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Processing Delicate Tissues for Scanning Electron Microscopic Imaging of an Embryo, Eggshell, and Fungal culture

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Reg: submission of the revised manuscript entitled **"Processing Embryo, Eggshell, And Fungal Culture for Scanning Electron Microscopy"** by Jessica Gibbons and Poongodi Geetha-Loganathan.

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TITLE:

Processing Embryo, Eggshell, and Fungal Culture for Scanning Electron Microscopy

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KEYWORDS:

SEM, painted turtle, eggshell, fungi, shell membrane, maxillary prominence, mandible, carapace, limb buds, hyphae, fungal spores

SUMMARY:

Here, we present detailed processing protocols for imaging delicate tissue samples using scanning electron microscopy (SEM). Three different processing methods, namely, hexamethyl disilazana (HMDS) chemical drying, simple air drying, and critical point drying are described for preparing rigid eggshells, embryos at early developmental stages, and fungal cultures respectively.

ABSTRACT:

Although scanning electron microscopy (SEM) is being widely used for the ultra-structural analysis of various biological and non-biological samples, methods involved in processing different biological samples involve unique practices. All conventional practices described in the literature for processing samples still find useful applications, but subtle changes in the sample preparation can alter image quality, as well as, introduce artifacts. Hence, using a unique sample preparation technique specific to the type of tissue analyzed is required to obtain a good quality image with ultrastructural resolution. The focus of this study is to provide the optimal sample preparation protocols for imaging embryos, rigid eggshells, and fungal cultures using SEM. The following optimizations were recommended to yield good results for the three different delicate biological samples studied. Use of milder fixatives like 4% paraformaldehyde or 3% glutaraldehyde followed by dehydration with ethanol series is mandatory. Fungal mycelium on agar blocks obtained by slide cultures yields a better ultrastructural integrity compared to cultures taken directly from agar plates. Chemical drying of embryos with HMDS provides drying without introducing surface tension artifacts compared to critical point drying. HMDS prevents cracking caused by shrinkage as samples are less brittle during drying. However, for fungal culture, critical point drying provides acceptable image quality compared to chemical drying. Eggshells can be imaged with no special preparation steps except for

thorough washing and air drying prior to mounting. Preparation methodologies were standardized based on acceptable image quality obtained with each trial.

INTRODUCTION:

Scanning electron microscope (SEM) ultrastructural analysis and intracellular imaging supplement light microscopy for three-dimensional profiling of prokaryotes, plants, and animals. The high spatial resolution of an SEM makes it one of the most versatile and powerful techniques available for the examination of microstructural characteristics of specimens at the nanometer to micrometer scale. Desiccated specimens are resolved to compositional and topographical structures with intense detail, which provides the foundation for developing valid conclusions about functional relationships[1-9]. When interpreting SEM images of biological specimens, it is a great challenge to distinguish between native structures and the artifacts that are created during processing. SEM is generally operated at very high vacuums to avoid any interference from gas molecules affecting the primary, secondary or backscattered electron beams emitted from the sample[10-11]. Also, biological materials are susceptible to radiation damage due to their poor or non-conducting properties. It is essential for the specimens loaded into the SEM to be completely dry and free of any organic contaminants to eliminate any possible outgassing in a high vacuum environment[10-11]. As biological specimens are mostly composed of water, additional preparative techniques are required to ensure that the native structures are retained.

The resolution obtained is based on optimizing preparation methods specific to specimen types and instrumental parameters utilized. Thus, it is necessary to avoid using generalized processing steps for all tissue types. Some biological specimens will require less stringent processing to preserve their structure while more time and care might be needed for delicate types of samples to avoid the introduction of drying artifacts, such as shrinkage and collapse. Sample preparation is a critical step in SEM imaging; the findings of morphometric studies are remarkably influenced by specimen preparation procedures[12-13]. Common preparation steps for many biological samples are fixation, dehydration and coating with a metal such as gold, platinum or palladium to convert their surfaces to be conductive for SEM analysis. The nature and combination of steps used will vary depending on the type of the tissue, and the specific goals of the study. Charge build-up, sensitivity to vacuum and electron beam damage pose problems when processing soft delicate biological samples, necessitating additional processing steps to retain the native structure of the object. Using conventional methods such as osmium tetroxide fixing, and dehydration cause shrinkage and the collapse of delicate tissues[14-17]. The aim of the study is to establish elegant methodologies derived by combining ideas from earlier studies with modifications to prepare and image soft delicate tissues (e.g., reptile embryos, eggshell of painted turtles, and fungal cultures).

Selection of a suitable fixing method is the first most important step for microscopic analysis of biological specimens. Fixing the tissues immediately after isolating from an organism is essential to prevent alteration in their morphology due to decomposition. An effective fixative should terminate cellular processes by permeating the cells quickly and maintaining the effect irreversibly to stabilize the structure of the sample to withstand both subsequent processing

steps and examination under the SEM[17-18]. Although several chemical and physical fixation methods are known, chemical fixation is more commonly used for biological specimens to avoid any cellular changes due to autolysis, putrefaction, and drying effects. There are numerous fixative chemical formulations discussed in literature[17,19-23], fixatives that work by denaturing and coagulating biological macromolecules, and those that fix by covalently cross-linking macromolecules. Alcohols are used as denaturing fixatives that preserve ultrastructure very poorly and are used mostly for light microscopy and not recommended for electron microscopic analysis. Cross-linking fixatives like formaldehyde, glutaraldehyde, and osmium tetroxide create intermolecular and intramolecular crosslinking between macromolecules within the tissues, providing excellent preservation of ultra-structures[11,24-26]. Biological samples are sensitive to temperature. The temperature at the beginning of fixation is recommended to be 4 °C to reduce the lateral mobility of membrane proteins, to slow the diffusion of intercellular molecules, and to slow the rate of fixation[11]. The time required for fixing tissues largely depends on the size of the sample and the speed at which the fixative diffuses and reacts with the components of the specimen. An overnight fixation in 4% paraformaldehyde or 3% glutaraldehyde in PBS at 4 °C is the preferred method for SEM analysis of specimens used in this study for their sequential penetrative properties, which allow smaller delicate samples to be processed[17-20,27]. A post-fixation step with osmium tetroxide is eliminated not only due to its toxic nature but also found to implement no added advantage to improve image quality for the samples analyzed in this study.

Biological samples contain fluids that interfere with the SEM operation; hence, the samples need to be dried before inserting it in the SEM sample chamber. Once dehydration is ensured, the solvent must be removed from the tissue without creating artifacts into the specimens due to the surface tension/drying. Three different drying methods were commonly used during processing tissues for SEM imaging: air drying, critical point drying, and freeze drying samples[28-31]. Few studies report all three drying methods producing identical results with animal tissue samples[28-31]. A general practice used for smaller specimens are chemical dehydration by ascending concentration series of alcohol and hexamethyldisilazane (HDMS), but larger specimens are dried using a critical point drying (CPD) instrument[32]. During the drying process, considerable forces formed in small cavities that are passed through the specimen by a liquid/gas interface; this can even lead to a complete collapse of the hollow structures[33]. Any deformation occurring due to the treatment could then be mistaken as a native structural feature of the specimen. Thus, the generalized phenomenon for processing should be eliminated and a unique drying process should be standardized for each type of tissue especially when delicate tissue specimens are analyzed.

