Journal of Visualized Experiments

Complementary Use of Microscopic Techniques and Fluorescence Reading in Studying Cryptococcus-Amoeba Interactions

--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE58698R4
Full Title:	Complementary Use of Microscopic Techniques and Fluorescence Reading in Studying Cryptococcus-Amoeba Interactions
Keywords:	Amoeba, Cryptococcus, Fluorescence, Interactions, Model, Microscopy, Phagocytosis.
Corresponding Author:	Olihile M. Sebolai University of the Free State Bloemfontein, SOUTH AFRICA SOUTH AFRICA
Corresponding Author's Institution:	University of the Free State
Corresponding Author E-Mail:	SebolaiOM@ufs.ac.za
Order of Authors:	Uju L. Madu
	Olihile M. Sebolai
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA
UFS UV

Tuesday, 23 April 2019

Dr Jaydev Upponi, Ph.D.
Science Editor: JoVE, Immunology and Infection

Dear Dr Upponi,

I have just uploaded a file of our revised manuscript i.e. 58698_R4, entitled "Complementary Use of Microscopic Techniques and Fluorescence Reading in Studying *Cryptococcus*-Amoeba Interactions" by Madu and Sebolai, for your consideration. All authors approved the final version of the submitted manuscript.

Future correspondence should be addressed to Dr. Olihile M. Sebolai (+2751 401 2004; sebolaiom@ufs.ac.za). I hope you will find all to be in order.

With sincere appreciation,

O M Sebolai

Dr Olihile M. Sebolai (Ph.D.)
Corresponding author
Senior Lecturer and Fulbright Fellow

205 Nelson Mandela Drive/Rylaan
Park West/Parkwes
Bloemfontein 9301
South Africa/Suid-Afrika

P.O. Box/Posbus 339
Bloemfontein 9300
South Africa/Suid-Afrika
T: +27(0)51 401 9111
www.ufs.ac.za

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

TITLE:

Complementary Use of Microscopic Techniques and Fluorescence Reading in Studying *Cryptococcus*-Amoeba Interactions

AUTHORS AND AFFILIATION:

Uju L. Madu[1], Olihile M. Sebolai[1]

[1]Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Park West, Bloemfontein, South Africa

Corresponding Author:

Olihile M. Sebolai (sebolaiom@ufs.ac.za)

Email Address of Co-Author:

Uju L. Madu (lynda.madu@yahoo.com)

KEYWORDS:

amoeba, *Cryptococcus*, fluorescence, interactions, microscopy, model, phagocytosis

SUMMARY:

This paper details a protocol for preparing a co-culture of cryptococcal cells and amoebae that is studied using still, fluorescent images and high-resolution transmission electron microscope images. Illustrated here is how quantitative data can complement such qualitative information.

ABSTRACT:

To simulate *Cryptococcus* infection, amoeba, which is the natural predator of cryptococcal cells in the environment, can be used as a model for macrophages. This predatory organism, similar to macrophages, employs phagocytosis to kill internalized cells. With the aid of a confocal laser-scanning microscope, images depicting interactive moments between cryptococcal cells and amoeba are captured. The resolution power of the electron microscope also helps to reveal the ultrastructural detail of cryptococcal cells when trapped inside the amoeba food vacuole. Since phagocytosis is a continuous process, quantitative data is then integrated in the analysis to explain what happens at the timepoint when an image is captured. To be specific, relative fluorescence units are read in order to quantify the efficiency of amoeba in internalizing cryptococcal cells. For this purpose, cryptococcal cells are stained with a dye that makes them fluoresce once trapped inside the acidic environment of the food vacuole. When used together, information gathered through such techniques can provide critical information to help draw conclusions on the behavior and fate of cells when internalized by amoeba and, possibly, by other phagocytic cells.

INTRODUCTION:

Microbes have evolved over time to occupy and thrive in different ecological niches such as the open physical boundaries of the soil and water, among others[1]. In these niches, microbes often engage in the direct competition for limited resources; importantly, for nutrients that they use

for supporting their growth or space, which they need to accommodate the expanding population[2,3]. In certain instances, some holozoic organisms like amoeba may even predate on cryptococcal cells as a way of extracting nutrients from their biomass[4,5]. In turn, this allows such organisms to establish territorial dominance via controlling the population numbers of its prey. Because of this predatory pressure, some prey may be selected to produce microbial factors, such as the cryptococcal capsule[6], to reconcile the negative effects of the pressure. However, as an unintended consequence of this pressure, some microbes acquire factors that allow them to cross the species barrier and seek out new niches to colonize[7], like the confined spaces of the human body that are rich in nutrients and have ideal conditions. The latter may explain how a terrestrial microbe like *Cryptococcus* (*C.*) *neoformans* can transform to become pathogenic.

