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*Title:* Scanning Electron Microscopy (SEM) protocols for problematic plant, oomycete and fungal samples (JoVE55031R1)

*Authors*: Bello, M.A., Ruiz-León, Y., Sandoval-Sierra, J.V., Rezinciuc S. and Dieguez-Uribeondo, J.

Dear Editorial Board members,

We are submitting the reviewed manuscript JoVE55031R1. We would like to thank the anonymous reviewers for their valuable comments and suggestions. They helped to improve the content and the format of our paper considerably.

One of the major concerns of the reviewers was the content of sections 4, 5 and 6, describing the CPD, coating and SEM protocols. We do agree that most of the steps we had originally depend on the instrument used and could cause problems if performed in different models. We reworded those sections in order to remove instrument-specific instructions.

Additionally, the visualization part was modified and focused in one section (plants) as suggested by the editor. However, few steps from sections 2 and 3 were also included, since those include important modifications for oomycetes and fungi. We are open to modify this selection if required.

The paper was changed following the advice of the reviewers. Specific changes and explanations are detailed in the reply to each reviewer (see below in red). The changes in the manuscript are highlighted and the figures were changed directly on the TIF files.

Yours sincerely,

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Author of the manuscript, M.A. Bello optimized the protocols for plant material. Also she selected, edited and organized the figures. M.A. Bello is author of photographs of Figure 6.

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Author of the majority of images, Y. Ruiz-León has optimized the protocols for oomycetes, fungi and other samples, selected the images and reviewed the contents of the manuscript.

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**Editorial comments**

The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55031\_R1\_060916.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.  
  
1. Formatting:  
-Author names – Middle initials should be placed after the First name. Done.  
-References – Please provide DOI where available. Done.  
  
2. Grammar:  
-Line 121 – “one of the most diverse and widespread group” Corrected.  
-5.2 – “even coatings”. Corrected.  
  
3. Visualization: Protocol is highly discontinuous. In order to create a complete, linear narrative, please highlight preparation of one of the three types of samples (section 1, 2 OR 3) and make sure to highlight all steps that are essential to performing the protocol. For section 4, for example, substantial additional material should be highlighted (4.2, 4.4, 4.6-4.8, 4.10-4.12, 4.15) to form a complete narrative. Please re-evaluate the length of the protocol after adjusting the highlighting.

The visualization part is focused now in section 1 (Studies of developing and fully formed plant structures). Additionally we highlighted some parts in sections 2 (Study of cyst behaviour of *Saprolegnia* on different surfaces) and 3 (Study of herbarium fungal spores of *Phellorinia* *herculanea* under SEM) to illustrate the differences between plant, oomycetes and fungi procedures, particularly in the fixation stages. Because sections 4 (CPD treatment), 5 (coating) and 6 (SEM manipulation) are specific to the model of the instruments, we think it would be more interesting for the public to watch the samples manipulations rather than the instrument settings. However, we are open for further suggestions.

4. Additional detail is required:  
-2.1.1 – What is the composition of PG-1 and PGA? If purchased, please include in the materials table. Are the mycelia removed from the agar and placed in petri dishes lacking media? Please clarify. Clarified in section 2.1.1.

-6.2.1, 6.2.2 – How are these actions performed? Manually or via software?

The protocol for section 6 was modified following the reviewers advice. All instructions in this section should follow the manufacturer instructions. Most of the instructions detailed in 6.2.1 and 6.2.2 are performed manually or with the software, but this can change in different SEM models.   
  
5. Branding: Line 460 – thermopar. Done.  
  
6. Results: Figure 2, 3 – Scale bars are not required. Removed.

**Reviewers' comments**  
  
Editor’s Note:  We do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.

