas. In doing so, the solvent molecules enter the gas phase more or less simultaneously throughout the sample, avoiding the formation of a liquid–gas interface and the accompanying large surface tension forces.

Despite the widespread use of critical-point drying, it is not ubiquitous. In Japan, the standard method for drying wet samples is to freeze-dry them from tertiary butanol (t-butanol). In this method, samples are dehydrated with ethanol and then infiltrated with t-butanol. Infiltrated samples are frozen and then put under a vacuum, which sublimates the frozen alcohol, meaning it goes from the solid to the gas phase and thereby avoids a liquid–gas interface and the concomitant surface tension. Because t-butanol freezes at about 25°C, freezing and sublimation are readily accomplished.

Freeze-drying from t-butanol gained popularity in Japan following the publication in 1988 of a paper by Inoué and Osatake [1]. These authors examined mouse tissues (pancreas, diaphragm, ciliated trachea, and red blood cells) and concluded that micrographs obtained from the t-butanol method were as good as, and sometimes better than, those obtained from critical-point drying. Attesting to the impact of this paper, when checked in September 2013 on the Web of Science, it had been cited more than 260 times, mainly by Japanese laboratories, in which this method is applied to diverse kinds of wet sample.

Despite widespread use in Japan, freeze-drying from t-butanol appears to be little used elsewhere. For example, two recent comprehensive reviews of biological sample preparation for scanning electron microscopy omit the method altogether [2, 3]. Frankly, it is puzzling that freeze-drying from t-butanol could be standard operating procedure in one country and almost unheard of in another.

To explore the use of t-butanol firsthand, we built an apparatus for freeze-drying, and we prepared samples for imaging with scanning electron microscopy. We examined sections of a plant seedling stem and the glans penis of a bat. Here, we show

that these samples freeze-dried from t-butanol look as good as, or better than, those prepared by critical-point drying.

