Chapter 6

Specimen Preparation and Observations of *Magnaporthe oryzae* Appressorial Cells Under Electron Microscopy

Raquel O. Rocha and Richard A. Wilson

Abstract

Electron microscopy (EM) allows characterization of the morphology and ultrastructure of a cell. However, challenges concerning cryo sample fixation are still one of the main roadblocks to its widespread adoption. In this protocol, we describe two alternative EM preparation methods employed to study *Magnaporthe oryzae* appressoria on artificial hydrophobic surfaces.

Key words Electron microscopy, Sample preparation, Surfaces, SEM, TEM, Imaging, Appressorium, *Magnaporthe oryzae*

1 Introduction

The rice blast pathosystem has emerged as a leading model for understanding plant-pathogen interactions due to advances in molecular genetics combined with cell imaging of both the pathogen and the host [1, 2]. The development of powerful new microscopic technologies and techniques, particularly those based on live-cell imaging and the use of fluorescent probes [3], has significantly enhanced our understanding of *Magnaporthe oryzae* physiology and infection-related development both on and in the host plant cells [4–10]. Incorporating new microscopic techniques into our workflow will continue to be critical to efforts aimed at decoding the links between the *M. oryzae* genome and cellular structure and functioning.

Among the leading technologies for cellular characterization, electron microscopy (EM) has grown to be an essential tool for characterizing the morphology and ultrastructure of a cell. By using accelerated free electrons, with shorter wavelengths than visible light, EM can generate better high-resolution images of smaller structures than light microscopy [11, 12]. Over time, access to electron instrumentation has increased to broader academic

Stefan Jacob (ed.), *Magnaporthe oryzae: Methods and Protocols*, Methods in Molecular Biology, vol. 2356,
https://doi.org/10.1007/978-1-0716-1613-0_6,

environments. Unfortunately, cryo sample fixation remains the biggest challenge to the use of electron microscopes. Fixation protocols relying on freezing samples are considered to be of the highest quality, generating fewer artifacts. However, specific equipment is required to prepare, maintain, and image cryofixated biological samples [13].

Here, we provide cost-effective alternative approaches to cryofixation that are suitable for rapidly preparing samples of *M. oryzae* appressoria for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis. Because appressorial morphogenesis from spores relies on sensing and attachment to hard hydrophobic surfaces, sample preparation of this particular stage needs to permit the removal of the appressorial structure from the hard surface while limiting alterations to its three-dimensional structure due to handling.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents.

2.1 SEM Analysis

2.1.1 Hydrophobic Surfaces for Appressoria Formation

1. Cellophane membranes (*see* **Note 1**).
2. Glass coverslips.

2.1.2 SEM Sample Preparation

1. Water-agar medium, 3% agar (w/v) in water.
2. Bath sonicator.

2.2 TEM Analysis

2.2.1 Hydrophobic Surfaces for Appressoria Formation

1. Glass coverslips.
2. Sof Sole® Water Proofer (*see* **Note 2**).

2.2.2 TEM Sample Preparation

1. 4% agarose in water (melt agarose and keep it at 40 °C until use).
2. 2.5% glutaraldehyde fixative in water. Glutaraldehyde fixatives are prepared from EM grade 25% glutaraldehyde, found in sealed ampoules.
3. 2% osmium tetroxide in water. Prepared from EM grade osmium obtained in sealed glass ampoules.
4. 30%, 50%, 75%, 95%, and 100% ethanol in water.

