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Preparation of Prokaryotic and Eukaryotic Organisms Using Chemical Drying for Morphological Analysis in Scanning Electron Microscopy (SEM) --Manuscript Draft--

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July 10, 2018

JoVE
1 Alewife Center, Suite 200,
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Attn: Ronald Myers, PhD., Senior Science Editor

Dear Dr. Myers:

I am pleased to submit an original research article entitled "Preparation of Prokaryotic and Eukaryotic Organisms Using Chemical Drying for Morphological Analysis in Scanning Electron Microscopy (SEM)" for consideration for publication in *JoVE*. We previously described methodologies we use in our lab in a recent publication (Trotter MB, Stephens, TD, McGrath JP, Steinhilb ML. The *Drosophila* model system to study tau action. *Methods Cell Biol.* (2017) 141: 259-286) and I was invited to submit an article that uses some of these techniques for *JoVE*.

In this manuscript, we describe how to fix, wash, dehydrate, dry, mount, sputter coat, and image three organisms: cyanobacteria (*Toxifilum mysidocida*, *Golenkina* sp., and an unknown sp.), two euglenoids from the genus *Monomorphina* (*M. aenigmatica* and *M. pseudopyrum*), and the fruit fly (*Drosophila melanogaster*). The purpose of this protocol is to describe a fast, cheap, and simple method to obtain detailed information about the structure, size, and surface characteristics of specimen that can be broadly applied to a large range of organisms for morphological assessment.

We believe that this manuscript is appropriate for publication by *JoVE* because it describe methods for examining specific morphological details of three representative types of organisms that would be broadly applicable to examining features of many organismal and tissue types.

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose. If you feel that the manuscript is appropriate for your journal, we suggest the following reviewers:

Dr. Brian Leander, University of British Columbia (bleander@mail.ubc.ca) – SEM expertise
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Thank you for your consideration!

Sincerely,

A handwritten signature in blue ink, appearing to read "Michelle Steinhilb", is written over a light blue circular stamp.

Michelle L. Steinhilb, Ph.D.
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TITLE:

Preparation of Prokaryotic and Eukaryotic Organisms Using Chemical Drying for Morphological Analysis in Scanning Electron Microscopy (SEM)

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KEYWORDS:

Scanning electron microscopy (SEM), cyanobacteria, euglenoid, fruit fly, *Drosophila*, critical point drying (CPD), hexamethyldisilazane (HMDS), t-butyl alcohol (TBA)

SUMMARY:

SEM analysis is an effective method to aid in species identification or phenotypic discrimination. This protocol describes the methods for examining specific morphological details of three representative types of organisms and would be broadly applicable to examining features of many organismal and tissue types.

ABSTRACT:

Scanning electron microscopy (SEM) is a widely available technique that has been applied to study biological specimens ranging from individual proteins to cells, tissues, organelles, and even whole organisms. This protocol focuses on two chemical drying methods, hexamethyldisilazane (HMDS) and t-butyl alcohol (TBA), and their application to imaging of both prokaryotic and eukaryotic organisms using SEM. In this article, we describe how to fix, wash, dehydrate, dry, mount, sputter coat, and image three types of organisms: cyanobacteria (*Toxifilum mysidocida*, *Golenkina* sp., and an unknown sp.), two euglenoids from the genus *Monomorphina* (*M. aenigmatica* and *M. pseudopyrum*), and the fruit fly (*Drosophila melanogaster*). The purpose of this protocol is to describe a fast, inexpensive, and simple method to obtain detailed information about the structure, size, and surface characteristics of specimens that can be broadly applied to a large range of organisms for morphological assessment. Successful completion of this protocol will allow others to use SEM to visualize samples by applying these techniques to their system.

INTRODUCTION:

A scanning electron microscope (SEM) uses a focused beam of high-energy electrons to generate an image from secondary electrons that shows the morphology and topography of a sample¹. SEM can be used to directly determine the physical size of a sample, the surface structure, and the three-dimensional shape, and offers greater resolution and larger depth of field compared to light microscopy. Another form of electron microscopy (EM), transmission electron microscopy (TEM) uses focused electrons that pass through the sample, generating images with fine details of internal structure. While TEM has higher resolution than light or SEM and can be used to resolve structures as small as single atoms, it has three major disadvantages: extensive sample preparation, a small field of view, and a shallow depth of field^{2,3}. Although other visualized protocols exist using SEM to examine specific cells, organelles, or tissues⁴⁻¹⁰, this protocol is unique in that we describe methods that can be broadly applied to a large range of organisms for morphological assessment.

SEM has found broad applications for examining inorganic materials including nanoparticles^{11,12}, polymers¹³, and numerous applications in geological, industrial, and material sciences¹⁴⁻¹⁶. In biology, SEM has long been used as a method for examining biological samples ranging from individual proteins to whole organisms^{17,18}. SEM is of particular value because morphological surface details can be used to inform scientific discovery. SEM analysis is a fast, inexpensive, and simple method to obtain detailed information about the structure, size, and surface characteristics of a wide range of biological samples.

Because an SEM normally operates under high vacuum (10^{-6} Torr minimum) to support a coherent beam of high-speed electrons, no liquids (water, oils, alcohols) are permitted in the sample chamber, as liquids prevent a vacuum from forming. Thus, all samples examined using SEM must be dehydrated, typically using a graded ethanol series followed by a drying process to remove the ethanol. There are several methods of drying biological tissues for use in the SEM, including air drying, lyophilization, use of a critical point drying (CPD) device, or chemical drying using t-butyl alcohol (TBA) or hexamethyldisilazane (HMDS)¹⁹⁻²². Most often, selection of a drying method is empirical since each biological sample may react differently to each drying method. For any given sample, all of these methods may be appropriate, so comparing the advantages and disadvantages of each is useful in selecting the appropriate method.

While air drying a sample at room temperature or in a drying oven (60 °C) is the simplest method, most biological samples show drying-induced damage such as shriveling and collapse, resulting in distortion of the specimen. The process of lyophilization also removes water (or ice) from a sample, but requires samples to be flash-frozen and placed under vacuum to remove the ice *via* the process of sublimation, potentially damaging the sample. In addition, the user must have access to a lyophilizer. The most commonly used method for dehydrating samples for SEM is critical point drying (CPD). In CPD, the ethanol in a sample is replaced with liquid carbon dioxide (CO₂) and, under specific temperature and pressure conditions known as the critical point (31.1 °C and 1,073 psi), CO₂ vaporizes without creating surface tension, thereby effectively maintaining the morphological and structural features of the sample. While CPD is generally the standard method, it has several drawbacks. First, the process requires access to a critical point dryer, which

is not only expensive, but also necessitates the use of liquid carbon dioxide. Second, the size of the sample that can be dried is limited to the chamber size of the critical point dryer. Third, the exchange of liquids during CPD can cause turbulence that can damage the sample.

Chemical drying offers many advantages over CPD and serves as a suitable alternative that is becoming widely used in SEM sample preparation. The use of chemical dehydrants such as TBA and HMDS offers a fast, inexpensive, and simple alternative to other methods, while still maintaining the structural integrity of the sample. We recently showed that there was no difference in the integrity of the tissue or the quality of the final image captured when using CPD or TBA as the drying method in adult *Drosophila* retinal tissue²³. Unlike CPD, TBA and HMDS do not require a drying instrument or liquid CO₂ and there is no limitation on the size of the sample to be dried. In addition to obtaining the chemicals, only a standard chemical fume hood and appropriate personal protective equipment (gloves, lab coat, and safety goggles) are required to complete the drying process. While both TBA and HMDS are flammable, TBA is less toxic and less expensive (approximately 1/3 the cost of HMDS) than HMDS.

