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PROTOCOL NOTE

A NEW TECHNIQUE TO PREPARE HARD FRUITS AND SEEDS FOR ANATOMICAL STUDIES[1]

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- *Premise of the study:* A novel preparation technique was developed to examine fruits and seeds of plants with exceptionally hard or brittle tissues that are very difficult to prepare using standard histological techniques.
- *Methods and Results:* The method introduced here was modified from a technique employed on fossil material and has been adapted for use on fruits and seeds of extant plants. A variety of fruits and seeds have been prepared with great success, and the technique will be useful for any excessively hard fruits or seeds that are not able to be prepared using traditional embedding or sectioning methods.
- *Conclusions:* When compared to existing techniques for obtaining anatomical features of fruits and seeds, the protocol described here has the potential to create high-quality thin sections of materials that are not able to be sectioned using traditional histological techniques, which can be produced quickly and without the need for harmful chemicals.

Key words: anatomy; fibers; microtomy; sclereids; thin sectioning; wafering.

A variety of methodologies exist for preparing plant organs for anatomical studies, many of which are sample dependent (Johansen, 1940; Sass, 1958; Feder and O'Brien, 1968; Carlquist, 1982; Ruzin, 1999; Keating, 2014). Herbaceous materials can be prepared and analyzed using traditional wax-embedding protocols with exceptional results, but hard tissues such as some fruits and seeds can produce less-than-optimal results when prepared with the same methods, and frequently fail to produce publishable data. Traditionally, hydrofluoric acid (HF) has been employed as a softening agent and to remove silica bodies in various organs (e.g., Liao and Wu, 2000), but HF is incredibly caustic, may be difficult to access due to health and safety concerns, and can take more than a month to prepare materials (see Carlquist, 1982 for details). Boiling or autoclaving have also been proposed as effective softening techniques, but highly sclerified tissues common in fruits and seeds remain notoriously difficult to section (Ruzin, 1999). Carlquist (1982) detailed a protocol originally described by Kukachka (1977) using the softening agent ethylenediamine (ETD) with great success to soften wood prior to embedding, but noted that this technique has limitations when working on materials with a mixture of cell types. In the author's own experience preparing fruits and seeds for anatomical studies, many hardened and mature tissues fail to produce the desired results using the ETD method, which warranted the development of an alternative preparation technique.

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In preparing fossil plant material for anatomical studies, it is common practice to embed anatomically preserved fossils in a clear resin and then grind and polish the specimens for viewing with reflected light microscopy (Jones and Rowe, 1999). Alternatively, fossils may be prepared by producing serial thin sections or "wafers" of material using a diamond wafering blade on a lapidary saw (Stein et al., 1982; Hass and Rowe, 1999). These wafers are then mounted onto a microscope slide, ground and polished, and observed using reflected or transmitted light when the specimen is ground thin enough (<1 mm; Benedict et al., 2008). Results of this technique show fine anatomical details of a variety of fossil organs from various ages and sediments (e.g., Manchester, 1994; Hass and Rowe, 1999; Pigg et al., 2004, 2008, 2014; Rothwell and Ash, 2015).

At present, it appears that this wafering technique has not been applied and described in detail for use with nonfossil plant materials. It is the aim of this paper to describe, in detail, the use of the wafering technique modified for extant fruit and seed material.

METHODS AND RESULTS

Producing slides of fruits and seeds for anatomical studies can be difficult due to the often heterogeneous nature of the various tissues in these plant organs. The method proposed here is a modification of techniques used in studying fossil plant material, including embedding techniques (Stein et al., 1982; Jones and Rowe, 1999), and thin sectioning and wafering techniques (Stein et al., 1982; Hass and Rowe, 1999).

The process involves three fundamental steps: (1) embedding specimens in Liquid Bio-Plastic synthetic resin (Ward's Science, Rochester, New York, USA), (2) creating thin sections on a low-speed lapidary saw, and (3) grinding and polishing specimens to obtain anatomical details (Appendix 1). Liquid Bio-Plastic synthetic resin has been used traditionally to prepare whole mounts of specimens for study and teaching (Burger and Seif, 1979), but it is also an ideal resin to create thin sections as it is completely transparent and hard when cured. The resin is mixed with its catalyst (see Table 1 for ratios) and poured as three separate layers, with the specimen embedded in the middle layer (Fig. 1A–F).