5. Low Viscosity Embedding Media Spurr's Kit (Electron Microscopy Sciences, Inc., USA).
6. Leica EM UC7 Ultramicrotome.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 SEM Sample Preparation

1. Suspend 3 g of agar in 100 mL distilled water. Heat to boiling to dissolve the agar completely. Sterilize the medium by autoclaving.
2. Place a layer (no more than 10 mm thick) of liquid water-agar on top of the glass coverslip. Wait until medium solidifies.
3. Gently lay the cellophane membrane on top of the water-agar (Fig. 1) (*see* **Note 1**). Make sure that the full extension of the membrane is in contact with the medium below.
4. Inoculate the spore suspension of *M. oryzae* (1×10^5 spores/mL) on top of the cellophane membrane. Leave the spore-cellophane-agar-coverslip assembly supported in a Petri dish containing a wet filter paper. Allow the appressoria to form for 24 h in the dark at 24 °C.
5. After the incubation time, take the spore-cellophane assembly and sonicate it for 30 min (*see* **Note 3**). Be careful to maintain the orientation of the cellophane membrane with the appressoria uppermost.
6. After sonication, carefully transfer the cellophane membrane and place it on a Petri dish, the appressoria-containing surface facing up. Leave the membrane to air dry for up to 24 h.
7. Place samples on a double-sided adhesive conductive tab on an aluminum SEM sample-mounting stub.
8. Sputter coat samples with chromium (~5 nm thick) using a Denton Vacuum Desk V sputter coater. The samples are now ready for imaging (Fig. 2).

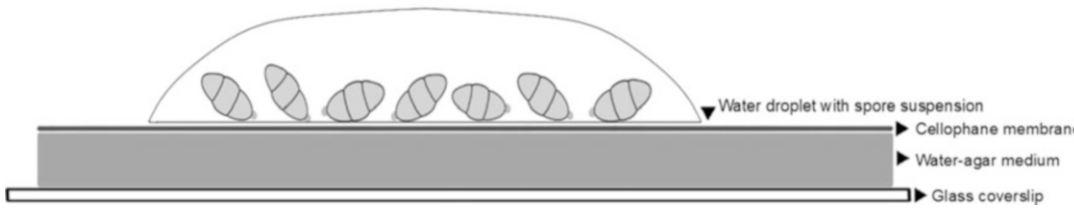

Fig. 1 Schematic representation of sample assembly prior to SEM preparation and analysis. Spores of *M. oryzae* are inoculated on top of the cellophane membrane and allowed to form appressoria

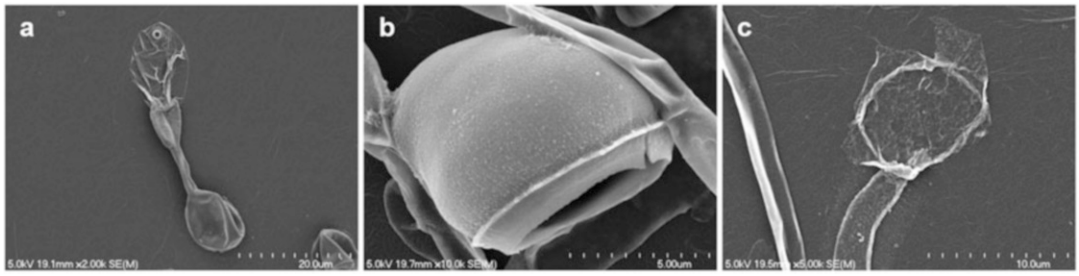

Fig. 2 SEM images were taken after following the described protocol. (**a**) Spore and appressorium attached to the cellophane membrane. (**b**) Appressorium detached from the cellophane membrane with visible mucilage ring and unmelanized pore. (**c**) The imprint of an appressorium formed on the surface of cellophane. Note the attachment of both appressorium and germ tube