To this end, it is important to study the initial contact that cryptococcal cells may have with amoeba and how this may select them to become pathogenic. More specifically, this may give clues on how cryptococcal cells behave when acted upon by macrophages during infection. It is for this reason that amoeba was chosen as a model for macrophages here, as it is relatively cheap and easy to maintain a culture of amoeba in a laboratory[8]. Of interest was to also examine how cryptococcal secondary metabolites viz. 3-hydroxy fatty acids[9,10] influence the interaction between amoebae and cryptococcal cells.

A simple way of perceiving the interaction between amoeba and its prey with the naked eye is to create a lawn using its prey on the surface of an agar plate and spot amoeba. The visualization of plaques or clear zones on the agar plate depicts areas where amoeba may have fed on its prey. However, at this macro level, only the outcome of the process is noted, and the process of phagocytosis is mechanized cannot be observed. Therefore, to appreciate the process on a cell-to-cell basis, there are several microscopic methods that can be used[11,12]. For example, an inverted microscope with an incubation chamber can be used to video record a time-lapse of events between a phagocytic cell and its target[13]. Unfortunately, due to the cost of a microscope with a time-lapse functionality, it is not always possible for laboratories to purchase such a microscope, especially in resource poor-settings.

To circumvent the above limitation, this study presents a sequential exploratory design that evaluates the interaction of *C. neoformans* viz *C. neoformans* UOFS Y-1378 and *C. neoformans* LMPE 046 with *Acanthamoeba castellani*. First, a qualitative method is used that precedes a quantitative method. Still images are captured using an inverted fluorescence microscope, as well as a transmission electron microscope to depict amoeba-*Cryptococcus* interactions. This was followed by quantifying fluorescence using a plate reader to estimate the efficiency of amoeba to internalize cryptococcal cells. When reconciling findings from these methods during the data-interpretation stage, this may equally reveal as much critical information as perusing a phagocytosis time-lapse video.

PROTOCOL:

Cryptococcus neoformans and some *Acanthamoeba castellanii* strains are regarded as biosafety level-2 (BSL-2) pathogens; thus, researchers must take proper precautions when working with

these organisms. For example, laboratory personnel should have specific training and personal protective equipment (PPE) such as lab coats, gloves, and eye protection. A biological safety cabinet (level-2) should be used for procedures that can cause infection[14].

1. Cultivation and standardization of fungal cells (modified from Madu et al.[15])

1.1. Streak out the test fungal strains (i.e., *C. neoformans* UOFS Y-1378 and *C. neoformans* LMPE 046) from stock cultures (no older than 9 months) on yeast-peptone-dextrose (YPD) agar plates. Information on YPD agar's ingredients can be found in **Table 1.**

NOTE: *C. neoformans* UOFS Y-1378 has been shown to produce 3-hydroxy fatty acids, while *C. neoformans* LMPE 046 does not produce 3-hydroxy fatty acids. Refer to **Supplemental File 1** for information on how the presence of these molecules is determined.

1.2. Incubate the agar plates for 48 h at 30 °C.

NOTE: A plate can be stored for up to 2 months at 4 °C before it can be discarded or used to make a stock culture[16].

1.3. Scrape off a loopful of cryptococcal cells (*C. neoformans* UOFS Y-1378 or *C. neoformans* LMPE 046) from the 48 h-old plate and inoculate into a 250 mL conical flask containing 100 mL of the chemically-defined YNB broth (6.7 g/L) supplemented with 4% (w/v) glucose. Information on YNB broth's ingredients can be found in **Table 2**.

1.4. Incubate the flasks at 30 °C for 24 h while agitating at 160 rpm on a rotary shaker.

1.5. After a 24 h incubation period, count the fungal cells using a hemocytometer and adjust the cell number to 1 x 10^6 cells/mL with PBS at pH 7.4.

NOTE: The prepared *C. neoformans* UOFS Y-1378 inoculum was used in steps 3.1 and 3.2, while *C. neoformans* LMPE 046 inoculum was only used in step 3.2.

2. Cultivation and standardization of amoeba cells (modified from Madu et al.[15])

2.1. Thaw a stock culture of *Acanthamoeba castellanii* and bring it to room temperature (RT).

NOTE: Amoeba was prepared based on the modified protocols of Axelsson-Olsson et al.[8] and Schuster[17].