## Materials and Methods

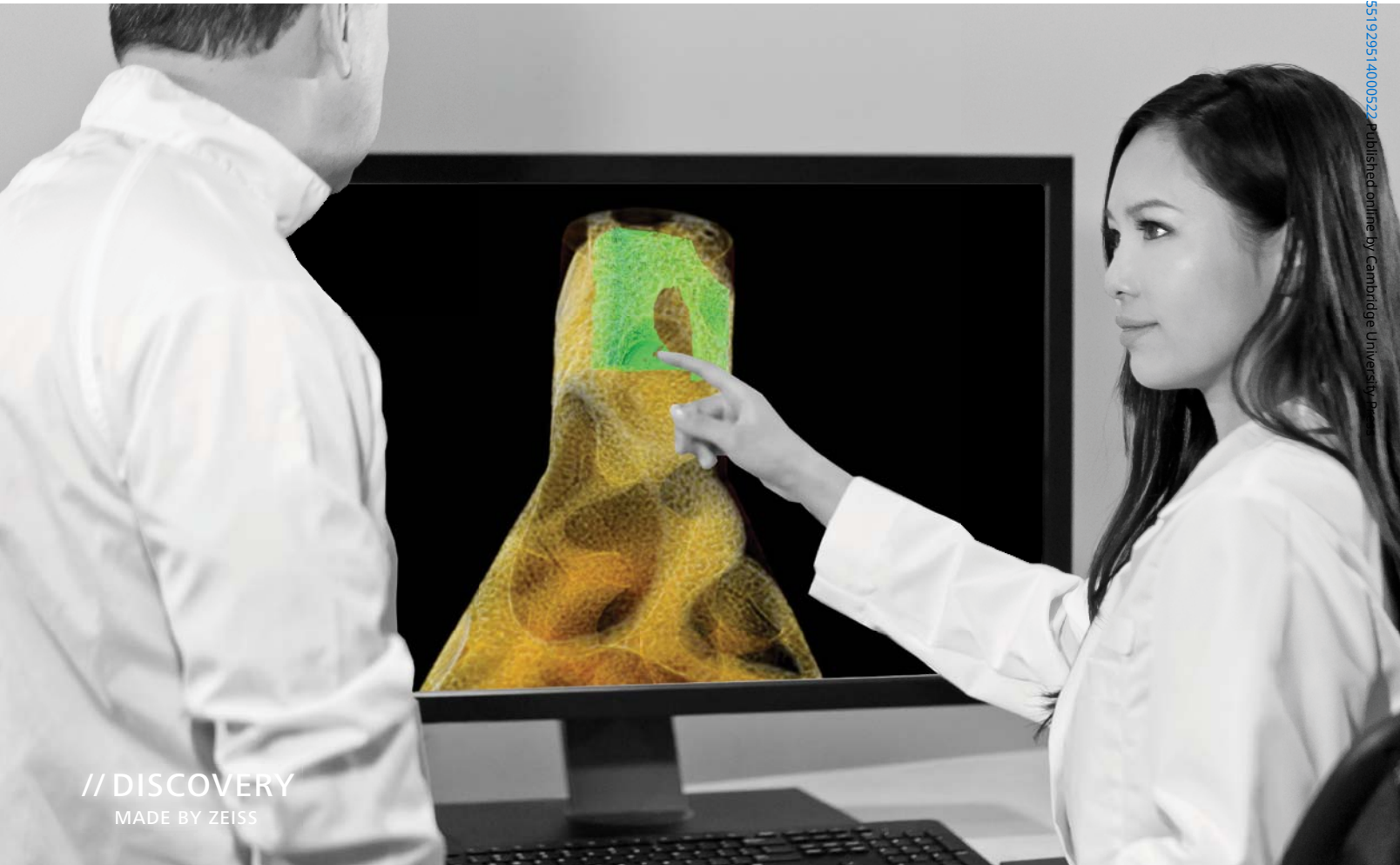
Cucumber (*Cucumis sativus* L., cv Burpee's Pickler) seeds were sown on filter paper moistened with tap water and grown in nominal darkness at ~25°C for four to five days, until the hypocotyl (seedling stem) was 5 to 10 cm long. At that point, 5 mm sub-apical stem segments were cut and glued to the stub of a Vibratome with gel-type “super glue” and sectioned at a nominal thickness of 100 μm in several hundred microliters of phosphate-buffered saline (PBS). Sections were collected in the well of a six-well micro-plate and incubated in PBS supplemented with 0.2% Triton-X100 for one hour with vigorous orbital shaking. Then they were dehydrated with gentle orbital shaking in a graded ethanol series with three final incubations in 100% ethanol, one going overnight.

Free-living, male red bats (*Lasiurus borealis*) were captured, killed, frozen, and donated by Evan Pankuuk (Arkansas State University's Institutional Bio-safety Committee approval No. 135349-1). Adult, reproductively active bats were chosen, based on the degree of tooth wear, wing-bone fusion, testis size, and coloration of the epididymal sheath. The entire reproductive tract was removed. To approximate the placement of spines during an erection, the tissue was inflated by injecting formalin into the corpora with an insulin syringe and tying the tract just above the site of injection. Then it was incubated in 10% buffered formalin (pH 7) for at least 48 hours. Next, the distal portion of the glans penis was dissected from the rest of the reproductive tract and dehydrated in a graded series of ethanol, 1 hour per step, with slow rotation, including three changes of 100% ethanol, the last of which went overnight.

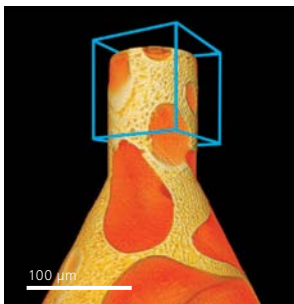
The population of samples was then divided. For critical-point drying, samples were loaded into a carrier and placed, submerged in ethanol, in the chamber of a Tousimis Samdri PVT 3-D instrument. The chamber was flushed with water-free, liquid carbon dioxide for ~10 to 20 minutes at ~0°C, heated to 40°C (1,300 psi), let stand for 30 minutes, and then slowly (30–60 minutes) returned to atmospheric pressure.