In several trials conducted using various combination of all the above-mentioned processes, we standardized the methods that can be used for SEM analysis of three delicate tissues: reptile embryos, eggshells of painted turtles, and fungal cultures. Developmental biologists and morphologists describe normal and abnormal morphogenesis during embryo development in representative vertebrate animals. Investigations on gene signaling pathways depend on the morphological description of novel structures. To avoid any abrupt change in the vertebrate embryo structure during SEM analysis, we recommend chemical drying following dehydration.

Chemical drying using HMDS is the relatively newest drying method and the advantages include relative quickness, ease of use, lost cost, and the limited expertise and equipment needed[9]. CPD is a commonly used drying technique using passaging CO_2 across the specimens at a specific temperature and pressure. We identified that HDMS is suitable for drying soft delicate tissues and allows larger samples to be processed compared to critical point drying, which caused extensive deformation to embryonic tissues. Several methods have been used to prepare samples for SEM imaging to study the morphological characteristics of fungi[34]. Fungal specimens are commonly fixed in osmium tetroxide followed by ethanol dehydration and critical point drying, which may provide satisfactory results, although the toxic effects of osmium tetroxide[6-7,35] and losing fungal materials while changing solutions during processing are pronounced disadvantages. The sample preparation technique using air-drying without fixation has also been practiced[36] but results in shrunken and collapsed structures, and observation of such specimens can easily be misinterpreted while characterizing the species. Fungal hypha loses its integrity in contact with liquids and an even drying may not be achieved to restore the structure. Due to this effect, freeze-drying is commonly used for drying soft tissues like fungal mycelium. Freeze drying works well for clean materials but the presence of any salts or secretion will obscure surface detail that will be identified only at the SEM viewing stage. We coupled the slide culture method with glutaraldehyde fixing and critical point drying to yield structural details of intact fungal hyphae and spores. Although CPD drying caused shrinkage in embryos, it resulted in well preserved mycelial structures when coupled with glutaraldehyde fixation. The eggshell is of primary importance to the embryo of oviparous animals by not only acting as a protective covering but also providing mechanical stability, permeability to gas and water, and a calcium reserve for the developing embryo. Freshwater turtle eggshells are classified as “rigid” based on their structure, and due to their availability have received significant attention from biologists[1-7, 37-38].

We detail simple methods for easy examination of eggshell and shell membranes of painted turtle that can be applied to any rigid eggshell species. Preparation methodologies were evaluated based on resulting image quality and reduced potential artifacts.

PROTOCOL:

NOTE: Painted turtle (*Chrysemys picta*) eggs used in this study were collected during the nesting season of May through June 2015-16 from Rice Creek Field Station, Oswego New York with permission obtained from the New York State Department of Environmental Conservation (DEC).

1. Chemical drying method to process embryos for SEM

1.1. Collect turtle eggs from field sites during the nesting season. Prepare the incubation chambers in advance, made of plastic boxes with lids (L x W x H) 6.7 cm x 25.4 cm x 10.2 cm filled with bedding medium prepared with a moist mixture of vermiculite and peat moss (1:1 ratio). Make 4-6 holes of approximately 0.25 cm along the sides of the boxes and on the lid to allow aeration.

1.2. Gently remove the soil from the nest to uncover the eggs. Wipe the surface of turtle eggs with diluted iodine tincture (1:25,000) to control microbial contamination during incubation. Place the clutches separate from each other, clutch size of painted turtle may vary from 5-9 eggs and place a maximum of 8-9 eggs per box.

NOTE: Carefully handle the eggs during collection, wiping, labeling and placing inside the boxes. Position and alignment of eggs need to be the same as they were laid, any movement will inhibit embryo development.

1.3. Manually bury the eggs half in the bedding, cover and place the box inside the incubator set at 30 °C. Incubate the eggs for 10-17 days to obtain the embryonic stages 12, 13, and 18 respectively used in this study. Add distilled water to partially wet the bedding medium every other day to avoid dehydration and to maintain the moisture level for normal development of the embryos.

NOTE: Incubation and staging embryos are according to a complete developmental table published earlier[39].

1.4. Fix the embryos by making the first cut on one side of the dorsal eggshell and yolk membrane together, vertical to the long axis using pointed scissors. Insert the scissors into the yolk carefully to avoid cutting the embryonic disc. Now cut the lateral side of the egg along the long axis and then cut the other side of the egg along the short axis.

1.5. Peel open the excised piece with the embryo side up using forceps. Cut the other lateral side of the eggshell and place the excised piece into phosphate buffered saline (PBS) at a pH of 7.4).

NOTE: The turtle egg is filled with highly viscous egg yolk and the dorsal side of the embryo adheres to the eggshell membrane. The eggshell membrane near the embryo changes with the incubation from translucent white to an opaque, chalky white. This allows one to locate the embryo in the center of the long axis and be seen as a dark calcified spot from the exterior[40-41].

1.6. Use a stereomicroscope to isolate the embryos along with the yolk membrane by peeling them from the eggshell using forceps. Remove extra-embryonic membranes using forceps and micro-scissors. Transfer the embryo using an embryo spoon to fresh PBS in a Petri dish to wash any blood or yolk.

1.7. Use clear 12-well plates to fix the embryos overnight in 4% paraformaldehyde in PBS at 4 °C or in 2-3% glutaraldehyde in PBS. Place one to three embryos in each well depending on the size of the embryos. Ensure complete infiltration (samples will appear white) for older embryos by extending fixation time for 2-3 days. Rinse embryos 3x with fresh PBS for 5 min each rinse.

NOTE: Avoid damaging the surface of the embryo by using polystyrene inserts with polyester mesh bottoms for 12-well plates to transfer specimens from one solvent to another.

1.8. Dehydrate samples using a series of ethanol concentration in distilled water: 30%, 50%, 70%, 80%, 95%, and 100% and treat samples for 1 h in each dehydration solution. Repeat the step with 100% ethanol twice to ensure complete dehydration. If not used immediately, store samples in 70% ethanol at -20 °C for a longer period.

1.9. Dry embryos using a series of hexamethyldisilazana (HMDS) to 100% ethanol concentration: 1:2, 2:1 and 100%. Leave the samples in each solution for 20 min and keep the Petri dish partially covered during the process.

NOTE: Carry out all steps involving HMDS in fume hood with necessary personal protection gear as HMDS is highly toxic.

1.10. Leave the embryos in the final 100% HMDS solution covered completely or partially in a fume hood overnight aiding in evaporation of HMDS, leaving samples ready for mounting and sputter coating. Cover the dish to eliminate dust settling over the samples.

NOTE: The tissue will appear white after complete drying and partially dried samples will look yellow in color, leave these tissues in the fume hood for a longer time.

1.11. Choose the size of aluminum stubs and carbon adhesive tape per size of the sample analyzed. Mount the dried samples carefully on a standard aluminum pin stub (12.7 mm x 8 mm) using double stick carbon conductive tape (12 mm).

1.12. Introduce mounted samples into the chamber of the sputter coater to coat the specimen with a very thin film of gold to eliminate the charge effect. Gold plate the specimens for 60-120 s at a 35 mA sputter.