In this article, we describe how to fix, wash, dehydrate, dry, mount, sputter coat, and image three types of organisms: cyanobacteria (*Toxifilum mysidocida*, *Golenkina* sp., and an unknown sp.), two euglenoids from the genus *Monomorphina* (*M. aenigmatica* and *M. pseudopyrum*), and the fruit fly (*Drosophila melanogaster*). These organisms represent a wide range in size (0.5 µm to 4 mm) and cellular diversity (single-celled to multicellular), yet all are easily amenable to SEM analysis with only small variations needed for specimen preparation. This protocol describes the methods for using chemical dehydration and SEM analysis to examine morphological details of three types of organisms and would be broadly applicable to examining many organismal and tissue types.

PROTOCOL:

1. Preparation and Fixation

1.1. Prepare cyanobacteria.

1.1.1. Grow unialgal cultures in F/2 media^{24,25} at a temperature of 28 °C on a 14:10 h light dark cycle. Transfer sufficient culture to a 1.5 mL microcentrifuge tube such that after allowing 15 min to settle, the bacterial pellet is approximately 0.05 mL in size. Remove the media and replace with 1.5 mL of fixative (1.25% glutaraldehyde, 0.1 M phosphate buffer pH 7.0), gently invert several times, and incubate overnight at 4 °C.

1.1.2. Transfer the fixed cells into a 10 mL filtration rig (**Figure 1**) using a polycarbonate 25 mm filter with 0.8 or 0.2 µm pores, depending on the size of the cells. Seal the side arm and funnel with rubber stoppers to contain the culture in the funnel. Remove the fixative by gentle vacuum on the filtration flask, after removing both stoppers.

Note: If the cell density on the polycarbonate filter is not optimal, adjust the amount of starting culture used.

1.2. Prepare single cell algae (euglenoids).

1.2.1. Grow unialgal cultures in AF-6 media²⁶ with 150 mL L⁻¹ of commercially-available soil-water medium added to the media, at a temperature of 22 °C on a 14:10 h light dark cycle. Transfer 2 mL of low density (OD₆₀₀ < 0.5) culture into a 10 mL filtration rig (**Figure 1**) using a polycarbonate 25 mm filter with 8 µm pores. Seal the side arm and funnel with rubber stoppers to contain the culture in the funnel.

Note: If the cell density on the polycarbonate filter is not optimal, adjust the amount of starting culture used.

1.2.2. Add three drops of 4% osmium tetroxide (OsO₄) directly to the culture and allow to incubate for 30 min. Remove the fixative by gentle vacuum on the filtration flask, after removing both stoppers.

Note: OsO₄ is an acute toxin (dermal, oral, and inhalation routes), corrosive to the skin, damaging to the eyes, and a respiratory sensitizer. OsO₄ should be handled in a chemical fume hood using appropriate personal protective equipment including gloves, lab coat, and eye protection.

1.3. Prepare *Drosophila melanogaster* (fruit fly)²³.

1.3.1. Anesthetize adult flies using 100% carbon dioxide. Place anesthetized adults (about 10 to 30 flies) in a small plastic screw cap vial or 1.5 mL centrifuge tube.

1.3.2. Immerse anesthetized flies in 1 mL of fixative (1.25% glutaraldehyde, 0.1 M phosphate buffer pH 7.2) for 2 h (or overnight) at 4 °C. If the flies float to the surface of the fixative, add a few drops of 2.5% polyethylene glycol tert-octylphenyl ether to weaken the surface tension of the fixative allowing for total submersion of the tissue. Remove the fixative using a glass pipet.

2. Washing and Dehydration

2.1. Wash and dehydrate cyanobacteria.

2.1.1. Wash the fixed sample three times with 5 mL of 0.1 M phosphate buffer pH 7.0 at room temperature for 10 min each. Keep the flask and funnel stoppered to hold the wash. Remove each wash by gentle vacuum on the filtration flask, after removing both stoppers.

2.1.2. Dehydrate the sample while maintaining continuous immersion using a graded ethanol series, for 10 min in a volume of 5 mL in the funnel. The graded ethanol concentrations are: 25%, 50%, 75%, 95%, and 2 changes of 100% ethanol. Keep the flask and funnel stoppered to contain

the ethanol in the funnel, preventing loss both *via* evaporation and passive flow through the filter.

2.1.3. Remove ethanol by gentle vacuum on the filtration flask, after removing both stoppers. Transfer the filter/sample to a disposable aluminum weighing dish containing just enough 100% ethanol to cover the sample before drying.

2.2. Wash and dehydrate single cell algae (euglenoids).

2.2.1. Wash the fixed sample three times with 5 mL of deionized water at room temperature for 10 min each. Keep the flask and funnel stoppered to contain the wash in the funnel. Remove each wash by gentle vacuum on the filtration flask, after removing both stoppers.

2.2.2. Dehydrate the sample while maintaining continuous immersion using a graded ethanol series for 10 min in a volume of 5 mL in the funnel. The graded ethanol concentrations are: 25%, 50%, 75%, 95%, and 2 changes of 100% ethanol. Keep the flask and funnel stoppered to contain the ethanol in the funnel, preventing loss both *via* evaporation and passive flow through the filter.

2.2.3. Remove the ethanol by gentle vacuum on the filtration flask, after removing both stoppers. Transfer the filter/sample to a disposable aluminum weighing dish containing just enough 100% ethanol to cover the sample before drying.

2.3. Wash and dehydrate *Drosophila melanogaster* (fruit fly).

2.3.1. Wash the fixed sample three times with 1 mL of 0.1 M phosphate buffer pH 7.2 at room temperature for 10 min in a 1.5 mL microcentrifuge tube. Remove each wash with a glass pipette, being careful not to remove the flies.

2.3.2. Dehydrate the sample using a graded ethanol series, for 10 min in a volume of 1 mL in a microfuge tube. The ethanol concentrations are: 25%, 50%, 75%, 80%, 95%, 100%. Remove the ethanol with a glass pipette, being careful not to remove the flies.

2.3.3. Retain the sample in the 1.5 mL microcentrifuge tube with just enough 100% ethanol to cover the sample before drying.

3. Drying

3.1. Perform chemical drying using hexamethyldisilazane (HMDS)²⁰.

3.1.1. Replace the 100% ethanol solution with a 1:2 solution of HMDS and 100% ethanol for 20 min. Replace the 1:2 solution with a 2:1 solution of HMDS and 100% ethanol for 20 min. Replace the 2:1 solution with 100% HMDS for 20 min. Repeat once.

Note: HMDS is flammable and an acute toxin (dermal route). HMDS should be handled in a chemical fume hood using appropriate personal protective equipment including gloves, lab coat, and eye protection.

3.1.2. Transfer the sample in HMDS, if in a 1.5 mL microcentrifuge tube, into a disposable aluminum weighing dish. Once in the aluminum weighing dish, replace the 100% HMDS with just enough fresh 100% HMDS to cover the sample.

3.1.3. Transfer the sample to a plastic or glass non-vacuum desiccator with fresh desiccant (5-6 cm deep) and place into in a chemical fume hood. Alternatively, place the sample directly in a chemical fume hood to dry with a loose lid, such as a box, to prevent debris from falling on the sample. Allow the sample to dry for 12 to 24 h.

3.2. Perform chemical drying using t-butyl alcohol (TBA)²⁷.

3.2.1. Replace the 100% ethanol solution with a 1:1 solution of TBA and 100% ethanol for 20 min. Replace the 1:1 solution with 100% TBA for 20 min. Repeat twice. Keep the solution at 37 °C so that TBA does not freeze.

Note: 100% TBA has a freezing point of 25.5 °C; the 1:1 solution will not freeze at room temperature. TBA is flammable, causes serious eye irritation, is harmful if inhaled, and may cause respiratory irritation, drowsiness, or dizziness. TBA should be handled in a chemical fume hood using appropriate personal protective equipment including gloves, lab coat, and eye protection.