3.2 TEM Sample Preparation

1. Spray glass coverslips with Sof Sole® Water Proofer spray. Leave the coverslips to air dry for 48 h.
2. Inoculate the spore suspension of *M. oryzae* (1×10^5 spores/mL) on the top of the coverslip. Leave the spore coverslip assembly supported in a Petri dish containing a wet filter paper. Allow the appressoria to form for 24 h in the dark at 24 °C.
3. After the incubation time, dry out the water droplet on the top of the coverslip with sterile filter paper. Dry the water droplet by its side to reduce interferences with appressoria surroundings.
4. Add 450 μL of the 2.5% glutaraldehyde fixative. Fix the samples for 1 h at room temperature (*see* **Note 4**).
5. Remove the glutaraldehyde solution by pipetting and drying out the remaining liquid with sterile filter paper.
6. Add 150 μL of 4% agarose (melted and at 40 °C) to the drop site. Wait until agarose is completely solidified.
7. Scrape off the coverslip using a sterile razor blade to remove appressoria enrobed with agarose (*see* **Note 5**).
8. Cut the agarose, as displayed in Fig. 3. Transfer appressoria-agarose blocks to 1.5 mL tube.
9. Add 1 mL of 2% osmium tetroxide fixative and incubate at room temperature for 2 h.
10. Properly discard the osmium tetroxide solution and wash the blocks with de-ionized water three times.
11. Dehydrate the block with washes in 1.5 mL of ethanol in increasing concentrations of 30%, 50%, 75%, 95%, and 100%, 10 min each.

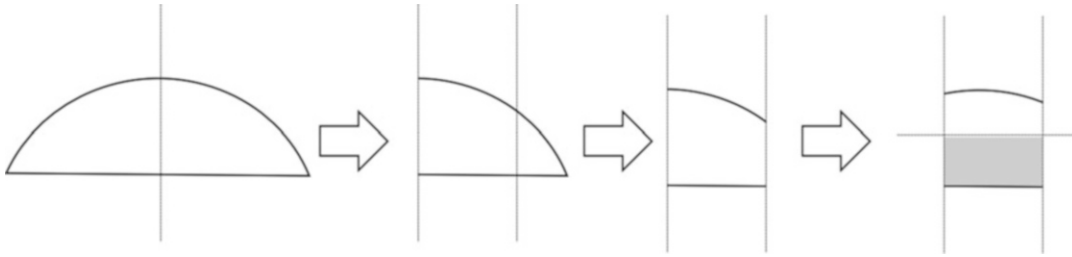

Fig. 3 Schematic representation of how the solidified agarose droplet should be cut before osmium tetroxide fixation. The appressoria should be located only at the base of the agarose droplet, and thus, only this portion should be carried forward to further experimental steps

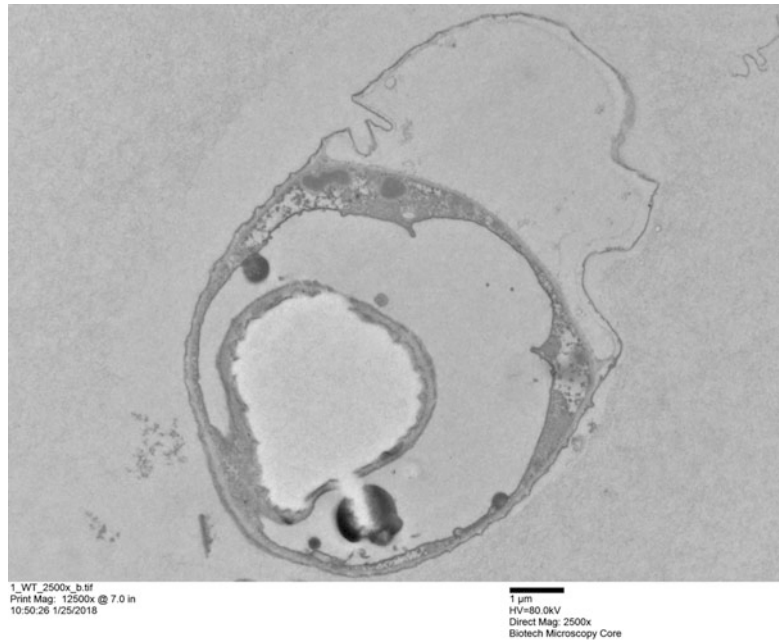

Fig. 4 TEM image of an appressorium taken after following the described protocol. Note the appressorial pore in the middle of the structure

12. Samples are embedded in blocks using Low Viscosity Embedding Media Spurr's Kit (Electron Microscopy Sciences, Inc., USA).
13. 100 nm thick sections are cut on an Ultramicrotome. Stain sections using uranyl acetate and lead citrate. Samples are now ready for imaging (Fig. 4).