TABLE 1. Liquid Bio-Plastic reference chart for embedding specimens. The proportions are adapted from Ward's Science (n.d.).

Estimated thickness (in inches) of Bio-Plastic layer to be poured	% catalyst by volume	CC of catalyst per 100 mL of Bio-Plastic (mL)	Drops of catalyst per 100 mL of Bio-Plastic[a]
≤1/4	2	2	80
1/4–1	1	1	40
1–2	0.25–0.5	0.25–0.5	10–20

[a] Using medicine or eye dropper.

First, a thin supporting layer (2–3 mm thick) is created to provide a base for the specimen to rest on (Fig. 1A), then the specimen-retaining layer is created to secure the specimen in the desired orientation (Fig. 1B), and a covering layer (2–3 mm thick) is poured to seal the specimen in the block (Fig. 1C).

The resin blocks with the specimens adequately embedded are then cut into serial thin sections, or wafers, using a lapidary low-speed saw with a diamond cutting edge (e.g., IsoMet Low Speed Saw; Buehler, Lake Bluff, Illinois, USA), which uniformly cuts through the specimen and resin to yield wafers 0.5–1.0 mm thick, depending on the type of blade and lapidary saw used. The wafers are then cleaned, dried, and affixed to a traditional microscope slide using any standard mountant typically used to affix fossil wafers to slides (e.g., Manchester, 1994 [Elmer's epoxy; Elmer's Products Inc., Westerville, Ohio, USA]; Pigg et al., 2004 [UV-cured adhesive, UV-154; T.H.E. Company, Lakewood, Colorado, USA]; Rothwell and Ash, 2015 [Devcon 5 Minute Epoxy; ITW Devcon, Danvers, Massachusetts, USA]). The specimens are then photographed to document general morphology and three-dimensional features that could be lost when grinding specimens for transmitted microscopy. The specimens are then taken through a series of coarse- to fine-grit sandpaper or polishing papers (e.g., 3M Wetordry 6-pc assorted 1–30-µm aluminum oxide/silicon carbide polishing papers; 3M, St. Paul, Minnesota, USA) and ground down to a minimal thickness (<100 µm). Upon completing grinding and polishing, specimens can be made into permanent slides by adhering a coverslip with a long-term mounting medium (e.g., Eukitt; Sigma-Aldrich, St. Louis, Missouri, USA; see Ruzin [1999] for a list of common mounting media or Brown [1997] for a review of various media) and then photographed using transmitted light microscopy (Fig. 2A–H). The author has prepared a variety of plant specimens in this manner for approximately 10 yr and no deformation of tissues as a result of dehydration has been observed; these slides should last as long as traditionally prepared microscope slides.

The major advantages of this technique include: (1) a decrease in processing time (traditional ethylenediamine embedding can take 1–2 wk to embed material, whereas this protocol takes a maximum of 3 d); (2) it does not involve the use of caustic softening agents; (3) it does not remove any chemicals or structures from the specimens (e.g., silica bodies, tannins, lipids) that are commonly removed with polar or nonpolar solvents used in traditional wax-embedding; and (4) it circumvents the breaking or tearing of hard or brittle tissues by avoiding the use of a blade to slice through a tissue sample, which is a common problem in almost any other traditional histological technique. It has been successfully used on fruits and seeds of gymnosperms and angiosperms, including Ephedraceae (Ickert-Bond and Rydin, 2011), Fabaceae (Taylor et al., 2009), Hamamelidaceae (Benedict et al., 2008; Fig. 2G–H; Table 2), Juglandaceae (Taylor et al., 2009), Meliaceae (Pigg et al., 2014), Sapindaceae (Fig. 2D–F; Table 2), and Zingiberales (Benedict, 2012; Benedict et al., 2015; Fig. 2A–C; Table 2), and could potentially be used on any organ that is exceptionally brittle or hard.