3.2.2. Transfer the sample in TBA, if in a 1.5 mL microcentrifuge tube, into a disposable aluminum weighing dish. Once in the aluminum weighing dish, remove TBA and replace with just enough fresh 100% TBA to cover the sample. Freeze the TBA at 4 °C for 10 min.

3.2.3. Transfer to a vacuum desiccator (bell jar) with frozen gel packs to keep TBA frozen. Evacuate and maintain vacuum with a rotary pump to allow the sample to dry by vacuum sublimation of the frozen TBA for at least 3 h or overnight.

4. Mounting

4.1. Mount cyanobacteria and euglenoids.

4.1.1. Label the bottom of either a 12- or 25-mm aluminum mounting stub to indicate what is being placed on top. Cut the polycarbonate filter with the dried cells into quarters with a clean razor blade or scalpel if using a 12 mm stub, or place the entire filter if using a 25 mm stub.

4.1.2. Place each filter on adhesive or a carbon adhesive tab secured to the top of a stub.

4.2. Mount *Drosophila melanogaster* (fruit fly).

4.2.1. Label the bottom of the aluminum mounting stub to indicate what is being placed on top. Place the dried flies in the desired position on adhesive or carbon adhesive tab secured to the top of a stub under a dissecting microscope with precision tweezers.

4.2.2. Apply silver conductive adhesive, *i.e.*, silver paint, around the outer edges of the stubs. Connect the silver paint to the flies using a toothpick to ensure conductivity. Do not allow the paint to touch the desired imaging area.

4.2.3. Place the stubs in a stub holder box and place the open stub holder box in a desiccator. Allow the silver paint to dry at least 3 h or overnight for best results.

5. Sputter Coating

5.1. Prepare the sputter coating apparatus by performing four to five flushes of argon gas. If the humidity is high (*i.e.*, the room air feels moist, usually about 50% relative humidity), perform six to seven flushes. Sputter coat the stubs following the manufacturer's instructions.

5.2. Coat samples based on specimen used:

5.2.1. For filters with cyanobacteria, set the timer to sputter coat for 180 s straight on.

5.2.2. For filters with eukaryotic algae, *i.e.*, euglenoids, set the timer to sputter coat for 120 s straight on, then 20 s on three sides at a 45° angle.

5.2.3. For large samples, *i.e.*, fly heads, set the timer to sputter coat for 60 s straight on, then 30 s on each side at a 45° angle.

Note: After coating is complete, stubs should be light gray in color.

6. Imaging

6.1. Image samples using a scanning electron microscope that includes a secondary electron (SE) detector.

6.1.1. For cyanobacteria, apply the following settings: 3-5 kV accelerator voltage (AC), 20-30 probe current (PC), and 5 mm working distance (WD). For euglenoids, use 3 kV AC, 30 PC, and 5 mm WD. For flies, use 5 kV AC, 30 PC, and 5 to 10 mm WD.

6.2. Adjust magnification depending on the size of the detail or object to be visualized. Adjust the stigmators, apertures, and focus until a clear image is produced. Capture the image using high-resolution settings according to the manufacturer's instructions of the SEM.

REPRESENTATIVE RESULTS:

Cyanobacteria are a prokaryotic group of organisms that are critical to the global carbon, oxygen, and nitrogen cycles^{28,29}. Of the estimated 6000 species of cyanobacteria³⁰, most have a mucilaginous sheath that cover and connect the cells together and to other structures³¹, which along with the shape, can be resolved microscopically³². Cell size, shape, and pigments present distinguish individual species and in aquatic habitats, can be visualized as cyanobacterial “blooms”²⁸. The modern cyanobacterial classification combines all morphological features possible using light microscopy, TEM, and SEM, as well as molecular data³³. Similar to the mucilage sheath of cyanobacteria, pellicle strips of euglenoid species aid in phylogeny determination. Euglenoids are aquatic flagellates that possess a great deal of morphological and behavioral diversity, and SEM analysis has proven useful in classifying distinct species. Specifically, euglenoids possess novel structures beneath their plasma membrane that are composed of parallel proteinaceous strips and microtubules, called the pellicle³⁴⁻³⁶. The number, arrangement, and size of pellicle strips are critical morphological comparators, and together with molecular phylogenetic data, are used to construct the phylogeny for Euglenozoa³⁷.

The first step in preparing both cyanobacteria and euglenoids requires filtering the organisms from their growth medium and is accomplished by assembling a filtration rig (**Figure 1A**). Aspiration is used to pull the medium through a polycarbonate filter, leaving the organisms on the surface of the filter. The pore size of the polycarbonate filter should be selected such that the intact organisms will not pass through (compare **Figures 1B** and **1C**). **Figure 2** shows representative results of three different genera of cyanobacteria. Two are freshwater and one is marine. Details of the cell, including shape and texture of the surface are visible, as well as a sheath of mucilage or projections from the cells. **Figure 3** illustrates how SEM is used to visualize the pellicle strips of two different euglenoid species. The helical arrangement of the pellicle strips that merge at the posterior end to form a tail aid in phylogeny determination when comparing *Monomorphina aenigmatica* (**Figures 3A** and **3B**) with *Monomorphina pseudopyrum* (**Figures 3C** and **3D**).

In addition to morphological characteristics that identify species, the external morphology of model organisms such as *Drosophila melanogaster* can be used to identify genetic modifiers using the photoreceptors neurons that comprise the compound *Drosophila* eye³⁸. Since the eye is a non-essential tissue in flies, it is possible to express toxic proteins in retinal cells and use SEM to examine the morphological changes that genetic modification has on the eye. Normal fly eyes are characterized by an ordered array of bristles and lenses (**Figure 4A**), however expression of proteins associated with Alzheimer’s disease create what is called the ‘rough eye’ phenotype (**Figure 4B**). Genes that suppress (**Figure 4C**) or enhance (**Figure 4D**) the rough eye phenotype may play a physiological role in disease progression and have potential for drug targeting³⁹. Also shown in **Figure 4** are examples of potential pitfalls to avoid including an example of the collapsed structure of the eye from improper drying technique (**Figure 4E**) and electron charging from insufficient sputter coating that appears during image acquisition (**Figures 4F** and **4G**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of the filtration rig and SEM of a polycarbonate filter. (A) This apparatus is used to separate small or single celled organisms such as cyanobacteria and

euglenoids away from their growth medium. Application of a vacuum to the side arm of the flask will pull the contents of the funnel through the polycarbonate filter and into the base of the filtration flask, leaving the organisms on the surface of the filter. (B) SEM of a 0.8 μm pore filter with no sample as the sample was lost because of mishandling. Scale bar = 20 μm . (C) SEM of a 0.8 μm pore filter with cyanobacteria present. Scale bar = 20 μm .

Figure 2: SEM micrograph of cyanobacteria. (A, B) *Toxifilum mysidocida*, a filamentous freshwater species from Colorado with a visible sheath of mucilage (M). Scale bars = 5 μm . (C, D) A freshwater *Golenkina* sp. from Ohio. Individual cells sometimes form colonies held together by a thin layer of mucilage (M). Filament-like protrusions (white arrows) extend for the individual cells. Scale bars = 10 μm . (E, F) Unknown filamentous species from a high salinity estuary in Texas coastal waters. Individual cell (black arrows) and dividing cells (white arrowheads) are visible. Scale bar in panel E = 2 μm . Scale bar in panel F = 1 μm .

Figure 3: SEM micrograph of the pellicle strips from two species of euglenoids. (A, B) *Monomorphina aenigmatica*. (A) Low magnification images of the entire cell. Scale bar = 5 μm . (B) High magnification of the posterior end. Scale bar = 3 μm . (C, D) *Monomorphina pseudopyrum*. (C) Low magnification images of the entire cell. Scale bar = 10 μm . (D) High magnification of the posterior end. Scale bar = 5 μm .