**Reviewer #1:**  
*Manuscript Summary:*  
N/A  
  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
I just have some suggestions for improvement of manuscript, as following:  
LONG ABSTRACT  
Page 2, line 74. Change the pharse: Floral meristems fixed with FAA (formalin - acetic acid - ethanol) and processed with the…Done.  
INTRODUCTION  
Page 3, lines 108, 109 ...of wood (Jansen, 1998). This reference was not included in the reference, as well as was not used the same pattern of the other references. Done.  
Page 3, line 130 - (Figure 1a, b). Done.  
Page 4, line 151 (Figure 1c, d). Done.  
PROTOCOL  
Page 4, line 173. Prepare the formalin-acetic-alcohol (FAA) fixative in. Change for  
Prepare the FAA fixative solution in Done.  
Page 5, line 198. Change - 70% alcohol by 70% ethanol. Done.  
line 198. Change put by place. Done.  
line 199. Change Fig. by Figure. Done.  
line 204. Change Put by place. Done.  
line 217. Change Fig. by Figure. Done.  
line 221. Change once by since. Done.  
line 224. Change put by place. Done.  
line 225. Change once by since. Done.  
line 228. Change put by place. Done.  
line 233. Change Fig. by Figure. Done.  
Page 6, line 255. Change fume cupboard by fume hood. Done.  
Line 275. Change once by since. Done.  
Page 7, line 302. Change Rapidly by quickly. Done.  
Page 9, lines 416 and 417. Change (Fig. 5 b,c) and (Fig. 5 d-g) by (Figure 5b, c) (Figure 5d-g). Done.  
Page 10, line 436. Change Calonge et al (2011) by Calonge 30. Done.  
Line 437. Change (Fig. 1 c,d) by (Figure 1c, d). Done.  
Line 441. Change with (b,d) by (b, d). Done.  
Line 465. Were pictures g-I captured under SEM? Please, check it. It is necessary to explain what the colors mean in these pictures. Done.  
Page 11, line 480. Change: Branched and headed hairs by Glandular and no-glandular trichomes on Done.  
  
*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #2:**  
*Manuscript Summary:*  
Dear authors,  
I was recently invited to review the manuscript JoVE55031R1 entitled "Scanning Electron Microscopy (SEM) protocols for problematic plant, oomycetes and fungi samples" and that you submitted for publication in JoVe.  
As I mentioned to the editor, I was not familiar with the format of the journal's article and based on my past experience, I think that the structure and the different sections of the text are overall well-written, and all the steps of the protocols are clearly explained. The materials needed are also relatively easy to come by and the experiments to replicate, and together with videos, I think it is perfectly suited for this journal and its format and can see myself using this paper as a reference for my class on microscopy.  
However, my main comments or concerns are the use of some terms or the description of structures, which could be done in a more accurate or consistent and coherent manner, to help the reader better connect and remember more easily the different protocols and the importance of each step, results, and corresponding figures together. Thanks to the reviewer comments included in the PDF version we were able to improve the descriptions and their consistency along the manuscript.

I also felt that, at times, the distinction on what was original and what was modified in the protocols could have been easier, and that the improvements you made to the original protocols could also be more strongly emphasized since you have good images showing the importance and benefits of fixation, dehydration, and coating. These protocols are not novel, they represent adaptations to three groups of organisms and few steps represent our original contribution. Asterisks were added to the steps were particular adaptations to the protocol are made.

For these reasons, I recommend your manuscript for publication to the editor, pending mostly minor changes. I also made more detailed comments and suggestions embedded in an electronic copy of the MS, which I think may help you better understand my decision and to revise your manuscript, but I could not find out how to upload it and asked the editor for help.  
If you have any questions or comments regarding my decision or revisions, please let me or the editor know, and I sincerely look forward to reading the revised or published version of your manuscript.  
  
Abstract and Intro: Both sections are okay, and with the exception of a few things that could be more consistent like mentioning some of the examples of your wet samples (pollen grains…) in your intro, the contents and sequences are logical and pretty straightforward, and the goals clearly explained. We initially considered as a “wet” material a wide range of samples, thinking in the origin of the sample, e.g. in the case of pollen grains the interior of a young anther, a wet stigma, etc. Samples such as those showed in Fig. 5 are processed following the standard protocol here described in sections 1.1–1.3. Because this can be confusing with the oomycetes protocol, where we manipulate suspensions, we modified the figure legend in Fig. 5.

Protocols: this part lists very clearly and thoroughly all the steps and operations, and while I found the details on the fixation and handling of the specimens justified and helpful, the sections on the CPD, Sputter Coater, and SEM seem a bit redundant with basic instructions found in their operation manuals, and may not always be relevant if the readers have a different piece of equipment. However, depending on the video contents, they would be perfectly appropriate to show all the steps from the preparation to the observation of the samples and I guess it is up to the journal to decide.

We reduced the sections 4, 5 and 6 (CPD, Sputter Coater, and SEM respectively), in order to remove irrelevant steps for the users. We indicated in these sections the conditions we are used to work with as examples.

Representative results: this part explains briefly the benefits of the protocols and the observations they allowed for each type of tissue and organisms, with proper references to the figures, and maybe could emphasize better on the new steps added to the protocols and their importance in general for preparing SEM samples. The results were reworded.