1.13. Mount the stubs to corresponding pore-plates by securely fastening the setscrews. Transfer the sample holder into or out of the sample chamber of SEM using the sample exchange tool. Image the samples at high vacuum mode with an accelerating beam voltage of 10 kV and emission current 10 µA.

NOTE: Always wear gloves while handling samples, sample holders, mounting stubs, and transfer tools to avoid grease contamination from hands to the SEM system.

1.14. Test alternative techniques listed below to compare the resolution of specimens obtained from above procedure.

1.14.1. Include a post-fixation with 1% osmium tetroxide for 1 h at room temperature after step 1.7.

1.14.2. Partially or completely remove the HMDS at step 1.10 for fast rapid evaporation of HMDS.

1.14.3. Process embryos after step 1.8 using critical point drying (CPD) by following steps 3.3-3.4.

2. Preparing the eggshell for SEM using an air-drying method

2.1. Collect and incubate the painted turtle (*Chrysemys picta*) eggs to fix the embryos as specified in steps 1.1-1.7.

2.2. Save eggshells in distilled water following embryo fixation in step 2.1. Clean the eggshells thoroughly by soaking in distilled water for at least 1 h to eliminate yolk and albumin contamination.

2.3. Air-dry eggshells after washing on delicate antistatic wipes in the fume hood overnight. Store dried eggshells in clean specimen bottles labeled by number and stage.

2.4. Mount, sputter coat and image the specimens by following steps specified in steps 1.11-1.13.

3. Critical point drying method for preparing fungal cultures for SEM

3.1. Establish slide cultures

3.1.1. Prepare potato dextrose agar (PDA) media for fungal cultures: Add 39 g of PDA power in 1 L of distilled water in an Erlenmeyer flask. Mix well by swirling the flask and autoclave the media at 121 °C for 30 min. Allow the media solution to cool and then add the antibiotic chloramphenicol (25 µg/mL) using a sterile micropipette.

NOTE: After sterilization, the agar solution should be hot and it will not solidify soon. Cool it enough so that it will not inactivate the antibiotics.

3.1.2. Mix it by swirling and pour plates (approximately 10-12 mL of media for each 10 cm Petri dish), carefully stack up and let solidify.

3.1.3. Use a sterile scalpel blade to cut out small blocks of agar about ½ to ¾ of an inch. Remove and place an agar block onto a clean glass microscope slide.

3.1.4. Place the slide in a clean Petri dish to prevent contamination and preserve moisture during incubation.

3.1.5. Raise the slide off the bottom of the Petri dish using a sterile toothpick to create surface tension between the plate and the slide to remove the glass slide without disrupting the delicate growth following incubation.

3.1.6. Use a sterile loop or a needle to transfer some of the fungi from the specimen inoculum to each of the four sides of the agar block on the slide.

3.1.7. Place a clean coverslip on the surface of the agar block following inoculation. Add a few drops of sterile distilled water to the Petri dish around the slide to ensure moisture for the growing fungi.

3.1.8. Seal the plate partially using paraffin film and incubate the plate at 30 ˚C for an appropriate length of time (for *Fusarium* species incubate for 36 to 48 h).

3.1.9. Remove the slide from the Petri dish and separate the tightly adhered coverslip from the agar block using sterile forceps. Fix the agar blocks in 3% glutaraldehyde in PBS overnight at 4 ˚C.

3.2. Dehydrate the samples by passing through an ethanol series: 10%, 25%, 50%, 75% and 90% with 15 min per change. Process the samples for final dehydration with two changes in 100% ethanol lasting 30 minutes each to ensure complete saturation.

3.3. Critical point drying: Place dehydrated samples in the chamber of the CPD apparatus. Seal and cool the chamber by opening the valves to allow liquid CO_2 in and vent ethanol out, until liquid CO_2 completely fills the chamber.

3.3.1. Seal and heat the chamber slowly to achieve a critical point when the chamber pressure exceeds 1000 psi and the temperature exceeds 31 °C, the liquid and gas phase of CO_2 is in equilibrium. Slowly drain the CO_2 from the chamber and the sample as gas to avoid effects of surface tension.

3.4. Perform mounting, gold plating and imaging the specimens by following steps specified in steps 1.11-1.13.

3.5. Perform comparative analysis by chemically drying the specimens from step 3.2 using HMDS by following steps 1.9-1.13.

REPRESENTATIVE RESULTS:

Figure 1 show scanning electron micrographic analysis of painted turtle (*Chrysemys picta)* embryos. Painted turtle eggs collected and incubated on a bedding medium, mounted on aluminum stubs following chemical drying were used for SEM imaging (**Figure 1A-E**). A lateral view of a stage 12 embryo shows the craniofacial structures; maxillary prominence extends beyond the mandibular and limits a well-marked nasal pit medially; five pharyngeal arches were also observed (**Figure 1F**). Newly formed somites at the posterior tail region of the embryo are

easily countable compared to the segmented body somites. Forelimb buds appear longer compared to hind limb buds and points more caudally than ventrally. A well-defined outgrowth of a carapace ridge is seen along the entire inter-limb flank region of the stage 15 embryo (**Figure 1G**)**.** At stage 18, painted turtles possess a short, feebly projecting snout. The lower beak rests within the upper jaw and is slightly upturned with a terminal hook that fits into the central notch of the upper beak (**Figure 1H**)**.** The upper jaw of the painted turtles shows a notched appearance forming a medial U-V shaped distal tip with a small cusp on each side, a tiny egg tooth begins to form at the tip of the upper jaw. Limb buds that appeared longer during earlier stages form a paddle-like structure during stages 13-14 with digital plate vaguely indicated. Limb mesenchyme demarcated by the apical ectodermal ridge is seen along the anterior-posterior margins (**Figure 1I-J**)**.**

Figure 2 shows ultra-structural analysis of *Chrysemys picta* eggshell and shell membrane using SEM imaging. Painted turtle eggshells were obtained as described above and were subjected to air-drying following washing with double distilled water (**Figure 2A,B**). Eggshells mounted on aluminum stubs with double-sided carbon tape (**Figure 2C**) were imaged using SEM. A lateral view of the shell showed outer calcareous eggshell layer firmly attaching to the inner filamentous shell membrane (**Figure 2D**). The outer surface of the eggshell consists of well-distinguished mineralized shell units, made of globular/spherical nodules arranged in groups and in between adjacent shell units a concentration of small rounded depressions or pores of various sizes were observed (**Figure 2E**). Nodules from each group of shell units meet at a connection junction, the center point (**Figure 2F**)**.** The outer surface was manually peeled using forceps to observe the surface of the shell membrane. Rows of central plaques were seen which provides the attachment point between the shell units and underlying multilayered fibrous membrane (**Figure 2G**).

Figure 3 shows the morphological characterization of ascomycete fungal isolate from slide cultures observed using SEM imaging. Scheme of slide culture setup established (**Figure 3A**) showing fungal growth on the agar block within two days of inoculation. Colonies growing rapidly with white to cream-colored aerial mycelium (**Figure 3B**). SEM imaging following critical point drying and sputter coating of agar slices revealed fungal hyphae, curved conidia, and rice like spores. Conidiophores were seen arising laterally from the septate aerial hyphae (**Figure 3C**). Macroconidia produced on shorter, branched conidiophores are moderately curved, with short, blunt apical and indistinctly pedicellate basal cells mostly septate (**Figure 3D**).