Figure 4: SEM analysis of phenotypic modification of the *Drosophila* eye. Compared to the normal external appearance of the ordered array of bristles and lenses of the fly eye (A), expression of the toxic protein tau causes the death of the photoreceptor neurons, generating the 'rough eye' phenotype (B). Expression of genetic modifiers that either suppress (C) or enhance (D) the tau rough eye provides evidence of genes that may play a role in tau neurotoxicity. Improper technique during drying steps can lead to a collapse of the structure of the eye (E) while insufficient coating of the sample will cause charging, which appears as either a white (F) or black (G) band during image acquisition, as shown by the arrow. Scale bar = 400 μm .

DISCUSSION:

Here we described a protocol using SEM to obtain detailed information about external morphological characteristics of three types of organisms that others can apply to examine features of many types of organisms or tissues. Within each step of the protocol, there are potential points of error that may arise and are discussed in detail below.

While the volumes for fixative and washes given here are specific, in general the fixative and washes should be 5-10x the volume of the specimen. All fixation, washing, and dehydration times are based on our experience, if problems arise, e.g., shriveling of the sample, you may need to increase the times at each step of dehydrating or drying. Depending on your single cell sample, you may need to do both a glutaraldehyde fixation and a post-fixation in osmium tetroxide. If a dual fixation is needed after step 1.1.2 of the cyanobacteria procedure cover with an equal amount 2% OsO_4 in the same buffer and proceed from step 1.2.2 of the single cell procedure. From work not shown here, we have found that *Drosophila* can either be directly placed into

fixative following anesthetization or can be frozen indefinitely at -20 °C in a microfuge tube and placed into fixative at a later time with no difference in finished image quality.

During the washing and dehydration steps, it is imperative to move through the graded ethanol series slowly and without skipping a noted concentration. If samples are dehydrated too quickly, it negatively impacts the underlying structural components in the tissue and specimen will shrivel, wither, or collapse. An example of tissue collapse is shown in **Figure 4E**. In addition, to prevent the introduction of artifacts such as clumping and flattening of morphological features, it is critical to leave just enough ethanol to cover the sample between steps during dehydration and drying.

When using the filtration rig, a drop of water on the base will hold the filter in place when assembling. Also, the filter should be placed with the shiny side up and all liquids should be added gently, being careful to handle the filter with care to prevent washing away the sample. Although we have found the results of drying using either HMDS or TBA to be of equal quality, we recommend using TBA: while both HMDS and TBA are flammable, TBA is about one third the cost and less toxic than HMDS.

During mounting when using silver conductive adhesive (also called silver paint), be careful when applying as your sample can easily be covered (buried) in the paint, thereby obscuring fine details in morphology. Sputter coating times may vary based on your sample, and values given here are based on our experience. One potential negative outcome of insufficient sputter coating is a phenomenon called charging, which often appears as a white or black line (sometimes a flash) in the final image (see **Figures 4F** and **G** for examples). If the amount of charging observed is minimal, it may be possible to mitigate the effects by adjusting various microscope parameters, such as lowering the accelerating voltage or beam current, increasing the condenser lens strength, and/or using a smaller objective aperture. Most SEMs used for biological samples have secondary electron detectors, however some SEMs may also be equipped with backscatter detectors or environmental secondary electron detectors, all of which can be adjusted to diminish or eliminate the effects of minimal charging. If the charging effects are more pronounced, it is likely that there is poor grounding of the sample or too little sputter coating to produce the secondary electrons, causing the charged electrons to build up in the specimen and be spontaneously released, producing distortion in the image during acquisition. Pronounced charging is most often resolved with a thicker coating or better grounding with silver paint. Because both too much and too little sputter coating can negatively impact the quality of the image, it is best to optimize the amount of coating applied by adjusting the variables of time (10 to 180 s) and pressure (70 to 150 mTorr) of the sputter coater to achieve an optimal coating. As the coating cannot be removed once applied to the sample, a test run is recommended with a single sample before coating all of the samples.

SEM parameters such as accelerating voltage (in kV), probe current (PC), and working distance (WD) must be adjusted to get the best image possible, as the settings given here are suggested starting parameters based on our experience. While the quality of the sample is dependent upon the care taken in preparation, the quality of the final image relies upon the experience and skill

of the SEM operator. Thus, we recommend working with an SEM technician or spending time developing your own SEM skills. All of the data (including sample preparation and imaging) shown in this paper were generated by undergraduate students in the Biology Microscopy program at CMU, with guidance from faculty and our microscopy technician.

The protocols described here include the use of potentially harmful chemicals, and the appropriate safety measures should always be observed to protect users, particularly students who may be handling these chemicals. While the protocols specify safety concerns associated with each chemical upon first use, users should always: (1) use a chemical fume hood, (2) have access to an emergency eye wash and safety shower, (3) use appropriate personal protective equipment including nitrile gloves, lab coat, and eye protection, and (4) know where to find written safety procedures inclusive of handling procedures, designated use areas, spill procedures, decontamination procedures, and waste disposal procedures.

