A Method for Preparing Spaceflight RNAlater-Fixed Arabidopsis thaliana (Brassicaceae) Tissue for Scanning Electron Microscopy

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A METHOD FOR PREPARING SPACEFLIGHT RNA-later-fixed
ARABIDOPSIS THALIANA (BRASSICACEAE) TISSUE FOR SCANNING
ELECTRON MICROSCOPY

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- **Premise of the study:** In spaceflight experiments, tissues for morphologic study are fixed in 3% glutaraldehyde, while tissues for molecular study are fixed in RNA-later; thus, an experiment containing both study components requires multiple fixation strategies. The possibility of using RNA-later-fixed materials for standard SEM-based morphometric investigation was explored to expand the library of tissues available for analysis and maximize usage of samples returned from spaceflight, but these technologies have wide application to any situation where recovery of biological resources is limited.

- **Methods and Results:** RNA-later-fixed samples were desalinated in distilled water, dehydrated through graded methanol, plunged into liquid ethane, and transferred to cryovials for freeze-substitution. Sample tissues were critical point dried, mounted, sputter-coated, and imaged.

- **Conclusions:** The protocol resulted in acceptable SEM images from RNA-later-fixed Arabidopsis thaliana tissue. The majority of the tissues remained intact, including general morphology and finer details such as root hairs and trichomes.

**Key words:** Arabidopsis thaliana; RNA-later; scanning electron microscopy; SEM; spaceflight.

Experimental flexibility is a luxury often taken for granted in typical laboratory science. Adapting a protocol in response to unfolding information as the experiment proceeds is a key element in discovery and is standard practice. In spaceflight, this luxury is not as accessible; crew time is highly regulated, orbital resources are limited, and protocols are tightly controlled once in place. In addition, the up-mass available to each researcher is limited, which restricts the choices of experimental treatments and orbital fixation options. These constraints often translate into hard choices that limit the scientific return on investment, including sacrificing the number of replicates to enable additional treatment and fixation scenarios. As a consequence, options that allow maximization of the data obtained from a single sample are highly valued.

Biological experiments are typically required to be segregated based on the analyses to be performed upon return from spaceflight. These segregated conditions are usually either molecular or morphological, due to the separate fixatives required for each application (e.g., Stutte et al., 2006). Investigations focused on transcriptome analyses, for instance, would not be likely to commit a portion of their limited up-mass on samples and associated fixation hardware for morphological applications, and would miss the opportunity to use both morphological and molecular perspectives. These limitations are keenly felt in scenarios where the transcriptome analyses reveal spaceflight-specific patterns of gene expression central to morphological patterns of cell growth and development. This scenario was encountered in a recent spaceflight experiment, in which telemetric imaging revealed interesting spaceflight-associated root morphologies (Paul et al., 2012). All material was fixed on orbit in RNA-later (Ambion, Grand Island, New York, USA) to facilitate transcriptome analyses on returned samples.

Although it is possible to partition an experiment into molecular and morphological components, using samples that correspond directly to those used in molecular applications would be of extreme value. A single fixation protocol better enables the full use of replicates to support a robust power analysis; all plant material allowed by the up-mass constraints can be dedicated to a single application per orbital treatment. Additionally, the plant material apportioned for microscopy can be taken directly from the pool of material whose balance represents a molecular replicate; thus, any morphologic change seen in these plants would directly reflect the effects of the altered gene expressions from those same plants. Terrestrial field studies could also benefit from this single fixative approach for similar reasons.

Despite its use as a molecular fixative, RNA-later is not commonly used as a morphologic fixative. RNA-later fixation produces unclear images with high background staining (Cadoret et al., 2013) and reduces immunofluorescent signals (Zaitoun et al., 2010; Paul and Ferl, 2011). This intracellular occlusion eliminates the possibility of clear subcellular localizations.

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However, the external morphology appears to be preserved in a fresh-like state, which suggests that RNA later may be acceptable for use in scanning electron microscopy (SEM). Two cases in the literature report the use of RNA later in a morphological investigation. Behrendt et al. (2011) reported that coral covered in crustose coralline algae was fixed in RNA later, postfixed in 2% glutaraldehyde, rinsed in sodium cacodylate, and postfixed again in OsO₄ prior to SEM investigations. Hofer et al. (2012) used SEM in their study on fungal organs of Clermontia species using RNA later as their main fixative with a hexamethydilsilazane drying step. In the current study, both of the above techniques resulted in significant tissue damage in Arabidopsis.