Figure 4 shows the comparative results for processing similar specimens using alternative techniques. Immersing heads of stage 15 embryos in HMDS and drying by gradual evaporation show well preserved craniofacial structures, almost no shrinkage or distortion is seen with extended times of slow evaporation from HMDS (**Figure 4A**). Embryos post-fixed with osmium tetroxide showed no distinguishable difference in image quality except for slight shrinkage of tissue compared to HMDS slow processing (**Figure 4B**). Drying embryos by removing HMDS partially or completely allowing fast evaporation results in structural distortion and shrinkage (**Figure 4C**), whereas slow evaporation resolved excellent surface structures of a similarly staged embryo (**Figure 4A**). The drying artifacts were seen in the embryos treated with the CPD

technique causing extensive shrinkage and destruction of tissue (**Figure 4D,E**). Agar slices from slide cultures show incomplete drying of fungal isolate with HMDS treatment compared to CPD (**Figure F**). Complete drying is achieved with CPD treatment of agar slices with a well dried, white specimen showing intact high-resolution structural features of fungal hyphae and spores (**Figure 4G-G'**). Agar blocks with fungal cultures appear shrunken and yellow in color making them not suitable for SEM imaging (**Figure 4H-H'**).

FIGURE LEGENDS:

Figure 1: Scanning electron micrographs of *Chrysemys picta* embryos prepared by chemical drying. (**A**) ***Chrysemys picta***, nesting during the breeding season at Rice Creek Field Station, Oswego, NY. (**B**) Well-constructed nest from outside. (**C**) Surface soil removed with care to exposure the eggs laid. (**D**) Eggs placed into the incubation chamber with bedding medium. (**E**) Mounting embryos onto aluminum stubs following chemical drying, E4 showing the sputter coated embryo. (**F**) Lateral view of stage 12 embryo showing facial structures, limb buds and somites. (**G**) Well defined carapace ridge and paddle-shaped limb buds are seen at stage 15. (**H**) Craniofacial structures of stage 18 embryo showing upper and lower jaw, note a small egg tooth formed at the tip of the upper beak. (**I**) Dorsal view of right forelimb at stage 13-14 and a close-up view of AER (**J**) at the dorsal-ventral boundary. Scale bars: F-H, 500 µm; I, 100 µm, and J, 10 µm. *Keys: mb, midbrain; np, nasal pit; mxp, maxillary prominence; pa, pharyngeal arches; h, heart; fl, forelimb; s, somite; hl, hind limb; t, tail tip; cr, carapacial ridge; et, egg tooth; oc, oral cavity; e, eye; uj, upper jaw; lj, lower jaw; m, mesenchyme, AER, apical ectodermal ridge.*

Figure 2: Ultrastructural analyses of the air-dried eggshell and the shell membrane of painted turtle eggs. (**A**) After fixing the embryos, eggshells were washed thoroughly in distilled water to remove yolk and albumin. (**B**) Clean eggshells were air dried at least overnight. (**C**) Mounting of an eggshell onto aluminum stubs. (**D**) Radial view of shell fragment showing outer calcareous layer and inner shell membrane. (**E**) Outer calcareous layer shows globular shell units (*su*) arranged in groups and pores seen in between these units (*asterisks*). (**F**) Magnified view of a nodule showing the center point (*c*) between the shell units. (**G**) Removal of calcareous layer shows the outer surface of the shell membrane with depressions left broken from shell units. Scale bars: E, 100 µm; F, 10 µm, and G, 20 µm.

Figure 3: Preparing fungal culture for SEM imaging. (**A**) Schematic diagram to show the setup of slide culture. (**B**) White colored fungal colonies seen on the agar block after two days of incubation at 30°C. (**C**) SEM image showing a section of mycelium of *Fusarium solani*, *black asterisks* marking the macroconidia in sporodochia, an *arrow* pointing to phialide, and *arrowheads* directed towards microconidia, scale bar, 10 µm. (**D**) Magnified image of septate chlamydospores, scale bar 5 µm. *Keys: ab, agar block; cs, coverslip; f, fungal culture.*

Figure 4: Comparative analysis of alternative techniques to process similar specimens. (**A**) Frontal view of painted turtle head dried with slow evaporation of HMDS (**A**), after post-fixing with osmium tetroxide (**B**), rapid faster evaporation of HMDS (**C**), and CPD (**D-E**). Including a post-fixation step using osmium tetroxide do not show any visible difference in image quality

compared to HMDS treatment without post-fixation. Deformations are seen after fast evaporation of HMDS, while gradual drying with slow evaporation provides a better surface structure. Different degrees of shrinkage and collapsed structures are seen in the regions of brain, eyes and facial prominences in embryos treated with CPD. (**F**) Mounting of slide cultures processed with HMDS (1), CPD (2) and sputter coated CPD treated specimen (3) on aluminum stubs. (**G-G'**) Complete drying seen as intact white colored agar slice with fungal inoculate achieved by CPD preserving structures of fungal mycelium and spores of *Fusarium solani*. (**H-H'**) Inadequate drying of slide culture, shrinkage and damaged structural integrity seen with HMDS preservation technique. Scale bars: A-E, 500 µm; G-H, 2 mm, and G'-H', 20 µm. *Keys: et, egg tooth; oc, oral cavity; e, eye; uj, upper jaw; lj, lower jaw; black asterisks,* macroconidia in sporodochia; *arrow,* phialide; and *arrowheads,* microconidia.

DISCUSSION:

In our study, different fixation agents, dehydration and drying methods were tested to prepare three different delicate biological samples for SEM imaging: embryos, eggshells, and fungal cultures. SEM is commonly used for surface analysis, so fixative penetration is less concerning, but it must be understood that poorly fixed internal structures will cause inward shrinking or/and collapsed surface structures. Extended fixation time should also be considered for larger tissue samples, replacing the fixative solution a few times depending on the fixation duration. Due to active interaction between the fixative and the tissue, the osmotic properties of cells would change considerably. Tissue fluids would dilute the fixative and thus an aldehyde effect contributing to the overall osmolality can be neglected as far as effects on cell volume are concerned. Also, formaldehyde would penetrate faster into tissues and is more effective as the cross-linker compared to glutaraldehyde. A buffered formaldehyde-glutaraldehyde mixture was also reported for fixing a wide variety of tissues, although for monolayers and cell suspensions diluted solutions were more suitable for fixing[15-16]. However, the diluted solutions are also hypertonic, thus the aldehydes are not themselves osmotically active. For delicate samples used in this study, PBS at pH 7.4 was used as a vehicle for the fixing agents, as phosphate buffers are thought to be more similar to the cytoplasmic environments of most biological samples. Further, the osmolarity is identified to be within the physiological range required for the sample while PBS is acting as a vehicle for the fixing agent. Post-fixation with osmium tetroxide is often recommended for various biological samples[16,22,42] but has been eliminated for all the biological samples used in this study. Samples without osmium tetroxide yielded crisp images and specimens fixed with osmium tetroxide yielded indistinguishable results (**Figure 4A**). Osmium tetroxide causes distortion of leaf tissue structures and yields poorer specimen preservation compared to glutaraldehyde and formaldehyde mixture[43], and it has been suggested that a build-up of osmium molecules could inhibit infiltration of dehydrating agents and transitional agents used in CPD, and the solvents like HMDS used for chemical drying. The effect is tissue-specific and excluding osmium tetroxide will not have any effect on the image quality for the type of delicate specimens analyzed in this study. For other types of tissues use of osmium tetroxide may be eliminated with extended primary fixation.