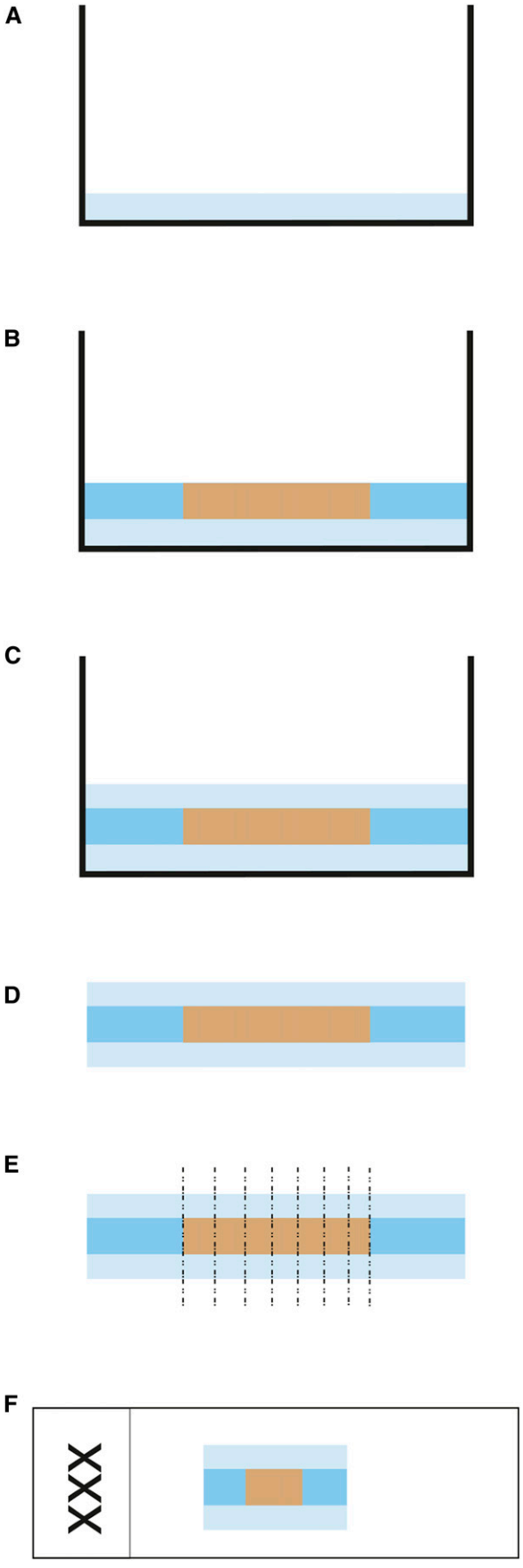

Fig. 1. Schematic of the embedding and mounting process. (A) Embedding box with initial specimen-supporting layer. (B) Embedding box with specimen and specimen-retaining layer added. (C) Embedding box with covering layer added. (D) Liquid Bio-Plastic and specimen removed from embedding box. (E) Liquid Bio-Plastic and specimen with hypothetical cutting planes. (F) A single section mounted onto a typical glass slide. Solid black lines represent embedding box. Light and dark blue shading represent Liquid Bio-Plastic embedding medium. Light brown shading represents the specimen. Dashed lines represent hypothetical cutting planes. Adapted and modified from Jones and Rowe (1999).

