Aug 21, 2018

JoVE

1 Alewife Center, Suite 200,

Cambridge, MA 02140

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 pint 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, µL, 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 OD600 < 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 flltration, 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.