HMDS dried turtle embryo specimens showed well-preserved surfaces, and less distortion or shrinkage compared to CPD (**Figure 4**). CPD minimizes the chance for distortion in cell

morphology and artifacts production due to the zero or minimal surface tension created during the process. However, CPD can cause a potential physical hazard for delicate fragile samples. In line with our observation, HMDS yielded similar or higher quality imaging by minimizing the surface tension caused due to drying based on previous studies published[44-47]. Compared to CPD treatment, HMDS preserved structural details of the organic meshwork excellently in etched bivalves and barnacle shells[48] and provided high resolution of the structural features of the complex internal organization of mermithid nematodes[49] and insect internal tissues[28]. The drying plate artifacts were seen in high quantities on the specimens prepared by the CPD technique as compared to the HMDS technique. The membrane blebs and pellet artifacts were increased in cervical cells prepared by using CPD technique[50]. HMDS reacts with water to produce hexamethyldisiloxane and ammonia, both of which evaporate from the object. HMDS is commonly used in gas chromatography to create silyl ethers of compounds such as sugars, amino acids, alcohols, and numerous other compounds. It is not known if HMDS reacts with some of these compounds in tissues. HMDS might crosslink proteins and stiffen the tissue during the drying process[28], although the exact reason that HMDS provides better preservation of embryos could not be explained except for tissue specificity. Based on the results of our investigation, CPD caused extensive shrinkage compared to HMDS, and is much more suitable for processing embryonic tissues, especially to analyze surface structure for early staged embryos. The mechanism by which HMDS works on tissue drying has not been elucidated, although slow drying with gradual evaporation in absolute anhydrous surroundings could be a reason for obtaining an excellent surface structure of the animals.

Small pieces of agar blocks with fungal colonies from previously established slide cultures were used in this study to avoid issues with maintaining the original fluffiness of the mycelial mat. CPD drying of agar pieces with fungal colonies resulted in a washing effect, while changes in the fixatives, buffers, and ethanol was found to be a better choice overcoming the difficulties with freeze drying. HMDS that works well for embryos of all stages was unable to resolve fungal cultures, while air drying with HMDS posed a significant problem: curling up and losing the structural rigidity. While CPD works well for fungal cultures, it induced instant damage to embryos especially at early stages in development, causing shrinkage on the surface. No charging or drying artifacts were observed when HMDS and CPD were used for embryos and fungal cultures, respectively. For rigid tissue like eggshell of turtles, no special processing is required except for washing and air drying at room temperature prior to mounting for imaging.

Regardless of the preparation method, gradual ethanol gradient steps should be used to reduce the potential for drying artifacts. Initially, both HMDS and CPD samples were found to have surface artifacts with shrinkage following the dehydration and drying protocols available in the literature. After a series of standardizations, we determined that the artifacts were the result of the alcohol dehydration. It is critical to use a slow dehydration process using more gradual ethanol increases during dehydration. Also, the duration of each iteration should be longer for embryos compared to fungal cultures. Further, an additional step at 100% ethanol ensured complete uniform saturation. HMDS can be removed completely or partially to allow air drying of samples, but extended exposure to HMDS and gradual air drying by evaporation were found to be effective for embryos to avoid disruption (**Figure 4A,C**) caused by large surface tension as

observed for microbial cell attachment[51]. Immersing specimens in HMDS and slow drying overnight revealed an excellent surface structure in *Daphnia species*[52] suggesting that soft delicate tissues could benefit from the slow drying process. Acceptable image quality for embryos can be obtained with slow chemical drying by gradually increasing the concentration of HMDS to ethanol following dehydration.

All samples were mounted onto aluminum stubs using a double-sided carbon tape to provide a conductive surface, a thin coat of colorless nail polish could also be used if the same sample needs to be imaged in a different orientation. Samples can be easily removed from the stubs when nail polish is used on the stub surface and positioning the samples in alternative planes is possible compared to using double-sided carbon tape. As the samples are nonconductive, it is necessary to sputter a thin layer of metal on the samples to increase conductance. A gold target was used in this study during sputter coating, and gold-palladium also yields similar results avoiding charging effects. The correct beam voltage is sample dependent, for lightly coated specimens, imaging with a field emission SEM at 2-5 kV will provide a good definition of surface structures. For tissues with adequate coating, 5 kV generally provides better signal without more beam penetration. For specimens that lack depth and are coated with gold alone, imaging at a higher voltage (10 kV) allowed better visualization of surface topography.

Preparation of delicate tissues for SEM poses distinctive challenges, and a range of sample preparation techniques can be used to overcome and minimize imaging artifacts. We provide comprehensive methods to process three different delicate biological tissue samples, for SEM imaging: embryos, rigid eggshells, and fungal cultures. In our investigation, subtle alterations to popular methods available from previously published studies were made, and we identified the most appropriate dehydration and drying methods specific to each delicate tissue type. These protocols could be adapted to obtain acceptable SEM image quality from similar biological samples.

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DISCLOSURES:

The authors have nothing to disclose

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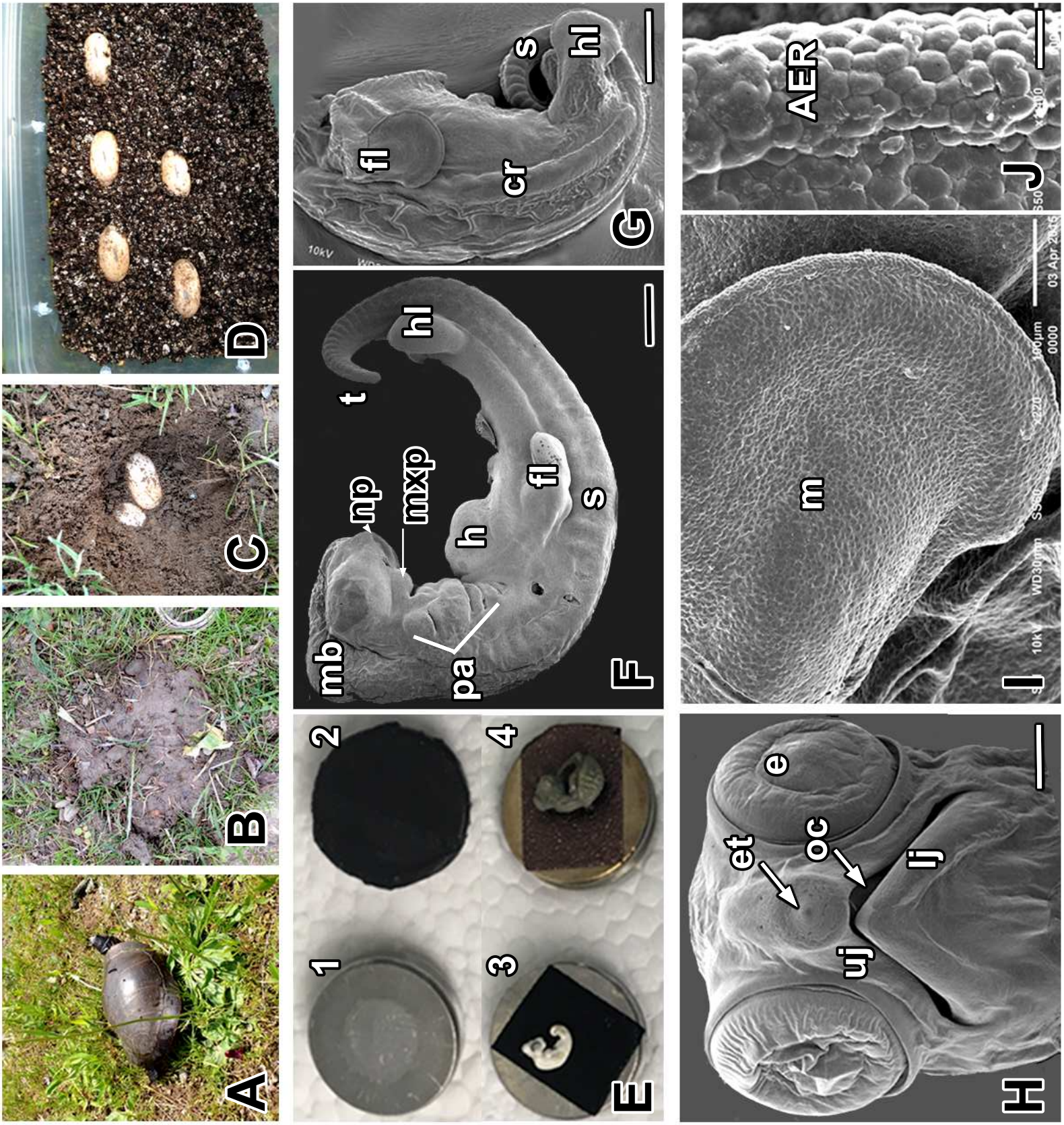

Figure 2

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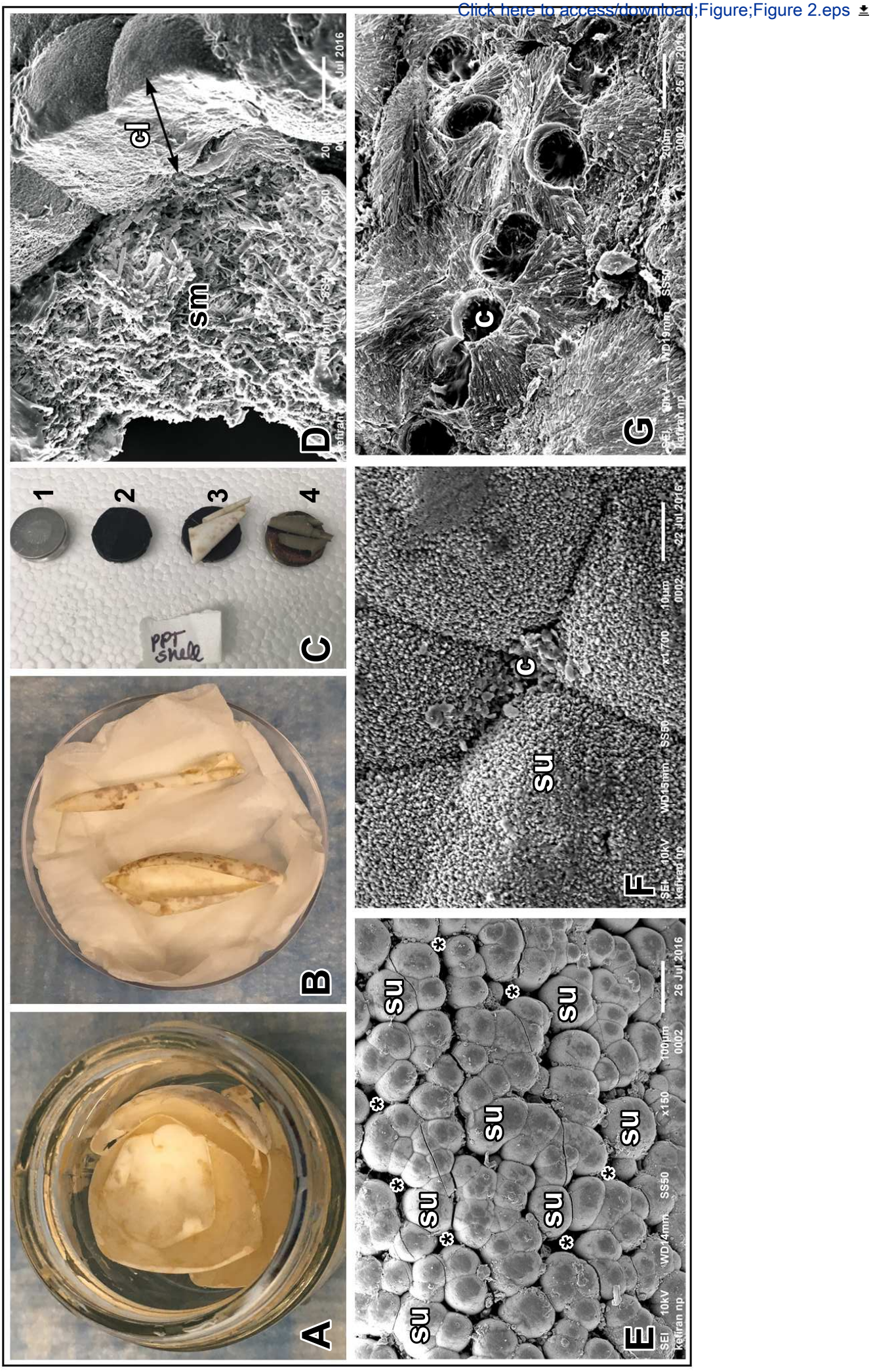