Figures: the quality and organization of the figures are very good and consistent with the text and the legends.  
Discussion: I am not familiar with the format of the paper and the absence of references in the discussion and the emphasis on the benefits of fixing and dehydrating tissues properly could have been stronger, but all the points made are valid and mostly consistent with the goals of the paper. We included some references in the discussion.  
  
*Major Concerns:*  
None  
  
*Minor Concerns:*  
See above and on attached pdf, if made available  
  
*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #3:**  
*Manuscript Summary:*  
The submitted manuscript "Scanning Electron Microscopy (SEM) protocols for problematic plant, oomycetes and fungi samples" outlines chemical fixation protocols followed by critical point drying and scanning electron microscopy of floral buds and fungal cysts and spores. These samples are difficult to fix as they are hydrated and require delicate handling and preparation for SEM.  
  
*Major Concerns:*  
1. While there is a need for adapted protocols for plant and fungal material compared to mammalian tissue, unfortunately the methodology presented in this paper is overall not novel as critical point drying has been around since the 1960s and the methods presented are not innovative or gold-standard for this field since the development of environmental microscopes.

We are aware of both, that we are not proposing a novel methodology and the development of the environmental microscopes. The JoVE editors do not require in depth or novel results for publication in this journal, only representative results that demonstrate the efficacy of the protocol. Although hundreds of studies using the SEM in plants, oomycetes and fungi are published, the procedures are almost always barely explained and limited to instruments and reagent suppliers due to the usual editorial policies. When we look into the JoVE format, we thought that finally we could have a publication detailing our updated SEM protocols with the organisms we work with, together with a visual aid. This is in itself the innovation, the format how a routine protocol adapted to our organisms is delivered to the audience. We visualized this manuscript as a guide for laboratories where it can be helpful, rather than a gold-standard for SEM technology.

It is true that our protocols are useless for environmental microscopes. However, they could have an impact in most of the studies involving electron microscopy because the SEM associated techniques are more widespread and accessible to many laboratories. Looking through Scopus, the largest database of peer-reviewed literature, the number of published studies with SEM from 2011 to 2016 (plants 4914, oomycetes 21 and fungi 2490) exceed with difference those performed with ESEM (plants 337, oomycetes 1 and fungi 250).

2. FAA is not the best fixative for microscopy as the methanol in formalin prevents optimal penetration of the tissue and slows fixation. Formaldehyde under inert gas, freshly prepared paraformaldehyde or glutaraldehyde are the standard fixatives used in microscopy.

According to Johansen (1940)1 and Feder and O’ Brien (1968)2, most botanical material can be fixed in FAA for morphological studies because of the rapid penetration of the material by the formaldehyde, which at the same time reacts slowly to the tissue components reducing the damaging effects of subsequent dehydration and inclusion. In the case of the glutaraldehyde, Feder and O’Brien (1968) claim that this fixative penetrates more slowly in the material causing cellular shrinking and plasmolisis. However, Ruzin (1999)3 states that glutaraldehyde is an excellent fixative when combined to plastic resins. In our experience with plant material for SEM examination, FAA is an appropriate fixative.

Of concern is the claim in the abstract that this is a "non-toxic" method when they use Formalin-Acetic-Alcohol (FAA) to fix their samples and also glutaraldehyde. These are known toxins and have multiple hazard classes associated with their MSDSs. I am uneasy about encouraging any use of FAA out of the fume hood (Page 4, Lines 166-168) even with a face mask as the vapours from the fixatives can lead to fixation of the eyeball and other mucous membranes. Beyond these safety and quality issues, there is a valid point in being able to use museum specimens that have been fixed in FAA for long term storage for SEM analysis where appropriate.

We acknowledge the reviewer this important comment about FAA safety. We eliminated the words “non-toxic” and also included an additional step with 70% ethanol (1.1.1) when there is not a fume cupboard available for the fixation. Also we rephrased the sentences were we claimed FAA was harmless.

3. As written, the paper does not flow easily from the introduction to the discussion.  
For example, the use of multiple types of specimen holders for samples in suspension was interesting and may be of use for other samples in suspension. However, it was a bit unclear from the text why the experiment was being done. A second image showing a different holder giving a better result than glass could be useful for comparing the sample holders.

The aim of testing several substrates was to compare the differential growth of the spines of the *Saprolegnia* cysts on different surfaces. This is important for identification purposes because the same species can display different spine morphologies (Fig. 6) depending on the substrate where they are growing. The glass substrate was “ideal” from the researcher's perspective, because the elongated and perfectly looped spines were relatively easier for counting and characterizing.