In conclusion, these organisms illustrate how information about the three-dimensional topography of the external surfaces of each is useful for phylogenetic grouping or modifier analysis. For cyanobacteria, features determined from the SEM can be used in combination with additional data from light microscopy, TEM, and molecular studies to identify, characterize, and name the cyanobacteria. For euglenoids, the number, width, arrangement (helical or straight), and ornamentation, if present, of the pellicle strips can be used with other morphological data, and if necessary molecular data, to identify, characterize, and name euglenoids. Moreover, other types of single-celled algae and protozoans or micro-invertebrates can be prepared and examined in a similar manner to determine external morphological features such as flagella, feeding structure, external ornamentation, or details of appendages in the case of micro-invertebrates. Finally, SEM has broad applications for examining morphological details, such as the eye of the model system *Drosophila*, to characterize genetic modifiers of disease pathology. The protocols described here utilize organisms that vary greatly with respect to complexity, yet all are amenable to SEM analysis to gather useful morphological information that is critical for scientific discovery spanning many different subdisciplines of biology.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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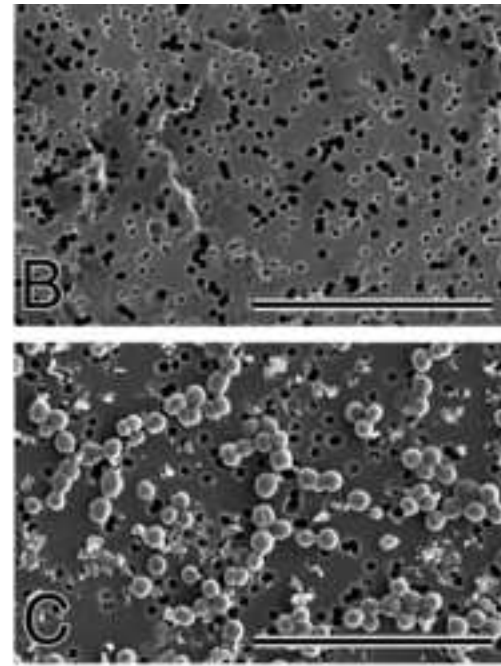
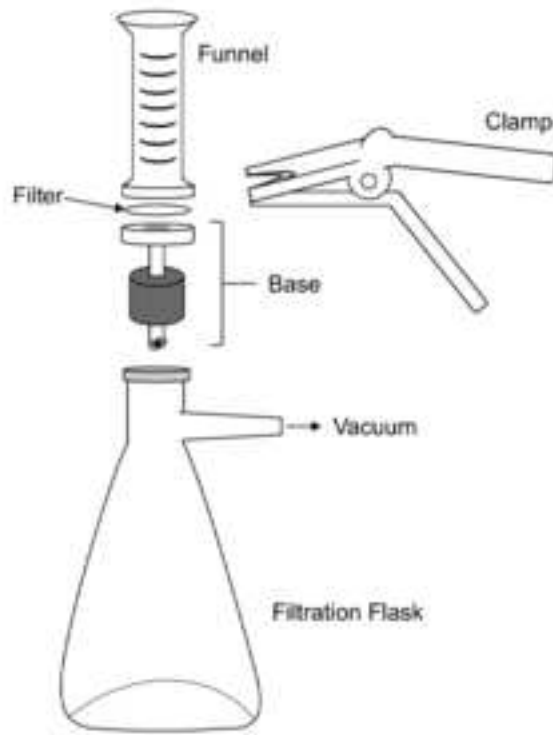
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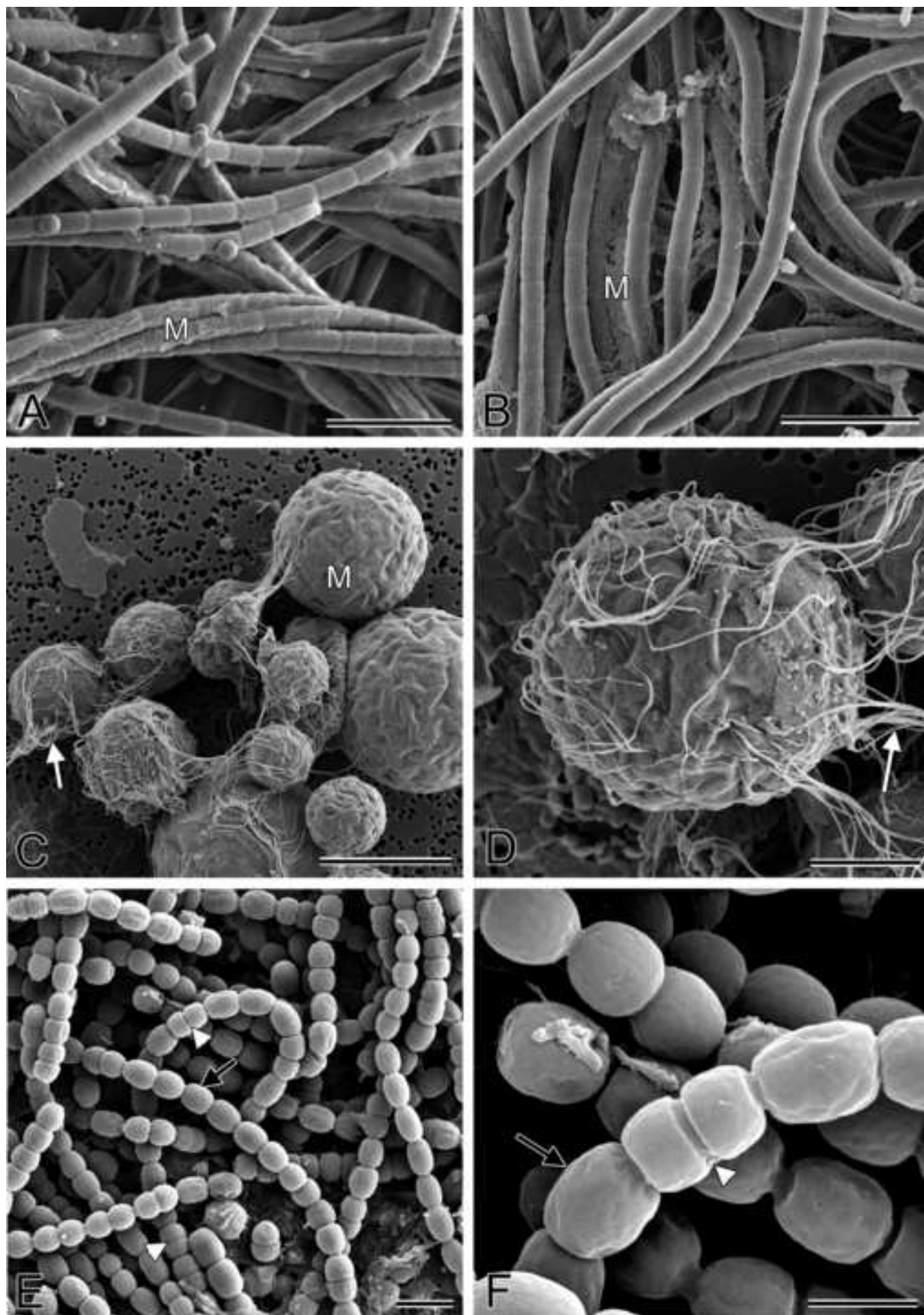
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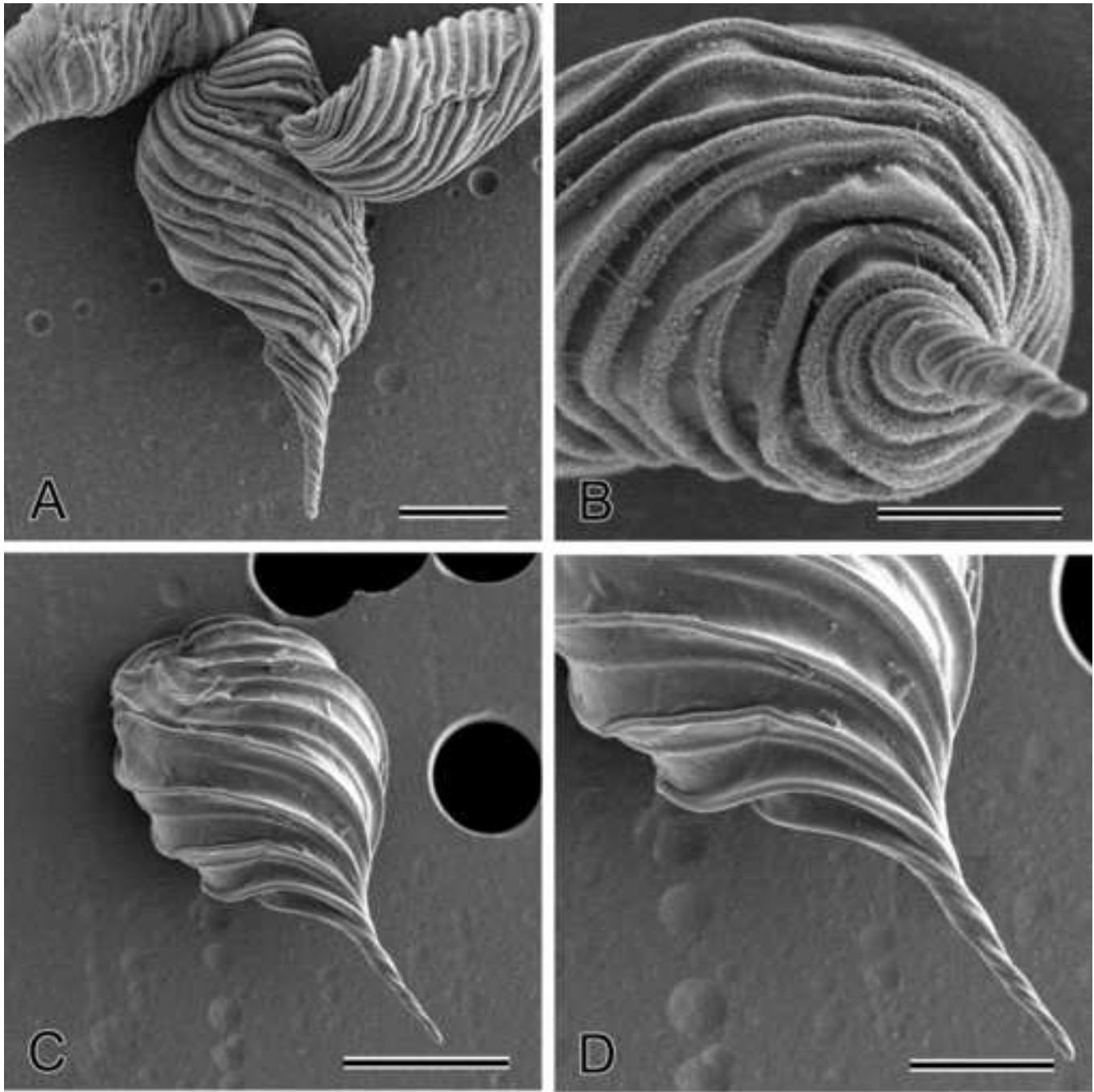
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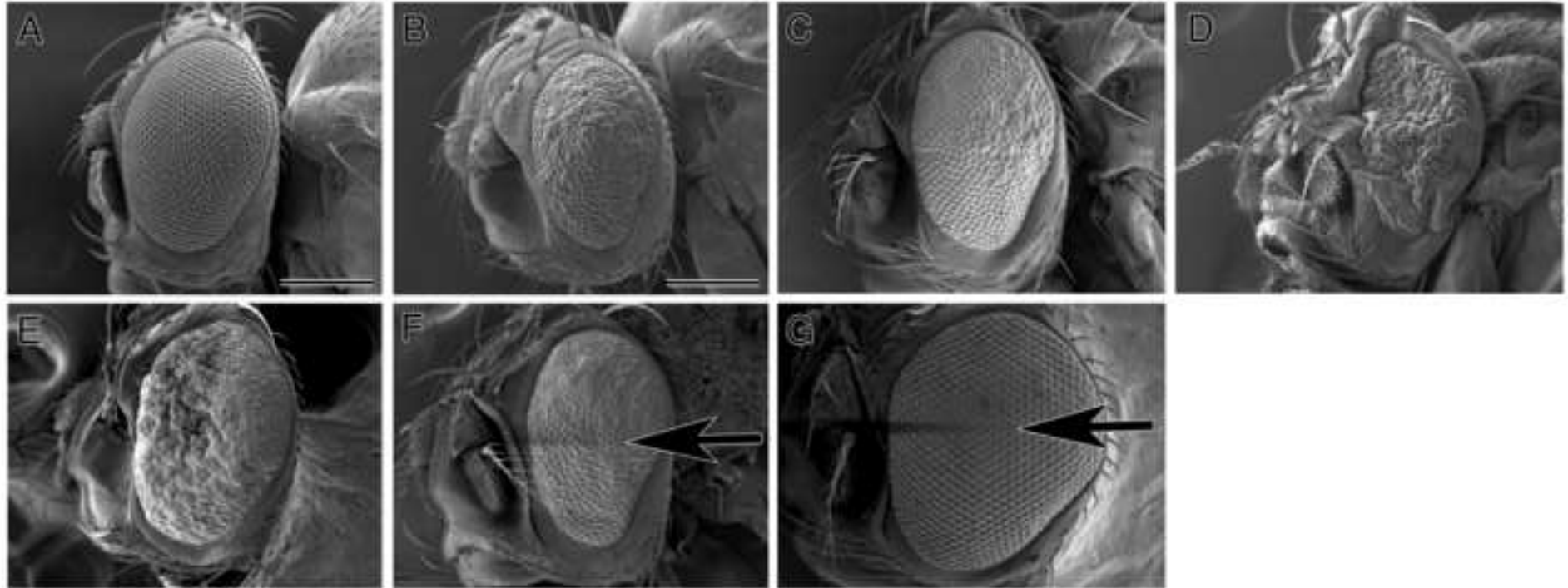
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570