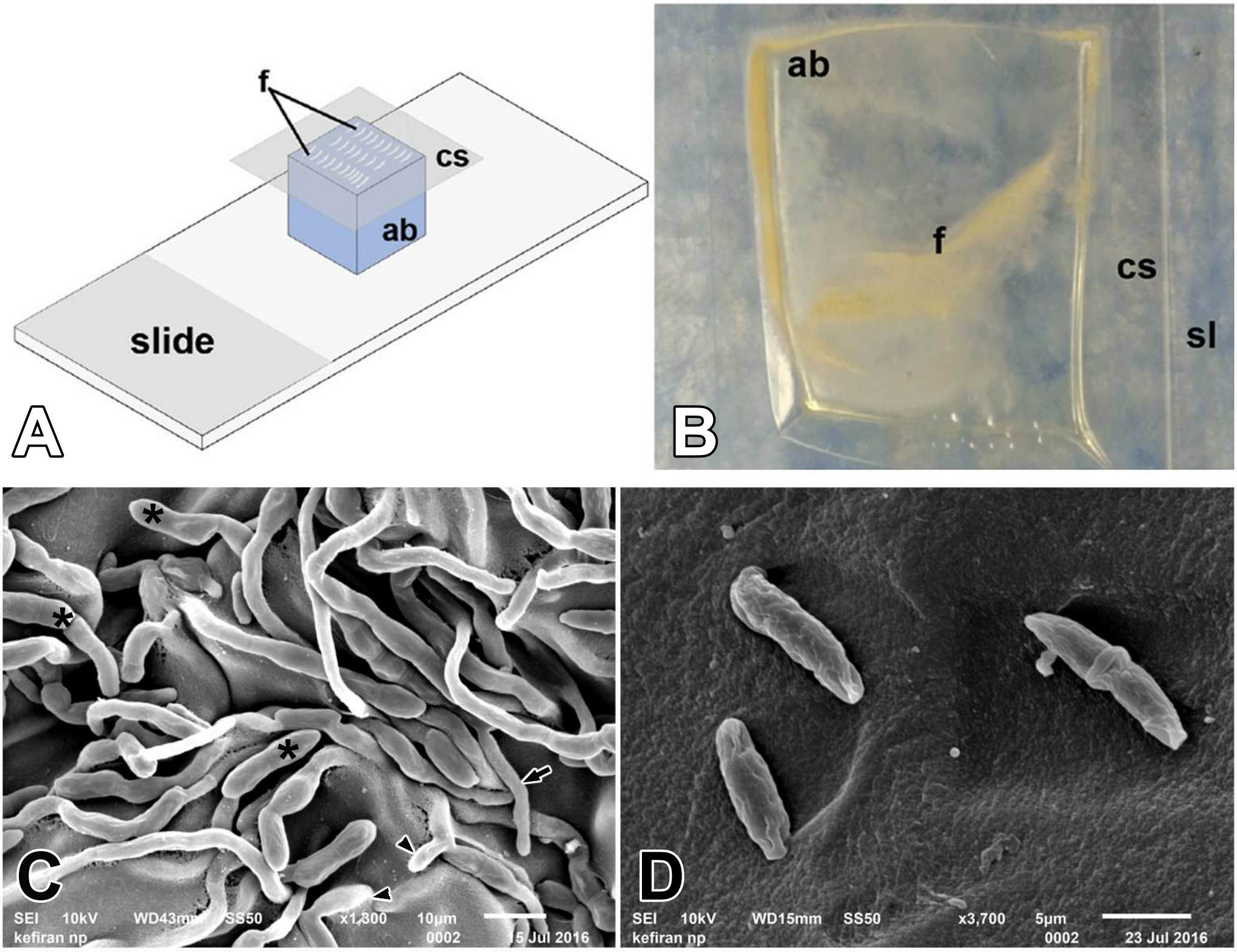

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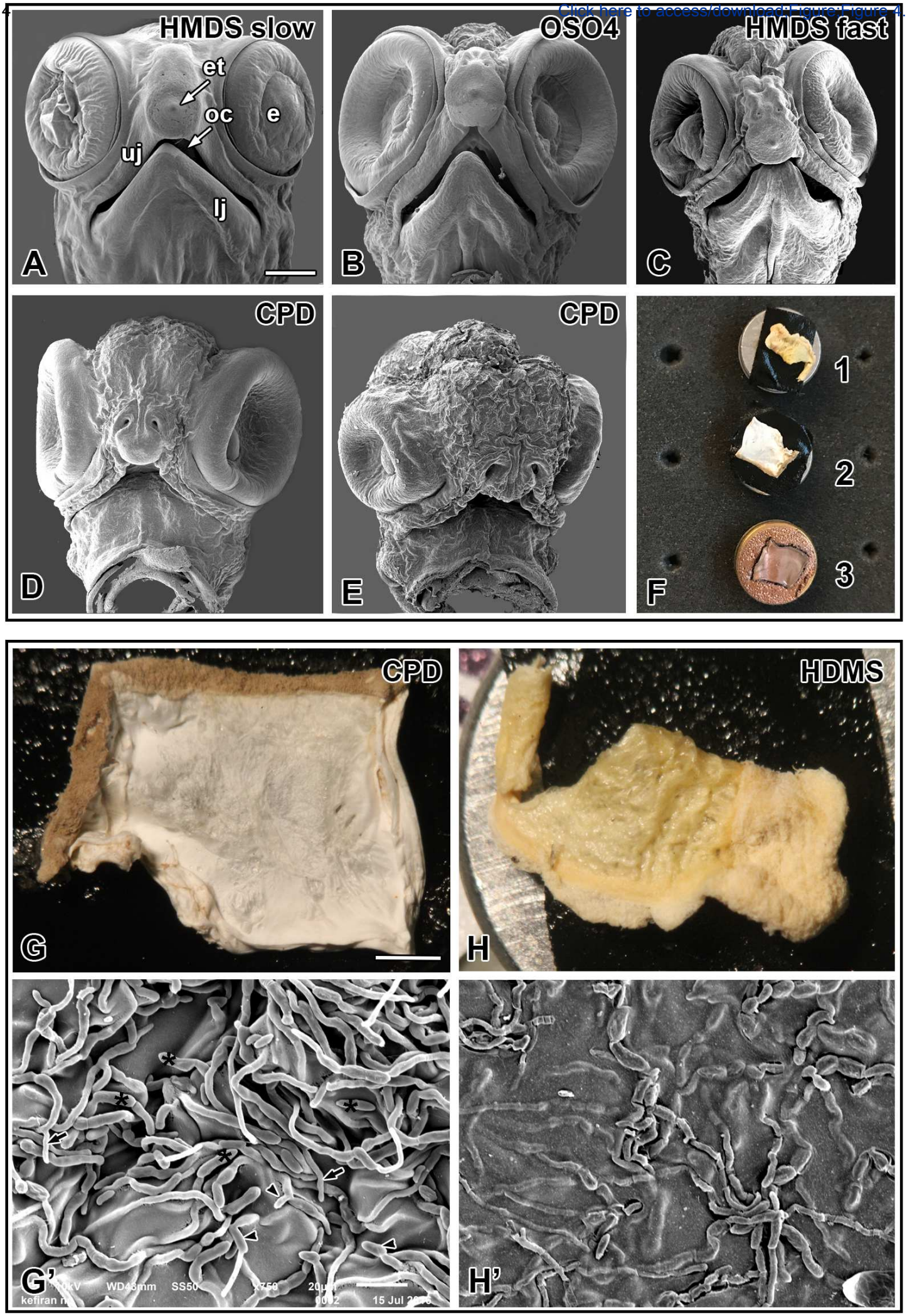
Figure 4



MATERIALS and EQUIPMENTS

Processing Delicate Tissues for Scanning Electron Microscopic Imaging an Embryo, Eggshell, and Fungal culture