The study of herbarium fungal spores follows a protocol of rehydration followed by dehydration. Was there a specific reason why the samples were rehydrated and then dehydrated when the samples were already dehydrated?

We rehydrated the originally dehydrated sample to be able to see the NURSE CELLS. Interestingly the spores of *Phellorinia* (Agaricales) do not require this rehydration step, ethanol dehydration or the critical point drier for characterization under the SEM. This is important to know since in other organisms such as myxomycetes4 the spores need ethanol dehydration and the CPD treatment for their observation with SEM.

4. For sections 4 (Use of the Critical Point Dryer) and 5 (Use of the Scanning Electron Microscope) the method for these instruments is outlined. These are sophisticated instruments that vary in their use depending on the manufacturer and the model. A generic protocol as outlined in the paper may cause more problems than a simple referral to the manufacturer's guidelines and these sections should be removed. Tips on sample mounting for the CPD and getting the optimal image for these types of samples under the SEM could be useful.  
  
*Minor Concerns:*  
Abstract  
Page 2, Line 60. The word "microphotography" could be replaced with micrography or something like sample preparation of difficult samples.Done *Page 2, Line 62. FAA is included but not the glutaraldehyde used in section 2. Please include glutaraldehyde in the abstract.* Glutaraldehyde is included now in the abstract and in table 1*.*

*Page 2, Line 66. Change "i.e." for "e.g." as diagnostic characteristics will vary depending on the sample.* Done *Page 2, Line 71. Remove "non-toxic". This is not a "non-toxic" method.* Removed *Page 2, Line 72. Dynamic processes are difficult to infer from fixed samples. Please attenuate the language.* Done *Introduction*Overall the introduction is a repetitive and could be tightened up.  
Protocols:  
Protocol 1.  
Page 4, Line 153. Replace "adult" with fully formed. Done  
Page 4, Lines 166-168. Remove "If this step is *glutaraldehyde* without a fume hood, do it in an open area using a face mask and keeping the containers closed when not in use." This is potentially a dangerous suggestion. Removed  
Page 4, Line 170. Usually samples are washed three times in the new solution (in this case 70% ethanol). This would be recommended to remove any residual FAA. Included in sección 1.1.4  
Page 5, Line 203. "Avoid undesirable particles" - Do you mean dust? Please explain further. Yes, we ment dust   
Protocol 2.  
Page 5, Line 228. Please clarify that the secondary cyst suspension comes from the sporulation step in section 2.1.1.

Yes they are. Sections 2.1.1 and 2.1.2 were rephrased in order to clarify the initial treatment of these samples.

Page 5, Line 229. It is unclear why we need a selection. This is not outlined in the abstract or introduction, neither is the use of glutaraldehyde.

We used different substrates in order to test if it was a differential growth of the spines, and if so, how this growth was in each case. The use of different substrates is outlined in the long abstract (line 75 page 2) and the introduction (lines 147-149, page 3).   
Protocol 3.  
Page 6, Line 255. Why would you rehydrate before dehydrating already dehydrated spores?

The initial rehydration of the tissue (step 3.1.1) is to visualize the NURSE CELLS. Originally taxonomists were focused in spore ornamentation, so the rehydration process for surrounding cells was ignored for these organisms. This initial rehydration step allows us to visualize the otherwise collapsed nurse cells.

Page 6, Line 261. Is there a reference for rehydrating fungal spores using the microwave? Are these spores potentially viable?

No it isn’t a previous reference. We haven’t tried the viability of the spores after the microwave rehydration, but as you can appreciate in fig 1d, the spore wall ornamentation is maintained.

Protocol 4.  
Remove the steps for the use of the CPD as each instrument is different depending on the manufacturer and model and not following the manufacturer's guidelines may lead to hazardous situations.  
Sample preparation and handling for the CPD is useful for this section.

Protocol 5.  
Again, remove the steps for the use of the sputter coater as each instrument is different depending on the manufacturer and model and it may lead to hazardous situations.  
Coating with gold and using a rotary stage to ensure even coatings on the specimens is a good suggestion.

Protocol 6.  
And again, remove the steps for the use of the SEM. These instruments can be very sophisticated and major (expensive) damage can occur if used inappropriately.  
Methods for optimising imaging conditions can be suggested such as slight tilting of the stage (as long as the sample is far away from the pole piece) or use of different kVs. While the use of the back scatter detector (Page 9, line 412) is appropriate, in some instruments, this requires the insertion of the correct detector (by an approved person) prior to loading the sample and is not always a quick and easy way of changing the imaging conditions.