2.2. Pipette 1 mL of the thawed culture and inoculate it into a 50 mL centrifuge tube containing 15 mL of ATCC medium 712. Information on ATCC medium 712's ingredients can be found in **Table 3**.

2.3. Manually shake it gently and immediately centrifuge for 5 min at 400 x *g* and 30 °C.

2.4. Aspirate the supernatant.

2.5. Resuspend the cells in 15 mL of ATCC medium 712 and incubate the tube at 30 °C for 14 days.

NOTE: Periodically check the cells, using a simple light microscope, to determine if they are in a trophozoite state. Once they are in a trophozoite state, start a fresh culture.

2.6. Pipette 1 mL from a culture that shows cells in a trophozoite state and use it to inoculate a sterile 50 mL centrifuge tube containing 15 mL of fresh, sterile ATCC medium 712.

2.7. Incubate the tube at 30 °C for 1 week while agitating at 160 rpm on a rotary shaker.

2.8. After a week, count the amoeba cells using a hemocytometer and adjust the cell number to 1 x 10^7 cells/mL with fresh, sterile ATCC medium 712.

2.9. Perform a viability assay using a trypan blue stain as detailed by Strober[18]. Proceed further with the cultures that show at least 80% viability.

3. Fluorescence staining of cells to study phagocytosis (modified from Madu et al.[15])

3.1. Gathering qualitative data through use of fluorescence microscope

NOTE: Perform this assay with *Acanthamoeba castellanii* and *C. neoformans* UOFS Y-1378.

3.1.1. Dispense a 200-µL suspension of standardized amoebae (1 x 10^7 cells/mL in ATCC medium 712) into chamber wells of an adherent slide and incubate for 2 h at 30 °C for cells to adhere to the surface.

3.1.2. While amoeba cells are settling down to adhere, stain the standardized *C. neoformans* UOFS Y-1378 cells that were adjusted to 1 x 10^6 cells/mL (in 999 µL of PBS) with 1 µL of fluorescein isothiocyanate in a 1.5 mL plastic tube.

NOTE: Prepare the stain by dissolving 1 mg of fluorescein isothiocyanate in 1 mL of acetone.

3.1.3. Gently agitate the *C. neoformans* UOFS Y-1378 cells on an orbital shaker set at 50 rpm for 2 h at RT and in the dark.

3.1.4. After 2 h, centrifuge at 960 x *g* for 5 min at 30 °C to pellet the cells.

3.1.5 Aspirate the supernatant to remove the PBS with the stain.

3.1.6. Add 1 mL of PBS to the tube for washing the cell pellet. Wash the cells by gently pipetting.

3.1.8. Centrifuge the cells at 960 x *g* for 5 min at 30 °C. Discard the supernatant. Repeat the washing step one more time.

3.1.9. Resuspend the washed cells in 1 mL of PBS.

3.1.10. Dispense a 200 µL suspension of the stained *C. neoformans* UOFS Y-1378 cells to chamber wells containing the unstained amoeba cells.

3.1.11. Incubate the prepared co-culture at 30 °C for an additional 2 h period.

NOTE: The co-culture can be incubated for different time points to suit the purpose of the experiment.

3.1.12. At the end of the co-incubation period, aspirate the contents of the wells.

3.1.13. Add 300 µL of PBS to the wells to wash the chamber wells and to remove any unbound co-cultured cells. Do this by gentle pipetting. Aspirate the contents of the wells. Repeat the washing step one more time.

3.1.14. Prepare the 3% glutaraldehyde solution by adding 3 mL of glutaraldehyde to 97 mL of distilled water.

3.1.15. Fix the co-cultured cells by adding 250 µL of 3% solution to the chamber wells and incubating for 1 h.

3.1.17. Aspirate the fixative and wash the chamber wells as detailed from step 3.1.13.

3.1.18. Dismantle the chamber wells using a tool that was provided with the chamber slides.

3.1.19. Add a drop of the antifade compound, 1,4-diazabicyclo-[2.2.2]-octane to the slide to prevent auto-bleaching. Cover with a coverslip and seal the sides with a nail polish to prevent evaporation.