For freeze-drying, samples were transferred to a 20 mL scintillation vial. The bat samples were incubated at room temperature in a transitional series (1:3, 2:2, 3:1 t-butanol: ethanol). Samples were incubated in three changes of 100% t-butanol at 50°C in a drying oven, with the first two incubations lasting several hours and the third going overnight. After a fourth change, the vial was placed at 4°C for ~2 hours

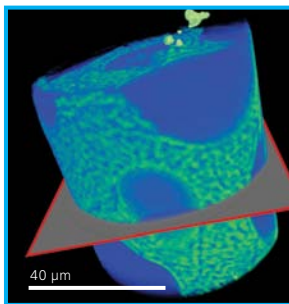
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and then freeze-dried. The vial contained about 1 mL of t-butanol, which took about 1 hour to sublime, at which point the pump was run for 30 minutes longer to ensure complete removal of the butanol. When pumping begins, the solid mass of frozen t-butanol is drawn up the vial. To prevent its escape, we used vials with a constriction (“neck”) at their opening.

We built a freeze-dryer by attaching a rotary vane pump (Alcatel No. 2002) to the sample chamber of a disused sputter coater. The sputter head was replaced by a polycarbonate bell. With no sample, the pump brought the vacuum in the chamber down to approximately 80 mTorr. To prevent the t-butanol from damaging the pump oil, we used a perfluorinated oil, Fomblin type 25/6, which is resistant to t-butanol. Also, to drive off any t-butanol dissolved in the oil, after each drying run, the pump was ballasted for 3 to 5 minutes.

After drying, samples were mounted on an aluminum stub with double-sided carbon tape known to have good conductivity. For bat samples, conductive graphite paint was applied around the base of the sample. Sections from the two types of preparation were mounted on a single stub so they could be coated and imaged side by side. Samples were sputter coated with platinum and imaged on a field emission scanning electron microscope (FEI Magellan) at an accelerating voltage of 1 kV and beam current of 50 pA.

## Results

The first samples tested were sections of the seedling stem of cucumber. The stem was sectioned while it was alive, a procedure that causes the cytoplasm to be lost and allows the surface structure of the cell wall to be imaged at high resolution [4]. The most striking difference between the methods was seen before sections were mounted (Figures 1a and 1b). The sections prepared by critical-point drying were strongly curved in two axes (parallel and perpendicular to the long axis of the stem). This double curvature made them difficult to flatten onto the stub. In contrast, the sections prepared by freeze-drying from t-butanol, although bent, were less strongly curved and far more readily flattened.

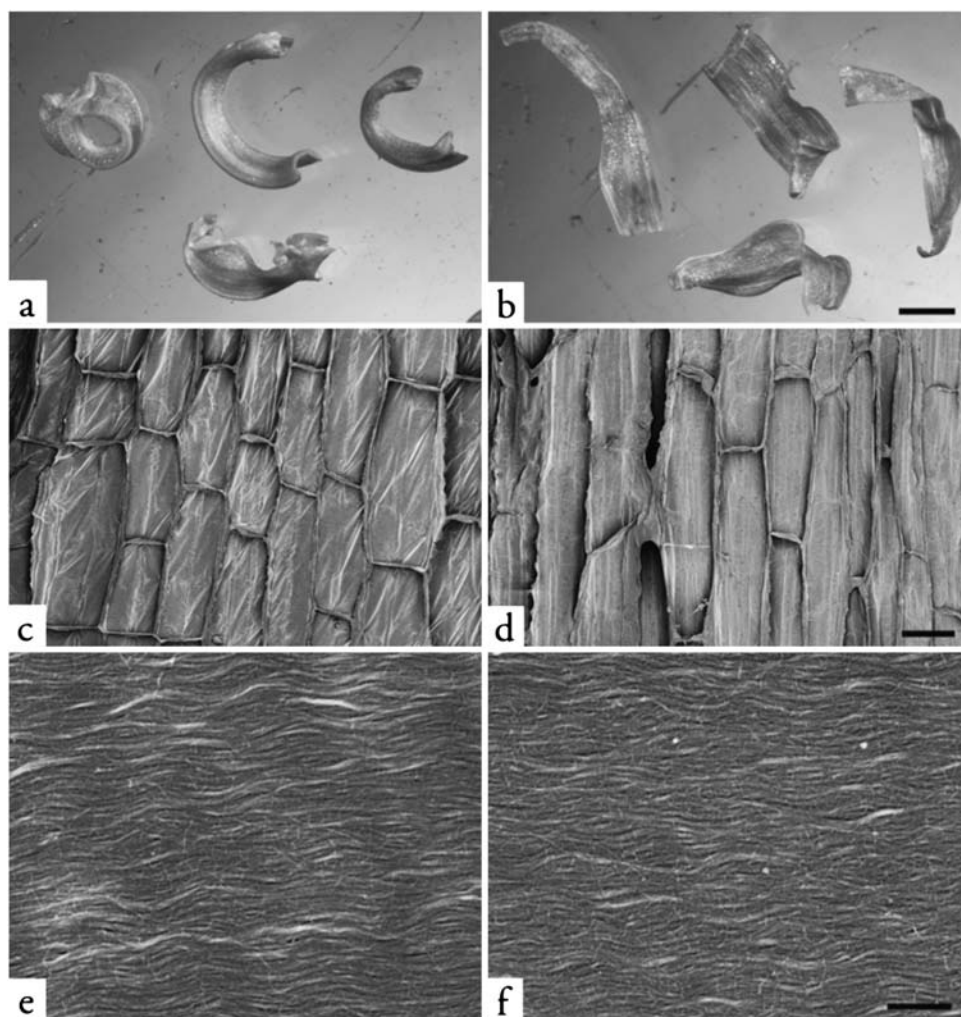
When viewed in the scanning electron microscope at low magnification, the cells appeared as empty boxes, recalling the monks’ cells of Robert Hooke (Figures 1c and 1d). Comparing the drying methods, large wrinkles were conspicuous in the critical-point dried sections, possibly because of the flattening process, but were minor or absent in the freeze-dried ones. Finally,