A







Name of Material/ Equipment

gold–palladium

Silver conductive adhesive 503

Whatman Nuclepore Track-Etched Membranes; diam.

25 mm, pore size 8 μm , polycarbonate

Whatman Nuclepore Track-Etched Membranes; diam.

25 mm, pore size 0.8 μm , polycarbonate, black

Whatman Nuclepore Track-Etched Membranes; diam.

25 mm, pore size 0.2 μm , polycarbonate

aluminum weighing dish

aluminum mounting stubs (12 mm)

aluminum mounting stubs (25 mm)

Adhesive tabs

conductive carbon adhesive tabs

F/2 media

AF6 media

Soil water medium

Polyethylene glycol tert-octylphenyl ether (Triton X-100)

Hummer 6.2 Sputter Coater

Hitachi 3400N-II SEM

Company

Ted Pella, Inc.

Electron Microscopy Sciences

Sigma

Sigma

Sigma

Fisher Scientific

Electron Microscopy Sciences

Electron Microscopy Sciences

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Electron Microscopy Sciences
Culture Collection of Algae at the University of Texas at
Austin

Bigelow - National Center for Marine Algae and Microbiota

Carolina Biological Supply Company

VWR

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Hitachi

Catalog Number	Comments/Description
91212	
12686-15	
WHA110614	
WHA110659	
WHA110606	
08-732-100	
75210	
75186	
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77825	
https://utex.org/products/f_2-medium	No Catalog number given - see link
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153785	
97062-208	
http://www.anatechusa.com/hummer-sputter-systems/4	No Catalog number given - see link
https://www.hitachi-hightech.com/us/product_list/?ld=sms2&md=sms2-1&sd=sms2-1-	The company doesn't appear to sell this

model any longer



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Dear Dr. Cao:

In the section below, please find our point-by-point responses to the editorial and reviewer comments for our manuscript submission entitled "Preparation of Prokaryotic and Eukaryotic Organisms Using Chemical Drying for Morphological Analysis in Scanning Electron Microscopy (SEM)".

We would like to thank the reviewers for contributing their valuable time to evaluate our manuscript. We sincerely appreciate their input and we feel the manuscript is substantially improved because of their comments, suggestions, and valuable insight.

Two reviewers had major concerns that we should include additional information to compare chemical drying (the primary focus of our protocols) with other common techniques that are widely used (especially critical point drying, CPD), including highlighting the advantages and disadvantages of these procedures. We substantially revised the introduction to address these suggestions and included additional information in the discussion to also expand upon these points. Reviewer #1 requested that we include a low magnification image of a pore filter that has retained the sample, so we added a panel to Figure 1 to show an SEM image of a filter with cyanobacteria present (now Figure 1C). All of the remaining minor concerns are addressed specifically below (the red text indicates our response to each item).

Thank you in advance for considering our resubmitted manuscript for publication in *JoVE*.

Sincerely,

Michelle L. Steinhilb

Manuscript title:

Preparation of Prokaryotic and Eukaryotic Organisms Using Chemical Drying for Morphological Analysis in Scanning Electron Microscopy (SEM)

Manuscript ID: 58761

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We ran spell and grammar check and resolved all issues.

2. Figures: Please change the color of panel labels, scale bars and arrows to make them easier to read.

We made the panel labels and scale bars larger, but we did not change the color. Since all of the images have both light and dark areas, we used black labels with a white stroke to make them as clear as

possible. If these still appear unclear, please provide specific directions (size/color/position/Figure number) on how to improve them and we will gladly make the necessary changes.

3. Figure 4: Please provide panel D of Figure 4.

We added panel D of Figure 4.

4. Please provide an email address for each author.

Additional email addresses were added for all authors.

5. Please use SI abbreviations for all units: L, mL, μ L, h, min, s, etc.

Changed all SI abbreviations to match format above.

6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Any missing spaces are now included.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols ([™]), registered symbols ([®]), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Millipore, Whatman, Triton-X 100, Hummer, Hitachi, etc.

We removed the commercial language including Millipore, Whatman, Triton-X 100, Hummer, and Hitachi and replaced them with generic terms instead.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Protocol was renumbered following the format specified.

9. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:

Line 104: What density is considered to be low to moderate? How long is it allowed to settle?

We changed this step to now read: ‘Transfer sufficient culture to a 1.5 mL microcentrifuge tube such that after allowing 15 min to settle, the bacterial pellet is approximately 0.05 mL in size. Remove the media and replace with 1.5 mL of fixative (1.25% glutaraldehyde, 0.1 M phosphate buffer pH 7.0), gently invert several times, and incubate overnight at 4 °C.’

We also added this note to the end of step 1.1.2 and 1.2.1: (Note: if the cell density on the polycarbonate filter is not optimal, adjust the amount of starting culture used).