Following the manufacturers guidelines, our SEM allows us a relatively easy interchange between the back scatter detector and the secondary electrons detector (SE). Although sometimes the resultant image has not the best quality, this change provides more information than a bad quality image taken with the SE. It is worth for unique but bad preserved samples. We rephrased the paragraph.

Representative Results  
Page 10, Lines 422-423. "Wet surfaces, which can be overcharged with the electron current if badly dehydrated and coated, can also be well preserved". I thought this was the point of the paper.

These samples used to be difficult to handle for image capture under SEM. An adequate combined treatment of fixation, dehidration and CPD allows better results. We rephrased the paragraph.

Page 10, Lines 430-436. Are these results comparable to other samples that have been observed or is there an alternative sample where a different holder gives a more optimal result?

The protocol for observation of cysts under the SEM has been tested in *Achlya, Aphanomyces, Dyctyuchus, Leptolegnia, Protoachlya* (Saprolegniales), *Lagenidium giganteum* (Lagenidiales), *Phytophthora palmivora* and *Pythium aphanidermatum* (Peronosporales). However, *Saprolegnia* is the only genus where there are cysts with growing spines (Fig. 6). Structures similar to the spines of *Saprolegnia* occur in *Dyctyuchus*, but in this case the structures don’t display differential growth on different substrates.

Figures  
Figure 1.  
Page 10, Line 449. Figure 1a) bud without treatment is as expected awful as is c) compared to d). Why does this need to be shown as this is the reason that CPD has been used since the 1960s?

We think it is good idea to show the samples processed with and without the proposed method in order to compare the results. In the particular case of the spores of this species (fig. 1c-d) the spores were observed without treatment and it was fine for taxonomists because the spore details were observed. However, with the here described protocol the surrounding cells were recovered, providing more information.

Figure 4.  
Fig. 4g) is not an SEM image. Please adjust the figure legend. Done  
Fig. 4h- i) Please remove the pseudo-colouring or put the non-pseudo-coloured image in beside the coloured image. It is difficult to see the nuances in the grayscale when this is done. Done  
Page 11, Line 481. Replace microphotographs with micrographs. Done.  
Page 11, Line 483-484. Pollen is not usually a structure from a wet microenvironment.

Pollen grains are difficult samples, particularly when they are attached to wet stigmatic surfaces. Inside the anthers, young stages of pollen development also occur under wet microenvironment. Nevertheless, we changed the heading of Fig. 4.

Figure 6.  
Images are pixelated and pseudo-coloured. Remove the pseudocolouring and provide higher quality images. Color was removed and the photograph resolution improved.

Discussion  
Page 11, Line 499. This is not a 'harmless' method. It is also only easy to follow if you have had appropriate and thorough training on the use of the CPD and SEM.

We do agree that any instrument for sample preparation requires certain training and a proper laboratory safety control. In several laboratories there are technitians in charge of the security and maintainance of these type of instruments, so they can provide the users the safety guidelines according to each institution. Once our protocol is optimized, it is relatively easy to follow. We reworded the first paragraph of the discussion mentioning safety with the CPD and the SEM and removed the adjective "harmless".

Page 11, Lines 501-502. These reagents are toxic enough to remove this whole point from the manuscript.

This is a method with evident reagent toxicity, but it is relatively less toxic than other protocols such as the one involving osmium tetroxide. The phrase was changed in the discussion.

Page 12, Line 535. This method cannot be used to look at 'dynamic' processes, just snapshots of differences between time points.

We do agree. This method provides the researcher the snapshots to reconstruct stages of structural growth rather than dynamic physiological processes. This part was reworded.

References cited in this reply:

1 Johansen, D.A. *Plant microtechnique*. McGrow-Hill, New York (1940).

2 Feder, N., O’Brien, T.P. *Plant microtechnique*: some principles and new methods. American Journal of Botany **55**, 123–142 (1968).

3 Ruzin, S.E., 1999. *Plant Microtechnique and Microscopy*. Oxford University Press, New York.

4 Wrigley de Basanta, D., Lado, C., García, J.M., Estrada-Torres, A. *Didymium xerophilum*, a new myxomycete from the tropical Andes. Mycologia **107**(1), 157-168, DOI: 10.3852/14-058 (2015).