higher-magnification images of the “floor of the cell” (that is, cell wall ultrastructure) from the two methods appeared to be indistinguishable (Figures 1e and 1f).

The next sample we examined was the distal portion of the glans penis of the red bat (*L. borealis*). This organ contains keratinized spines, and scanning electron microscopy has proven to be a useful method for examining their number, dimension, and orientation [5, 6]. In general, the two methods gave equivalent results (Figure 2). With each method, the epidermal surface was occasionally wrinkled or cracked, but to a similar extent, and the keratinized spines appeared all but identical. Despite size-matching the samples for comparison, there appeared to be more differences between samples than between methods. Thus, we might have missed subtle effects of the drying methods. But overall, critical-point drying and freeze-drying from t-butanol appear to process the bat penis equivalently.

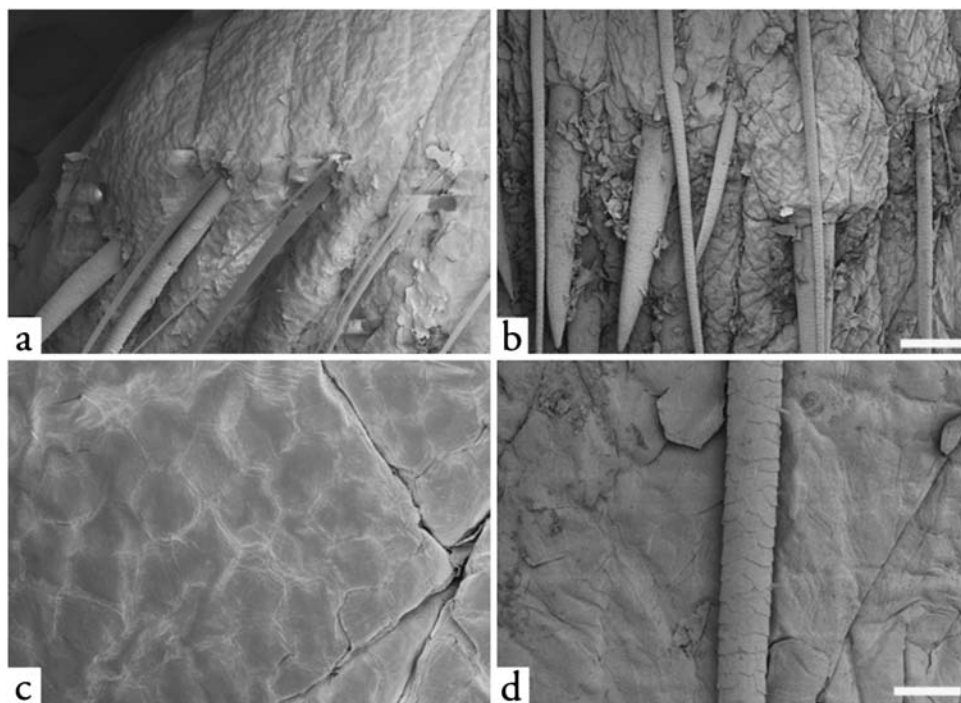
## Discussion

Freeze-drying from t-butanol has a few methodological considerations. Because of the high melting point of t-butanol (25°C), controlling the temperature of the sample chamber



**Figure 1:** Comparison of drying methods for cucumber hypocotyl sections: (a, c, e) critical-point dried; (b, d, f) t-butanol dried. (a, b) Bright-field light micrographs of sections immediately after drying. (c–f) are scanning electron micrographs comparing the structure of cells (c, d) and of cell walls (e, f). Scale bars=1 mm (a, b); 50 μm (c, d); 250 nm (e, f).





**Figure 2:** Comparison of drying methods for the bat penis: (a, c) Critical-point dried; (b, d) t-butanol dried. (a, b) are survey views showing the deployment of epidermis and spines. (c, d) are higher-magnification views. Scale bars = 100  $\mu\text{m}$  (a, b); 25  $\mu\text{m}$  (c, d).

is not particularly problematic, at least for the small volumes (1 to 2 mL) used here. To minimize the formation of liquid, we placed the pump in an air-conditioned room held at 21°C and put a small, frozen (-20°C) metal block in the chamber along with the sample vial.