3.1.20. View the co-cultured cells using the 100x objective lens (with oil) of a confocal laser-scanning microscope.

NOTE: It is important to take pictures in bright-field and fluorescence to view interaction between amoeba and cryptococcal cells. Wherever possible, the fluorescence can be super-imposed onto the bright-field images. Amoeba cells are typically larger in size (i.e., 45–60 µm), and the trophozoite cells have an irregular shape. Cryptococcal cells are 5–10 µm in diameter and have a globose to ovoid shape. When exposed to a laser, it is possible that unstained

amoeba cells may emit auto-fluorescence. Refer to Beisker and Dolbeare[19] and Clancy and Cauller[20] for methods to reduce autofluorescence.

3.2. Acquiring quantitative data through use of fluorescence plate reader

NOTE: Perform this assay with *Acanthamoeba castellanii* and *C. neoformans* UOFS Y-1378 or *C. neoformans* LMPE 046.

3.2.1. Dispense a 100 µL suspension of standardized amoebae (adjusted to 1 x 10^7 cells/mL in ATCC medium 712) into a black, adherent 96 well microtiter plate.

3.2.2. Incubate the plate for 2 h at 30 °C to allow amoeba cells to adhere to the surface.

3.2.3. While amoeba cells are settling down to adhere, stain the standardized *C. neoformans* UOFS Y-1378 cells that were adjusted to 1 x 10^6 cells/mL (in 999 µL of PBS) with 1 µL of pHrodo Green Zymosan A BioParticles in a 1.5 mL microcentrifuge tube. Stain C. *neoformans* LMPE 046 cells as well in a separate tube.

NOTE: The dye, unlike FITC, selectively stains cells that are trapped inside the acidic environment of a phagocytic cell[21,22]. For this technique, it is important to maintain the cryptococcal cells in a medium with a neutral pH (PBS) and amoeba in a medium with a neutral pH (ATCC medium 712). A medium with an acidic environment will result in a false positive reading of the relative fluorescence units, implying that a greater number of cryptococcal have been internalized.

3.2.4. Gently agitate cryptococcal cells on an orbital shaker set at 50 rpm for 2 h at RT and in the dark.

3.2.5. After 2 h, centrifuge the microcentrifuge tube at 960 x *g* for 5 min at 30 °C to pellet the cells. Aspirate the supernatant to remove PBS with the stain.

3.2.7. Add 1 mL of PBS to the tube to wash the pelleted cells. Wash the cells by gentle pipetting.

3.2.9. Centrifuge the cells at 960 x *g* for 5 min at 30 °C. Discard the supernatant. Repeat the washing step one more time.

3.2.10. Resuspend the pellet of washed cells in 1 mL of PBS.

3.2.11. Dispense a 100 µL suspension of stained cryptococcal cells to wells containing unstained amoeba cells.

3.2.12. Incubate the prepared co-culture at 30 °C for an additional 2 h period.

NOTE: The co-culture can be incubated for different timepoints to suit the purpose of the experiment.

3.2.13. At the end of the co-incubation period, measure the fluorescence on a microplate reader. Convert logarithmic signals to relative fluorescence units.

NOTE: The dye's excitation is at 492 nm and emission is at 538 nm. Consult Beisker and Dolbeare[19] and Clancy and Cauller[20] for methods to reduce autofluorescence.

4. Use of transmission electron microscopy to study phagocytosis (modified from van Wyk and Wingfield[23])

4.1. Add a 5 mL suspension of amoebae (adjusted to 1 x 10^7 cells/mL in ATCC medium 712) to a 15 mL centrifuge tube and allow them to settle for 30 min at 30 °C.

4.2 Add a 5 mL suspension of *C. neoformans* UOFS Y-1378 cells (adjusted to 1 x 10^6 cells/mL in PBS) to the same centrifuge tube that contains 5 mL of standardized amoeba cells.

4.3. Allow the tube stand for 2 h at 30 °C.

4.4. Centrifuge the tube at 640 x *g* for 3 min at 30 °C to pellet the co-cultured cells. Aspirate the supernatant. Do not wash the co-cultured cells.

4.6. Fix the co-cultured cells by resuspending the pellet in 3 mL of 1.0 M (pH = 7.0) sodium phosphate-buffered 3% glutaraldehyde for 3 h.

4.8. Centrifuge the tube at 1,120 x *g* for 5 min at 30 °C to pellet the co-cultured cells. Aspirate the supernatant.

4.10. Add 5 mL of sodium phosphate buffer to the centrifuge tube to wash the pelleted cells. Wash by gently pipetting the contents of the tube for 20 s.

4.12. Centrifuge the tube at 1,120 x *g* for 5 min at 30 °C to pellet the co-cultured cells.

4.13. Repeat steps 4.8–4.10. Aspirate the supernatant.

4.15. Fix the co-cultured cells again by resuspending the pellet in 3 mL of 1.0 M (pH = 7.0) sodium phosphate-buffered 1% osmium tetroxide for 1.5 h.