Line 116: What density is considered to be low?

We defined low density to be $OD_{600} < 0.5$

Line 124: Please specify the concentration of carbon dioxide used.

We added that the CO₂ is 100%

10. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Protocol steps were combined to contain 2-3 actions and a maximum of 4 sentences per step.

11. Please include single-line spaces between all paragraphs, headings, steps, etc.

Spaces were added between all paragraphs, headings, and steps.

12. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We are unsure how to calculate pages, but based on line numbering, it appears there are 44 lines per page, so 2.75 pages would be equivalent to 121 lines of text. We highlighted 98 lines of text.

13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

We highlighted complete sentences and every step includes at least one action. We did not include steps describing anesthetization, euthanasia, or growth of cultures.

14. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We included all of the relevant details required to perform the step. We did want to note that since the steps for 2.1.1-2.1.3 are the same as 2.2.1-2.2.3 just using a different organism, we only highlighted the steps for 2.1.1-2.1.3.

15. Discussion: Please also discuss any limitations of the technique and the significance with respect to existing methods.

We added additional information in both the discussion and introduction to address limitations of the techniques described in the context of existing methods.

16. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

The table was updated to (1) include more information about the Whatman filters; (2) have separate lines for items that we previously had listed together; (3) add Polyethylene glycol tert-octylphenyl ether (Triton X-100); (4) add the Hummer sputter coater and Hitachi 3400N-II SEM.

17. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Thank you for sending the updated JoVE EndNote Output Style. We used the new style to format the bibliography. The full name of journals is now displayed. Volume and issue numbers for references are included (note that some references do not possess either a volume or issue number, depending on the journal).

18. Please follow the book citation example below to reformat book references:

Kioh, L.G. et al. Physical Treatment in Psychiatry. Blackwell Scientific Pubs. Boston (1988).

Despite using the new EndNote Output Style that you sent, the output format for books does not match

the style you are requesting above. I tried to format them manually within the references section, however manual changes in EndNote are problematic. Would it be possible for you to send a new EndNote Output Style file for us to use?

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Koon et al. describe a methodology to prepare and image a range of specimens for SEM visualization, including unicellular, multicellular, prokaryotic, and eukaryotic organisms. This article is useful to a broad biological sciences audience and is well-suited for publication in JoVE.

Major Concerns:

No major concerns

Thank you

Minor Concerns:

- Fig. 1 - a low magnification image of a pore filter that has retained the sample would be helpful to include, so that the reader can directly compare to 1B.

Excellent suggestion – we added an SEM image of a pore filter that has cyanobacteria present (now Figure 1C) to compare directly to Figure 1B.

- The *Drosophila* eye images in Fig. 4 appear grainy and lack the clarity/focus of the other SEM images in Figs. 2-3.

We suspect that because this image is in landscape format, it may have been sized-down during the review process, causing the image to appear grainy. The original image is a .psd file at 350 ppi (JoVE requires 300 dpi).

Reviewer #2:

Manuscript Summary:

Overall: I was disappointed that the authors did not compare their results with those of other drying techniques. Since "Chemical Drying" was prominently featured in the title of the paper I fully expected the authors to make a comparison of HMDS and TBA drying techniques to those of CPD. If one of the selling points of this technique paper is to demonstrate that a wide variety of biological samples can be prepared with less effort, and less costly equipment, then making a direct comparison to alternative drying techniques (or at the very least discussing them) would seem to be in order.

This is an excellent suggestion and we significantly expanded the introduction to address this concern. In addition to describing more about CPD and comparing this drying technique to chemical dehydrants like TBA and HMDS in much greater detail, we also referenced our published work that shows no difference between TBA and CPD in *Drosophila*.

Major Concerns:

The drying techniques used are helpful but not novel. They show that these chemical drying techniques

can be used on a variety of organisms but without comparing the advantages and disadvantages to conventional SEM preparation techniques the paper loses its impact.

We agree that the techniques we describe are not novel, however there is not currently a good visual protocol that illustrates how to effectively use chemical drying for analysis in SEM. We purposefully selected a variety of organisms to make these protocols useful to a broad biological audience. The introduction now compares advantages and disadvantages of several techniques to boost the impact of this paper.

Minor Concerns:

Ln 14: Of the keywords the authors have chosen I feel that "phylogenetic grouping, species identification, phenotypic discrimination" are not appropriate as none of these represent a central aspect of the paper and are therefore unhelpful. However I do feel that "critical point drying, CPD" should be included as the researchers are looking for an alternative to CPD would be most interested in this paper.

We removed the keywords 'phylogenetic grouping', 'species identification', and 'phenotypic discrimination' from the manuscript and added 'critical point drying, CPD'.

Ln 26: 'specimen' to 'specimens' (same in abstract)

Changed 'specimen' to 'specimens' at both places mentioned in the Long Abstract.

Ln 33: 'cheap' to 'inexpensive' [the word cheap can translate into worthless in other languages]

Changed the word 'cheap' to 'inexpensive' in both the long abstract and the introduction.

Ln 43: eliminate 'higher magnification' since greater resolution is the principle advantage of SEM, not higher mag.

We eliminated the words 'higher magnification' – the sentence now reads: 'SEM can be used to directly determine the physical size of a sample, the surface structure, and three-dimensional shape, and offers greater resolution and larger depth of field compared to light microscopy'.

Ln 44: eliminate 'and focus' since an SEM does not form an image using an image forming lens (e.g. objective lens) there is technically no 'depth of focus' in an SEM. It does however create an image with a very large depth of field.

We eliminated the words 'and focus' – the sentence now reads: 'SEM can be used to directly determine the physical size of a sample, the surface structure, and three-dimensional shape, and offers greater resolution and larger depth of field compared to light microscopy'.

Ln 47: change 'scanning microscopy' to 'SEM' to avoid confusion with other scanning microscopies such as STM. The abbreviation SEM was already established on Ln 40. Use it!

Changed 'scanning microscopy' to 'SEM'.

Introduction: The authors fail to mention the principle reason why specimens must be dried in the first place. Namely the demands of a high vacuum environment that is required in a conventional SEM. Without this information the parts comparing SEM to TEM make no sense to those who are not already familiar with SEM and CPD.

We expanded the introduction to describe the high vacuum environment in better detail. We also added several paragraphs to further describe the types of drying methods available and their advantages and disadvantages.

Ln 55: Again, magnification is a meaningless term whereas resolution is not. ANY image can be magnified a million times (or even a billion for that matter). Instead tell the reader what the smallest objects are that can be visualized in an SEM. Tell us about resolution, not magnification.

We removed the words 'up to 1 million-fold magnification' and the sentence now focuses on the smallest objects that can be visualized – proteins. The sentence now reads: 'In biology, SEM has long been used as a method for examining biological samples ranging from individual proteins to whole organisms'.

Ln 69-92: Is all this necessary? The paper deals with a SEM preparation protocol, the organisms used are not really relevant. Mentioning them already (Ln 61-67) is sufficient. I would remove all of this from the Introduction.

We agree that it doesn't quite fit in the introduction, but we do feel that the information is critical for giving context to the organisms that are imaged and the utility of SEM for each (shown in Figures 2-4), therefore we removed these paragraphs from the introduction and used much this information in the Representative Results section.

Ln 106: Do not abbreviate 'fixative' to 'fix' Fix means to repair or to freeze in place. A 'fixative' is a solution used to accomplish this. Fixative is the correct term.

The word 'fix' and replaced with 'fixative'.

Ln 111: A 'gentile' is someone who is not Jewish. I believe the word you are searching for here is 'gentle'

The word 'gentile' was replaced with 'gentle'.

Ln 119: again, clean up the wording of 'fix' and fixative (which is used correctly in Ln 121)

Th word 'fix' was replaced with 'incubate'.

Ln 121: GENTLE

The word 'gentile' was replaced with 'gentle'.