In our initial trials, the rotary vane pump contained standard vacuum pump oil. After half a dozen runs, the minimal pressure achieved by the pump had risen by a factor of ten. Evidently, t-butanol damages standard pump oil. Although a fore-line trap may be added to condense the t-butanol before it reaches the pump, a freeze-dryer (Vacuum Devices VDF-21S) sold in Japan for this method uses a perfluorinated oil—Demnum S-65, Daikin Industries—which is unavailable in the USA. We therefore rebuilt the pump to contain Fomblin oil, which is similar to Demnum. As an additional precaution, the pump is ballasted after each run. These expedients appear to be maintaining pump performance.

Our finding that t-butanol is equivalent or superior to critical-point drying is consistent with previous reports. The results of Inoué and Osatake [1] were noted above. The t-butanol method has been adopted for drying adult drosophila by Phil Oshel (Central Michigan State University; personal communication). Apparently the first to report freeze-drying from t-butanol, Wheeler et al. [7], examined canine endocardia and found the freeze-drying to be equivalent to critical-point drying, but more convenient. Cantu-Crouch et al. [8] studied human lens epithelial cells and found those that were freeze-dried from t-butanol, compared to those critical-point dried, suffered fewer cracks across membranes and cell processes. Finally, critical-point dried fish sperm shrank to a significantly greater extent than those freeze-dried in t-butanol [9].

In contrast, Boyde [10] reported extensive shrinkage for a mouse embryo limb dehydrated in t-butanol. Mouse limbs might be particularly prone to shrinkage in t-butanol, but Boyde dehydrated the sample in t-butanol; whereas for freeze-drying, samples are customarily dehydrated in ethanol prior to infiltration with t-butanol [1, 7, 8, 9]. Boyde wrote that he presented the results for t-butanol dehydration at *Scanning Electron Microscopy 1978* (Los Angeles, CA) and “in many lectures on specimen preparation” [10]. Insofar as Boyde was a pioneer of methods for scanning electron microscopy in biology, his negative opinion could explain the limited use of t-butanol freeze-drying outside of Japan.

## Conclusion

Although we prepared only two kinds of specimen, our experience coincides with what is described in the literature: freeze-drying from t-butanol appears to be a valuable method for the scanning electron

microscopist. Freeze-drying is easy, and runs are consistent. Every sample and study is unique, but we encourage those needing to dry wet samples to consider using t-butanol.

## Acknowledgments

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