4.16. Remove the fixative (osmium tetroxide) by washing the co-cultured cells in a similar manner to removing 3% glutaraldehyde.

4.17. Dehydrate the TEM material (also known as the co-cultured cells) in a graded acetone series of 30%, 50%, 70%, 95% and two changes of 100% for 15 min each, respectively. To do so,

add 3 mL of the acetone solution to the pelleted cells and let it stand for 15 min. Then, centrifuge at 200 *x g* for 10 min at RT Discard the supernatant and add the higher percentage of the acetone solution.

4.18. Prepare the epoxy of normal consistency according to the protocol by Spur[24].

NOTE: The epoxy resin is used for sectioning.

4.19. Embed the TEM material into the freshly prepared epoxy resin. To do so, follow the steps below.

4.19.1. Add 3 mL of the freshly prepared epoxy to a tube that contains the TEM material resuspended in 3 mL of 100% solution of acetone. Allow the tube to stand for 1 h.

4.19.3. Centrifuge the tube at 200 x *g* for 10 min at 30 °C. Aspirate the epoxy-acetone solution.

4.19.5. Add 6 mL of the freshly prepared epoxy to the pellet in the tube. Allow the tube to stand for 1 h.

4.19.7. Centrifuge at 200 x *g* for 10 min at 30 °C. Aspirate all the epoxy-acetone solution.

4.19.9. Add 3 mL of the freshly prepared epoxy to the tube. Allow the tube to stand for 8 h.

4.19.11. Centrifuge at 200 x *g* for 10 min at 30 °C. Aspirate all the epoxy solution

4.19.13. Add 3 mL of the freshly prepared epoxy to the tube. Keep the TEM material in the epoxy solution overnight in a vacuum desiccator.

CAUTION: Epoxy resin is a radioactive material. Use PPE to handle the epoxy resin. The epoxy resin should also be handled in a fume hood. Researchers should follow safety regulations for discarding such material as specified by each country[25].

4.20. Polymerize the TEM material for 8 h at 70 °C.

4.21. On the ultramicrotome, trim small sections of approximately 0.1 mm x 0.1 mm and 60 nm thickness from the epoxy-embedded material with a mounted glass knife. Assemble sections on a grid and place the grids in a TEM sample holder box before staining.

4.22. Stain the sections with a drop of 6% uranyl acetate for 10 min in the dark. Ensure the sections are completely covered.

NOTE: Reconstitute the stain (6 g) in 100 mL of distilled water.

CAUTION: Uranyl acetate is a radioactive material. Use PPE to handle uranyl acetate. Uranyl acetate should also be handled in a fume hood. Researchers should follow safety regulations for discarding such material as specified by each country[25].

4.23. Rinse sections by dipping them five times into a beaker that contains 100 mL of distilled water.

NOTE: The distilled water should be disposed accordingly as it contains traces of uranyl acetate.

4.24. Stain the section with a drop of lead citrate for 10 min in the dark. Ensure the sections are completely covered.

NOTE: Lead citrate should be prepared according to the protocol by Reynold[26].

4.25. Rinse sections by dipping them five times into a beaker that contains 100 mL of distilled water.

4.26. Individually assemble the grids with stained sections on a TEM sample holder box.

4.27. View sections with a transmission electron microscope.

REPRESENTATIVE RESULTS:

Microbes are microscopic organisms that cannot be perceived with the naked eye. However, their impact may result in observable clinically evident illnesses, such as skin infections. When studying certain aspects of microbes, ranging from their morphology, byproducts, and interactions, being able to provide pictorial and video evidence is of the utmost importance.

We first sought to visualize the interaction between cryptococcal cells and amoeba. For this purpose, bright-field images that showed 2 h co-incubated cells were studied first. One image revealed a cryptococcal cell that was in the close proximity to amoeba. One of the amoeba cells was seen with extended pseudopodia to capture a cryptococcal cell **(Figure 1A)**. Next, a corresponding image in fluorescence was captured for referencing (**Figure 1B**). The green fluorescence on the surface of the stained cells aided in confirming the presence of cryptococcal cells. The unstained amoeba also auto-fluoresced. This, in addition to the apparent difference size and morphology, assisted in further distinguishing the two cell types.