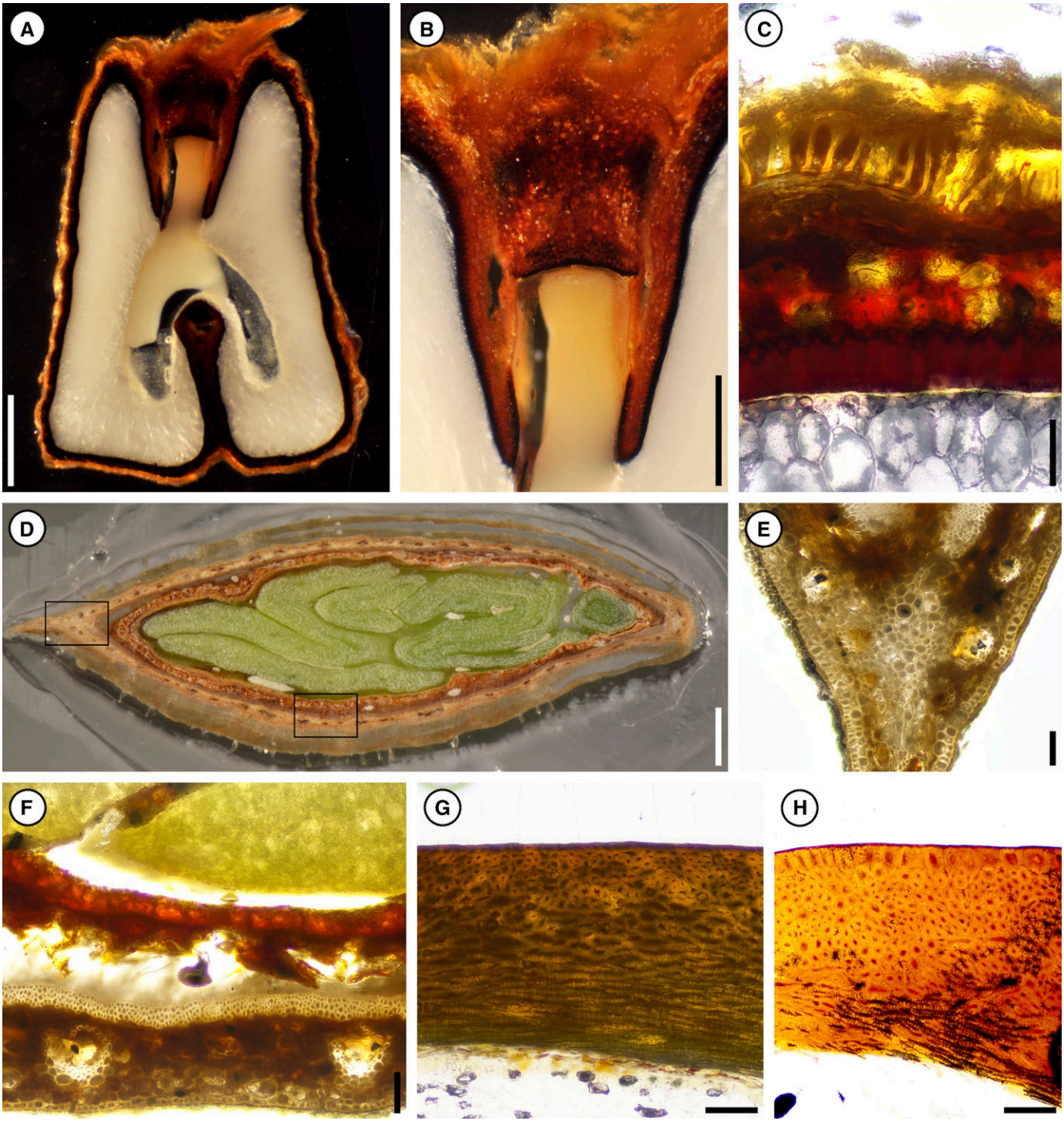

Fig. 2. Fruit and seed morphology and anatomy using the thin sectioning technique. (A–C) Longitudinal section of *Alpinia malaccensis* (Zingiberaceae). (A) Overview of longitudinal section of the seed before grinding. (B) Detail of the operculum and micropylar region of the seed before grinding. (C) Detail of the seed coat after grinding with a yellow, palisade exotesta, red to yellow mesotesta, and dark red sclerenchymatous endotesta. (D–F) Transverse section of a maple fruit (*Acer platanoides*; Sapindaceae). (D) Overview of a maple fruit in transverse section before grinding. (E) Detail of the fruit wall after grinding. (F) Detail of the fruit wall (at bottom), seed coat (red, middle), and embryo (top) after grinding. (G) Detail of the seed coat of *Distylium racemosum* (Hamamelidaceae) after grinding. (H) Detail of the seed coat of *Hamamelis virginiana* (Hamamelidaceae) after grinding. Scale bars: A, D = 1 mm; B = 500 µm; C = 50 µm; E–H = 100 µm.

TABLE 2. List of specimens sampled and voucher information.

Species	Voucher information[a]
Acer platanoides L.	*J. C. Benedict s.n.* (MICH)
Alpinia malaccensis (Burm. f.) Roscoe	*C. Saldanha 14771* (US)
Distylium racemosum Siebold & Zucc.	1973-20784 (K)
Hamamelis virginiana L.	*K. B. Pigg s.n.* (MICH)

[a]Herbarium abbreviations follow Index Herbariorium (Thiers, continuously updated).