Ln 129: change 'to cause the tissue to sink to the bottom of the tube' to 'to weaken the surface tension of the fixative allowing for total submersion of the tissue.'

Changed 'to cause the tissue to sink to the bottom of the tube' to 'to weaken the surface tension of the fixative allowing for total submersion of the tissue.'

Ln 138: 'the GRADED ethanol concentrations...'

Changed from 'the ethanol concentrations are' to 'the graded ethanol concentrations are'.

Ln 139: Do you mean 'to prevent evaporation of the ethanol'? If so then state it that way.

We clarified the sentence to now read 'Keep the flask and funnel stoppered to contain the ethanol in the funnel, preventing loss both via evaporation and passive flow through the filter.'

Ln 174: More detail is needed here. What size desiccator? How much fresh desiccant? Would a vacuum desiccator be appropriate? Why or why not?

We clarified this step by adding additional details – it now reads '3.1.3 Transfer the sample to a plastic or glass non-vacuum desiccator with fresh desiccant (5-6 cm deep) and place into in a chemical fume hood. Alternatively, the sample can be placed directly in a chemical fume hood to dry with a loose lid, such as a box, to prevent debris from falling on the sample. Allow the sample to dry for 12 to 24 h.'

Ln 178: The point about warming the TBA should be made earlier unless the 1:1 solution can be made below the freezing point of 25.5 °C.

The sentence was clarified and now reads '3.2.1 Replace the 100% ethanol with a 1:1 solution of TBA and 100% ethanol for 20 min. Replace the 1:1 solution 100% TBA for 20 min. Repeat twice. Keep the solution at 37 °C so the TBA does not freeze (100% TBA has a freezing point of 25.5 °C; the 1:1 solution will not freeze at room temperature).'

Ln 185: change 'alcohol' to 'TBA' to avoid confusion with ethanol (which is also an alcohol)

Changed 'alcohol' to 'TBA'.

Ln 217: I believe that CMU has a "Hitachi 3400N-II" SEM

We were asked to remove the trade name Hitachi from the manuscript, however we did correct the model number in the Material document.

Reviewer #3:

Manuscript Summary:

The authors present two easy and low-cost methods to process different sample types for conventional Scanning Electron Microscopy. They give a detailed description of consecutive steps of sample processing, also indicating artifacts that might be observed. Fundamental fixation, post-fixation and dehydration steps are included, with alternative drying procedures suggested for both prokaryotic and eukaryotic organisms.

Major Concerns: In the introduction at least a summary of other (widely accepted) techniques such as Critical Point Drying (CPD) and lyophilization should be included, to motivate the exploration of alternative methods. Aspects of instrumentation, sample size, continued gas supply and safety for students should be included.

We revised the introduction to include information about other widely accepted techniques, including CPD and lyophilization, as well as the rationale for selecting chemical dehydration over other methods. We also included more information about instrumentation, sample size, and gas supply. To address the concern about student safety, we added specific safety instructions within the protocol for HMDS, TBA, and OsO₄ upon first use, and added the following section to the Discussion:

Safety

The protocols described here include the use of potentially harmful chemicals, and the appropriate safety measures should always be observed to protect users, particularly students who may be handling these chemicals. While the protocols specify safety concerns associated with each chemical upon first use, users should always: (1) use a chemical fume hood, (2) have access to an emergency eye wash and safety shower, (3) use appropriate personal protective equipment including nitrile gloves, lab coat, and eye protection, and (4) know where to find written safety procedures inclusive of handling procedures, designated use areas, spill procedures, decontamination procedures, and waste disposal procedures.

Minor Concerns: Indicate where toxic substances are used, and that a fume hood is essential when working with reagents such as osmiumtetroxide (OsO₄) and hexamethyldisilazane (HMDS).

We more clearly indicated where toxic substances are used by expanding the information in the protocol for OsO₄, HMDS, and TBA – these now read:

1.2.2 Add three drops of 4% osmium tetroxide (OsO₄) directly to the culture and allow to incubate for 30 min. Remove the fixative by gentle vacuum on the filtration flask, after removing both stoppers. (Note: OsO₄ is an acute toxin (dermal, oral, and inhalation routes), corrosive to the skin, damaging to the eyes, and a respiratory sensitizer; OsO₄ should be handled in a chemical fume hood using appropriate personal protective equipment including gloves, lab coat, and eye protection)

3.1.1 Replace the 100% ethanol with a 1:2 solution of HMDS and 100% ethanol for 20 min. Replace the 1:2 solution with a 2:1 solution of HMDS and 100% ethanol for 20 min. Replace the 2:1 solution with 100% HMDS for 20 min. Repeat once. (Note: HMDS is flammable and an acute toxin (dermal route); HMDS should be handled in a chemical fume hood using appropriate personal protective equipment including gloves, lab coat, and eye protection)

3.2.1 Replace the 100% ethanol with a 1:1 solution of TBA and 100% ethanol for 20 min. Replace the 1:1 solution 100% TBA for 20 min. Repeat twice. Keep the solution at 37 °C so the TBA does not freeze (100% TBA has a freezing point of 25.5 °C; the 1:1 solution will not freeze at room temperature). (Note: TBA is flammable, causes serious eye irritation, is harmful if inhaled, and may cause respiratory irritation, drowsiness, or dizziness; TBA should be handled in a chemical fume hood using appropriate personal protective equipment including gloves, lab coat, and eye protection).

Also indicate that air-drying of specimens during dehydration steps, using vacuum filtration, should be avoided, since the surface tension of evaporating reagents will introduce artifacts of clumping and flattening of morphological features.

We added the following sentence to the discussion: ‘In addition, to prevent the introduction of artifacts such as clumping and flattening of morphological features, it is critical to leave just enough ethanol to cover the sample between steps during dehydration and drying’. In addition, we added more information in the context of the actual step by saying ‘while maintaining continuous immersion’ in steps 2.1.2 and 2.2.2.

Also briefly include how SEM parameters can be adjusted to avoid charging artifacts - e.g. lowering accelerating voltage, or using an alternative noise reduction algorithm (frame averaging vs pixel averaging) - or a mixture of detectors e.g. InLens with SE2 (Everhart-Thornley detector), depending on SEM manufacturer.

We modified the discussion to include these helpful suggestions, including about lowering accelerating voltage and various types of electron detectors. With respect to the suggestion about the noise reduction algorithm, we felt that a noise-reducing algorithm as suggested would not work, as charging is usually continuous and would not be able to get parts of the image to make a composite (pixel averaging) image. The section now reads:

Drying, Mounting, and Sputter Coating

During mounting when using silver conductive adhesive (also called silver paint), be careful when applying as your sample can easily be covered (buried) in the paint, thereby obscuring fine details in morphology. Sputter coating times may vary based on your sample, values given here are based on our experience. One potential negative outcome of insufficient sputter coating is a phenomenon called charging, which often appears as a white or black line (sometimes a flash) in the final image (see **Figure**

4F-G for examples). If the amount of charging observed is minimal, it may be possible to mitigate the effects by adjusting various microscope parameters, such as lowering the accelerating voltage or beam current, increasing the condenser lens strength, and/or using a smaller objective aperture. Most SEMs used for biological samples have secondary electron detectors, however some SEMs may also be equipped with backscatter detectors or environmental secondary electron detectors, all of which can be adjusted to diminish or eliminate the effects of minimal charging. If the charging effects are more pronounced, it is likely that there is poor grounding of the sample or too little sputter coating to reflect the electrons, causing the charged electrons to build up in the specimen and be spontaneously released, producing distortion in the image during acquisition. Pronounced charging is most often resolved with a thicker coating or better grounding with silver paint. Because both too much and too little sputter coating can negatively impact the quality of the image, it is best to optimize the amount and time the sample is sputter coated. As the coating cannot be removed once applied to the sample, we recommend a test run with a single sample before coating all of the samples.