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Agar	Fischer Scientific	S25127A	for slide cultures
Aluminum pin stub	Tedpella	16111	12.7 mm x 8 mm
BD Difco Dehydrated Culture Media: Potato Dextrose Agar	BD 213400	DF0013-17-6	Media for isolation and cultivation of Fungi, yeast and molds
Chloramphenicol	Fischer BioReagents	BP904-100	Antibiotic for media
Coarse Vermiculite	Greenhouse Megastore	SO-VER-12	bedding medium
Clear 12- well plate	Corning	07-201-589	for fixing embryo
Coverslips	Fischer Scientific	S17525B	for slide culture
Critical Point Dryer	Quorum CPD	EMS850	critical point drying
Culture dishes	Fischer Scientific	08 747B	DISH PETRI 100X10MM 12/PK
Ethanol	Fischer Scientific	A406P 4	dehydration agent
Forceps- Aquarius Tweezers	Tedpella	5804	style 4, length 108mm, widh x thickness 0.017 x 0.17 mm
Glutaraldehyde	Fischer Scientific	G151-1	fixative
Gold target for sputter coater	DENTON VACUUM	TAR001-0158	Gold Target, 2.375” D X .002”
Hexamethyldisilazana	Fischer Scientific	C19479-5000	chemical drying agent
Kim wipes	Kimtech	S-8115	cleaning
Microscope slides	Thermo Scientific	67-762-16	for slide culture
Microscopy Scissors	Tedpella	1327	Double pointed, stainless steel, 100 mm L (3-5/8").
Micro-scissors	Tedpella	1346	Vannas-type, straight, 80mm L

Moria Perforated Embryo Spoon	Fine Science Tools	10370-17	Length 14.5 cm, tip diameter 20 mm, spoon depth 5 mm
Netwell Inserts	Corning	0330B09	15 mm Inserts with 74 µm Mesh Size Polyester Membrane act as handy carriers during specimen processing into different solvents
Paraformaldehyde	Fischer Scientific	T353 500	fixative
Peat moss	Walmart- Miracle Gro	551705263	bedding medium
PELCO tabs double stick carbon conductive tape	Tedpella	5000	12 mm OD
Sputter coater	DENTON VACUUM	DESK V	thin metal coating
SEM	JEOL USA	JEOL JSM 6610LV scanning electron scope	electron microscopy

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Rebuttal

Editorial comments:
Please see below for changes made to the revised manuscript

Comment 1. The manuscript needs thorough proofreading. Please proofread well for any grammar or spelling issues.
Substantial revisions have been done to address the issue

Comment 2. Please make the title crisp. Maybe
Processing delicate tissues for scanning election microscopy?
Or processing embryo, eggshell and fungal culture samples for scanning electron microscopy?
The title has been changed using the later suggestion made by the editor.

Comment 3. We can only have 6-12 keywords.
Modified to include 11 keywords.

Comment 4. Please bring out clarity in the Introduction with respect to all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
So basically, why is SEM study required, what are the different ways of doing so, what are the limitations of the prevailing methods, why specific method suggested for specific type of material, are these methods previously known and citations if any, have these been used only for the samples mentioned by you or something else as well, what are the changes in your protocol.
Please include citations where ever applicable.
Comment 5, 6 & 7. Citation?
Comment 8. Any citation why this is recommended?
The introduction is extensively revised to address all the suggestions in comments 4-8. Corresponding citations were added to the reference listing.

Comment 9. Protocols should only be made up of actions steps in imperative tense, describing all specific details related to the steps.
Notes can be used for providing extraneous details, optional steps, or recommendations that are not critical to a step
We cannot have paragraph of text in the protocol section. Each step should not have more than 2-3 action steps.
Please make the steps crisper and remove redundancy throughout. Please write exactly how you would perform your experiment detailing all the steps of your protocol as discrete action steps.

All the suggestions were taken care, please see below for details

Comment 10. So, the bedding medium is different than vermiculite and peat moss?
Bedding medium consists of vermiculite and peat moss mix (1:1). To clarify, the sentence in step 1.1 is modified as “bedding medium prepared with a moist mixture of vermiculite and peat moss (1:1)”.

Comment 11. Is this vermiculite and peat moss mix? Please bring out clarity.
The dirt is not the bedding media but the nesting soil. This is clarified in step 1.2 as “Gently remove the soil from the nest to uncover the eggs”.

Comment 12. Converted to a note, please check.
Change is acceptable

Comment 13. Do you burry it half or fully? Incubate at what temp? Please write exactly how you perform your experiment.
Reworded the entire step to make it crisp. Please check.
Eggs need to be buried half into the bedding medium. Step 1.3 is revised to include this information along with a sequential description of the incubation process.

Comment 14. Need more details on how to do this part. Please include all the actions.
A detailed description of isolating a turtle embryo is added in steps 1.4 to 1.6. A note section included providing information on how to identify the position of the embryo from the exterior.

Comment 15. In a dish? Can you visually differentiate 12, 13, 18 stage embryo? Do you place all of them together or differenty? How many embryos are collected?
Yes, the staging of embryos for any species can be done even when placed together in the same dish by observing the developmental landmarks within the embryos. The staging for painted turtle is done using the staging series provide in Cordero et al., 2014. This information was added in the note section following step 1.3. Each egg will be opened individually to fix the embryos, so once the embryo is transferred in the Petri dish with PBS, the stage will be identified by comparing the morphological attributes to the staging series.

Comment 16. In which container and how?
Embryos are fixed in clear 12-well plates, this is also added to the table of materials

Comment 17. How will you ensure this visually?
Completely fixed specimens will appear white, this has been added within parenthesis to the step 1.7.

Comment 18. Do you take the embryo out or drain the liquid from the container and add PBS to it? All these are important details filming.

Transferring specimens to a series of solvents during processing was done by using Netwell polystyrene inserts with polyester mesh bottoms designed for 12-well plates. This is added as a note next to step 1.7 and the item is listed in the table of materials.

Comment 19. Again this is not a step and hence its been converted to a note instead.
Agreed on the change

Comment 20. Please convert this to a note instead.
Revised as suggested

Comment 21. Like what?
All the alternative techniques were numbered in step 1.14, this step was included to R1 to address the review's comment.

Comment 22. Does this need to be in quotes?
Term 'flexible' was removed in the current version of the manuscript, so no quotes needed.

Comment 23. Please make different step or substeps.
The line specified is made as a different step.

Comment 24. How?
Fungal samples were dried in a critical point dryer (the method is called as critical point drying), this is what been mentioned in the title of step 3

Comment 25. We cannot have paragraph of text. Please consider making substeps. All steps should be action steps describing how to do the procedure. Also please provide citations for each of the method used.
All the paragraphs within the protocol section are divided into sub-steps, each step now does not contain more than 3 action steps.

Comment 26. Prepare how? How much media per plate? Do you autoclave?
Step 3.1.1 and 3.1.2 along with a note section in between have been included to detail the preparation of PDA plates. The materials were also added to the table of materials.

Comment 27. Please expand during first time use and keep using the abbreviation throughout.
Expansion for CPD added to introduction 'line 121' where the abbreviation was used for the first time.

Comment 28. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

All the images presented in the manuscript are findings from our recent research; no images were used from any work published earlier.

Comment 29. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Some of the details can move to the introduction section to introduce the techniques used.

Several sections were removed from discussion and added to the introduction section. All the description seen in the current version explains the reasoning behind the claims made by the authors and to address the reviewer's comments.