CONCLUSIONS

Many methods exist for producing thin sections of various plant tissues for anatomical studies, but few methods exist for highly sclerified and heterogeneous tissues of fruits and seeds. The technique outlined here effectively prepares fruit and seed tissues from a variety of gymnosperm and angiosperm families for which traditional embedding/sectioning methods have failed. It has the advantage of dramatically decreasing the processing time of materials from weeks to months, to a maximum of three days. It eliminates the need for polar and nonpolar solvents, which leaves the chemical composition of materials processed intact. It also avoids tearing hard or brittle tissues by removing the need for a sharp blade to section material, which can damage the sectioning apparatus or the desired tissues.

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Appendix 1. Protocol for sectioning hard, heterogeneous fruits and seeds for anatomical studies.

Embedding specimens in Liquid Bio-Plastic

The simple principle behind embedding in a synthetic resin is creating a thin supporting layer, a specimen-retaining layer, and a covering layer. Detailed directions are provided with the Liquid Bio-Plastic kit (Ward's Science, Rochester, New York, USA), but a reference chart is provided for correct proportions of Liquid Bio-Plastic and its catalyst (Table 1).

Place material in uncatalyzed Liquid Bio-Plastic, cover, and leave overnight to remove air bubbles and allow resin to infiltrate the specimen (optional: place in vacuum). If the specimen has large air cavities, it is helpful to poke small holes in the specimen to increase infiltration of the resin; if the specimen floats, weigh it down so it can be fully submerged. Choose an appropriate mold for the specimen that will allow for at least 3 mm of resin to encase the entire specimen. Mix a small amount of Liquid Bio-Plastic (e.g., 10 mL) with the catalyst, pour a supporting layer into the mold, and set aside for at least 2 h to harden (ratios in Table 1). Remove the specimen from the uncatalyzed Liquid Bio-Plastic and place in the mold, on the hardened supporting layer. Mix enough Liquid Bio-Plastic with the catalyst to cover the specimen with 3 mm of material and add a label written in pencil to identify the specimen. For specimens that are buoyant in Liquid Bio-Plastic (specific gravity = 1.05–1.22 at 25°C), add enough to secure the placement of the specimen, but avoid wasting Liquid Bio-Plastic by pouring a thick layer and having the specimen float. If more Liquid Bio-Plastic is catalyzed than needed in this or any other step, unused molds can be prepared by making specimen-supporting layers of 2–3 mm; these should be stored away from dust. Wait 2 h for the specimen-retaining layer to harden. Mix another small batch of Liquid Bio-Plastic and catalyst and pour it over the specimen to achieve a covering layer of approximately 3 mm. Cover the mold and allow to rest overnight (Fig. 1A–F).

Place the mold in an oven at 60°C for 3–4 h for final curing (Ward's suggests that a 40–60-W incandescent light bulb oven made of an inverted light in a carton will achieve this temperature). Turn off the oven and allow the specimen to cool to room temperature, then remove the mold from the cast and label it on the opposite side of where cuts will be made for the thin sections (if a label was not added with the specimen).

Producing thin sections of the specimen

The following protocol has been used with IsoMet Low Speed Saws (Buehler, Lake Bluff, Illinois, USA) with thin diamond wafering blades (3.75–4″ in diameter; e.g., IsoMet 15LC No. 11-4254), or a Hi-Tech Diamond 6″ Trim Saw Machine (Hi-Tech Diamond, Westmont, Illinois, USA), but could easily be modified for use with any lapidary saw (~60–200 rpm) with a diamond blade.

Select the appropriate chuck or flange that will best hold the specimen to be cut with the lapidary saw. If needed, use coarse sandpaper or carborundum powder (180 grit) and water to grind unwanted edges off of the Liquid Bio-Plastic block prior to sectioning. If large portions of the block need to be trimmed, a coping saw may be used (wear personal protective gear). Place the specimen in the chuck and adjust the holding arm so the first cut will be at least 1–2 mm away from the specimen (if applicable, add weights to the holding arm to prevent the block from bouncing during the cutting). Make the first cut just before the specimen to ensure that the rotation speed, holding arm weight, and specimen orientation are adjusted correctly. In increments of approximately 0.5 mm (depending on sample size and apparatus configuration), cut thin sections of the specimen.