An alternative to fixation in SEM sample preparation is freezing. Cryo-SEM does not require prior fixation, but instead preserves tissues in a fresh-like state for imaging (Koroleva et al., 2010; Sarsby et al., 2012; Yu et al., 2013). However, few laboratories have access to such equipment, and the equipment and operation can often be expensive. Therefore, this study investigated the possibility of emulating cryo-SEM on a standard SEM, a “cryo-analog,” where tissue would first be returned to a fresh-like state for freezing prior to imaging.

METHODS AND RESULTS

For process development, test samples of 9–12-d-old Arabidopsis thaliana (Col-0) or Wassilewskija (WS) (The Arabidopsis Information Resource [TAIR], Columbus, Ohio, USA) were harvested into RNA later and fixed overnight at ambient room temperature. Fixed plants, along with freshly harvested individuals of the same age, were washed in distilled water with gentle rocking at ambient room temperature. Fixed plants, along with freshly harvested individuals of the same age, were washed in distilled water with gentle rocking at ambient room temperature. Fixed plants, along with freshly harvested individuals of the same age, were washed in distilled water with gentle rocking at ambient room temperature. Drying occurred via liquid CO₂, critical point drying (CPD; Bal-Tec CPD 030; Leica Microsystems). Eleven quick-cycled washes replaced most of the loading methanol with CO₂, followed by three 10-min infiltrations. One final exchange was performed and left overnight. Two additional 10-min infiltrations were performed, followed by CPD with controlled venting. Samples were in solution until the end of CPD. Dry samples in baskets were transferred to a desiccant-filled cryovial (Drierite; W. A. Hammond Drierite Company, Xenia, Ohio, USA) sealable container until being mounted on stubs using carbon adhesive and returned to a stub rack within a different desiccant-filled sealable container. Mounted samples were sputter-coated for 90 s with a gold-palladium alloy (Desk V HP; Denton Vacuum, Moorestown, New Jersey, USA) and imaged on a scanning electron microscope (Hitachi S-4000; Hitachi, Tokyo, Japan).

Initial observations were focused on roots, resolution of cell files, and retention of root hairs throughout the processing of the tissue (Fig. 2). Later tests observed trichomes and more durable tissues, such as adaxial leaf surfaces and seed coats (Fig. 2A–C). The fresh-frozen tissues, used as protocol development control material, retained finer details, including closed stomata and normal trichome morphology (Fig. 2D, E). RNA later-fixed test materials yielded similar images as the fresh-frozen plants, with minimal additional tissue damage (Fig. 2F–K).

Spaceflight materials and the corresponding ground controls, both fixed in RNA later, were processed using the protocol discussed above and detailed in Appendix 1. Tissues yielded images of acceptable quality, with minimal cell wrinkling and high retention of fine details (Fig. 2J, K). The focus of these samples was root structure, morphology, and root hair retention because of interesting molecular and genetic observations from a recent spaceflight experiment (Paul et al., 2012; Paul et al., unpublished).

Cost of the developed protocol is minimal, while the accessibility is high. Assuming the microscopy core to be used has standard SEM capability, the additional cost for this protocol is merely the cost of consumables. These components are readily available and likely already obtained by the laboratory interested in using this protocol. Another advantage of this protocol is a near retention of tissue morphology at a fresh harvest state. Not only are finer elements of tissues retained (Fig. 2D–I), but other tissues, such as the adaxial leaf epidermis, seed coat, and floral stalk (Fig. 2A–C), produce results comparable to those processed through dedicated morphologic means (e.g., Bhargava et al., 2013). The amount of time needed for this process is slightly longer than other standard SEM processing times, with an additional 4 h for the desalination washes and several days required for freeze substitution. Hand-plunged samples may be processed in batches in times to batch to throughput, being limited to the number of samples able to be held with a pair of forces without compromising structural integrity. Examples of other SEM protocols may be seen in Liu et al. (2008), Koizumi et al. (2010), Springer et al. (2010), and Yi et al. (2010). As a result of this protocol, samples fixed with RNA later retained many fine features including root hairs and trichomes, with a majority of cells inflated and displaying fresh-frozen-like morphology, regardless of whether they were harvested during spaceflight or on the ground.