4 Notes

1. Cellophane is a flexible surface that can be placed on top of the sample-mounting stub, which reduces the error generated by extra sample handling. However, pre-shape the size of the cellophane to not exceed the limits of the stub.
2. The use of waterproof spray creates a temporary hydrophobic surface on a glass coverslip. Appressoria are able to form in contact with the sprayed layer and are easily removed by scraping with a razor blade.
3. In order to visualize the pore at the base of the appressorium and/or the puncture generated by the penetration peg on the cellophane membrane, 30 min of sonication is the preferred method to detach appressoria from the cellophane surface without forming excessive artifacts.
4. After 1-h fixation in 2.5% glutaraldehyde at room temperature, the assembly can be transferred to a cold room until use for the next steps.
5. Agarose plays the role of a soft holder of the appressoria. The agarose protects the structures from handling-induced damages and keeps the appressoria evenly distributed throughout the agarose, without creating clusters of appressoria, which impede the location and distinction of the specimens during imaging.

Acknowledgments

Work in the Wilson lab is supported by the National Science Foundation (IOS-1758805).

References

1. Wilson RA, Talbot NJ (2009) Under pressure: investigating the biology of plant infection by *Magnaporthe oryzae*. Nat Rev Microbiol 7:185–195
2. Yan X, Talbot NJ (2016) Investigating the cell biology of plant infection by the rice blast fungus *Magnaporthe oryzae*. Curr Opin Microbiol 34:147–153
3. Hickey PC, Swift SR, Roca MG, Read ND (2004) Live-cell imaging of filamentous fungi using vital fluorescent dyes and confocal microscopy. Method Microbiol 34:63–87
4. Kankanala P, Czymmek K, Valent B (2007) Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. Plant Cell 19:706–724
5. Khang CH, Berruyer R, Giraldo MC, Kankanala P, Park SY, Czymmek K, Kang S, Valent B (2010) Translocation of *Magnaporthe oryzae* effectors into rice cells and their subsequent cell-to-cell movement. Plant Cell 22:1388–1403
6. Giraldo MC, Dagdas YF, Gupta YK, Mentlak TA, Yi M, Martinez-Rocha AL, Saitoh H, Terauchi R, Talbot NJ, Valent B (2013) Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*. Nat Commun 4:1996

7. Marroquin-Guzman M, Hartline D, Wright JD, Elowsky C, Bourret TJ, Wilson RA (2017) The *Magnaporthe oryzae* nitrooxidative stress response suppresses rice innate immunity during blast disease. Nat Microbiol 2:17054
8. Marroquin-Guzman M, Sun G, Wilson RA (2017) Glucose-*ABL1*-TOR signaling modulates cell cycle tuning to control terminal appressorial cell differentiation. PLoS Genet 13:e1006557
9. Sun G, Elowsky C, Li G, Wilson RA (2018) TOR-autophagy branch signaling via Imp1 dictates plant-microbe biotrophic interface longevity. PLoS Genet 14:e1007814
10. Li G, Qi X, Sun G, Rocha RO, Segal LM, Downey KS, Wright JD, Wilson RA (2020) Terminating rice innate immunity induction requires a network of antagonistic and redox-responsive E3 ubiquitin ligases targeting a fungal sirtuin. New Phytol 226:523–540
11. Nayfeh M (2018) Seeing atoms and clusters on surfaces. In: Micro and nano technologies, fundamentals and applications of nano silicon in plasmonics and fullerines. Elsevier, Amsterdam, pp 59–87
12. Corradini MG, DJ MC (2019) Microscopy/Food Applications. In: In Encyclopedia of Analytical Science, 3rd edn. Academic Press, Cambridge, pp 47–56
13. Hand E (2020) Cheap shots. Science 367: 354–358