In some seeds, the embryo or nutrient tissue may loosen during the cutting process. To avoid specimen loss, apply a thin layer of nail polish onto the cutting surface and allow it to dry prior to each cut. This will hold the various tissues in place during the cutting process. The nail polish can then be removed using acetone or another nail polish remover after the thin section has been cut. Number each thin section, remove oil with soapy water or ethanol, and select optimal cuts to be mounted on microscope slides. A water-based saw may also be used (e.g., Hi-Tech Diamond 6″ Trim Saw), but specimens should not be left in water long because fibers and sclereids may become disfigured and warp the wafer. Both sides of a section should be analyzed and the optimal side noted prior to mounting. Also, it is wise to take overview photographs of the specimens at this stage to document the overall morphology of the specimen (e.g., Figs. 1A–B, 2D) and to determine how much grinding is needed to obtain roughly a single layer of cells for analysis.

Mounting thin sections to glass slides, grinding, and polishing

Thin sections should be clean, dry, and mounted on slides with the optimal side facing the glass slide. A variety of mountants can be used to adhere thin sections to glass slides, but superglue (e.g., Elmer's Products Inc., Westerville, Ohio, USA) has been used previously with success and dries quickly. Various research laboratories use differing mounting mediums to attach fossil wafers to glass slides (e.g., Manchester, 1994 [Elmer's epoxy; Elmer's Products Inc.]; Pigg et al., 2004 [a UV-cured adhesive, UV-154; T.H.E. Company, Lakewood, Colorado, USA]; Rothwell and Ash, 2015 [Devcon 5 Minute Epoxy; ITW Devcon, Danvers, Massachusetts, USA]). The author has not done an exhaustive search of adhesives, but two general notes would be to: (1) avoid any heat-cured medium that requires >60°C, due to the risk of damaging cells or cell contents, and (2) avoid a highly viscous two-part epoxy that may produce air bubbles when mixed (e.g., Loctite Epoxy Quick Set; Henkel, Düsseldorf, Germany). Once the thin sections are mounted and the mountant is dry, grind the thin sections using a series of coarse- to fine-grit sandpaper or polishing papers (e.g., 3M Wetordry 6-pc assorted 1–30-µm aluminum oxide/silicon carbide polishing papers; 3M, St. Paul, Minnesota, USA). To ensure an even grind, place the polishing papers on a smooth, clean, flat surface and hold the glass slide parallel to the paper with your thumb resting on the edge of the slide, and index and ring fingers equally spaced on the slide. Slide the glass slide slowly across the polishing paper, keeping it as parallel to the paper as possible; be careful not to apply pressure to the slide, as that will increase the chance for uneven grinding.

Depending on the thickness of the thin section, a range and variety of coarse- to fine-grit papers can be used. With thin sections <0.5 mm, it is useful to start with 600 grit (30 µm) and grind until saw-blade marks have been removed from the Liquid Bio-Plastic and/or specimen. Then move to 1200 grit (15 µm) and gently grind until cells can be observed in the specimen. Finally, move to 1800 grit (9 µm) or 14,000 grit (1 µm), depending on your polishing series, to buff out all remaining streaks and produce a shine on the Liquid Bio-Plastic and your specimen. In some instances, the polished specimen is slightly darker than the unpolished specimen and may need to be ground more thinly with 1200- or 1800-grit paper and repolished with the finer-grit papers (do not return to 600-grit paper, as it is often too coarse). It is helpful to do grinding/sanding near a compound microscope to check repeatedly that the specimen is not destroyed by overgrinding. All of the materials the author has produced have natural pigments in most tissues and staining or bleaching is not necessary. If a specimen is too dark, this is often a sign that the specimen is too thick and should be carefully ground more thinly. If structures are not naturally pigmented, stain could be applied at this step, but care should be taken that the mountant or embedding medium (e.g., Liquid Bio-Plastic) does not also uptake the stain. The author has stained slides containing superglue, Liquid Bio-Plastic, and plant material; cells stained well with toluidine blue O (TBO) or safranin red, and the superglue and Liquid Bio-Plastic did not uptake the stain. Once the slide has been prepared to satisfaction, a permanent slide can be created by adding a coverslip with the mounting medium (e.g., Eukitt; Sigma-Aldrich, St. Louis, Missouri, USA) in the traditional manner, and photographed using transmitted light (Fig. 2A–H).