CONCLUSIONS

The protocol presented here provides distinct advantages for both spaceflight and ground-based experimental applications. Arabidopsis plants harvested on the International Space Station by astronauts were successfully retrieved from RNA later and processed to produce acceptable quality images without the use of a dedicated morphologic fixative (Fig. 2J, K). Field studies may also benefit from the use of this protocol. As in spaceflight, the amount of materials able to be carried and used during an expedition is limited. With the option of using one stable, safe chemical fixative for all desired studies to be conducted upon return, scientists in the field may be able to collect more data with fewer trips. This protocol may also assist laboratories with libraries of samples in RNA later by expanding the available techniques for study.

Alternative imaging techniques outside the scope of this investigation provide alternatives to this protocol. True cryo-SEM would likely be an acceptable alternative (examples may be seen in Koroleva et al., 2010; Sarsby et al., 2012; and Yu et al., 2013), though desalination of specimens prior to tissue processing would still be beneficial. A second option is...
a cryo-holder, seen in Tang et al. (2012), which may prove to be an acceptable alternative step in this protocol if the SEM being used can handle the LN₂ vapors. A third alternative is scanning ion conductance microscopy (SICM; Marti et al., 1988; Hansma et al., 1989; Ushiki et al., 2012). Due to the high ionic content of RNA later and the necessity for an ionic submersion liquid for SICM, it is possible that this instrument could generate comparable images without any tissue processing.
this process needs to be adapted to each sample being studied, it enhances flexibility in experiments when the main objective is not imaging.

The protocol reported here will likely require optimization for each desired tissue to be tested. Arabidopsis is a very fragile tissue, with limited flat surfaces and small organ sizes. Other plants that may be more robust may tolerate a rougher technique, while more fragile tissues (i.e., floral tissues) may need a more gentle approach. Some potential additional steps to enhance tissue integrity include a postfixation step or reversing CPD and sputter-coating. A postfixation step (as seen in Behrendt et al., 2011), which would take place after the desalination, could improve tissue integrity during the cryo-analog plunge, especially if fixatives for electron microscopy, such as Karnovsky’s or OsO₄, are used. Sputter-coating prior to CPD (as seen in Yi et al., 2010) may also improve the physical tissue integrity, though both mounting prior to CPD and pulling a vacuum during the coating process may prove difficult.

The use of this protocol provides more morphological opportunities for samples that would have previously been wasted due to improper fixation. It is designed to be a supplement to rather than a replacement of SEM standard practices. Although possibility was not tested due to lack of access to the necessary equipment.

LITERATURE CITED


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**APPENDIX 1. Generalized protocol for RNAlater-fixed sample preparation for SEM investigation.**

1. Wash samples in distilled H₂O for four 1-h increments.
2. Dissect samples to fit onto SEM stubs and place into SPI microporous specimen capsules (120–200 μm pore size; Structure Probe Inc., West Chester, Pennsylvania, USA).
3. Dehydrate in graded MeOH (10–100%, 10% intervals) at 10 min per step.
4. Plunge in LN₂-cooled ethane.
5. Immediately transfer to cryovials containing either 2% OsO₄ in MeOH or 0.5% glutaraldehyde in 0.1% tannic acid in acetone.
6. Freeze-substitution (LN₂ at 4°C) for 4–10 d.
7. Perform 11 quick washes (start with MeOH in chamber; cool 10°C for CO₂), followed by three infiltrating washes, leaving the final overnight.
8. Perform two additional infiltrations prior to critical point drying.
9. Critical point dry samples following manufacturer’s guidelines and transfer to dessicant-filled box.
10. Clean stubs (if necessary) with acetone sonication.
11. Mount samples on stubs with thick carbon adhesive.
12. Sputter-coat samples for 90 s in gold-palladium alloy.
13. Proceed to SEM imaging.

Chemicals needed: water, RNAlater (for fixation; Ambion, Grand Island, New York, USA), MeOH (for dehydration), LN₂, ethane, CO₂, Cryovials: OsO₄ and MeOH, or glutaraldehyde, tannic acid, and acetone.