Autofluorescence is a quality often observed when biological structures naturally emit light that they have absorbed (e.g., following exposure to a laser during confocal laser scanning microscopy)[27]. In **Figure 1C**, cryptococcal cells were noted (at the same timepoint of 2 h) that were already internalized by amoeba. The corresponding image in fluorescence was also captured for referencing (**Figure 1D**). Based on the evidence at hand, it is tempting to conclude that the amoeba killed the two trapped cells. However, phagocytosis is a dynamic process wherein the host, predator and pathogen, and prey employ different strategies to destroy or

evade each other[28]. The act of cryptococcal cells evading phagocytic cells is elegantly demonstrated by vomocytosis[29,30], which is a non-lytic expulsion process of trapped cells from macrophages. This daring move has been captured in time-lapse videos[29,30]. Unfortunately, this highlights the limitation of studying still images of fixed cells, as in our study, to elucidate a dynamic process like phagocytosis. To the point, a researcher may miss the interval when a cell escapes from its capturer.

To compensate for the above, the reading of relative fluorescence units was considered. In the current study, readings were taken after a 2 h co-incubation period and helped to compare the response of the two test cryptococcal strains [i.e., one that produces 3-hydroxy fatty acids (*C. neoformans* UOFS Y-1378) and the other that does not (*C. neoformans* LMPE 046)]. It was hypothesized that 3-hydroxy fatty acids may act as a virulence determinant that impair the uptake of cryptococcal cells, including phagocytosis by amoeba. For more information on the influence of 3-hydroxy fatty acids on amoeba, it is advised to refer to Madu et al.[15,31]. **Figure 2** shows the amount of cryptococcal cells that were internalized based on the reading of fluorescence units. When comparing the two cryptococcal isolates, it was clear that cells that produce the 3-hydroxy fatty acids were internalized less frequently compared to cells that do not produce 3-hydroxy fatty acids.

To enhance the qualitative data, transmission electron microscopy was included in the analysis (**Figure 3A**). Here, it was noted that the strain that produces 3-hydroxy fatty acids (*C. neoformans* UOFS Y-1378) had spiky protuberances on the capsule (**Figure 3B**), which may be used by the cell to release 3-hydroxy fatty acids to the outside environment.

It is important to note that the data (in **Figure 1, Figure 3**) convey the fate of cryptococcal cells as being internalized and not killed/phagocytized. To determine if the cells survived the phagocytic event, it is recommended to include an additional assay in which the researcher lyses the amoeba cells and prepares a spread plate agar to enumerate the cryptococcal colony forming units (CFU). By counting CFUs, Madu et al.[15] reported that cryptococcal cells producing 3-hydroxy fatty acids were also resistant to the phagocytic action of amoeba following internalization. Thus, these cells yielded a significantly higher survival rate when compared to cells that do not produce 3-hydroxy fatty acids.

Figure 4 shows the importance of TEM sample preparation and examination. In this instance, *C. neoformans* UOFS Y-1378 sections were purposefully overexposed to electron bombardment. At the end, the captured image cannot be used, as it compromises the quality of information that can be deduced. Taken together, the obtained information shows that by combining these different techniques, a researcher is able to deduce sufficient information to determine the fate of cryptococcal cells when co-cultured with amoeba.

FIGURE AND TABLE LEGENDS:

Table 1: Ingredients for making YPD agar. Add the required amount all the ingredients in 1 L of water. Heat while stirring to dissolve the ingredients completely. Once done autoclave prior to the use.

Table 2: Ingredients for making YNB broth. Add the required amount all the ingredients in 1 L of water. Heat while stirring to dissolve the ingredients completely. Once done autoclave prior to the use.

Table 3: Ingredients for making ATCC medium 712. Prepare the basal medium in 900 mL of water. Prepare the supplements separately and add to the basal medium. Once done adjust the pH to 7.4 with 1 N HCl or 1 N NaOH and autoclave. Filter sterilize 50 mL solution of 2 M glucose (18 g/50 mL) and add it aseptically to the complete medium prior to use.

Figure 1: Bright-field and corresponding fluorescent micrographs showing amoeba-*Cryptococcus* interactive moments. (**A**) An amoeba cell in close proximity to a *C. neoformans* UOFS Y-1378 cell can be seen. The corresponding fluorescent image is shown in (**B**). (**C**) Depiction of two *C. neoformans* UOFS Y-1378 cells that are trapped inside the amoeba food vacuole. The corresponding fluorescent image is shown in (**D**). This figure has been modified from Madu et al.[15]. A = amoeba; C = *C. neoformans*.

Figure 2: The results of the internalization assay of cryptococcal cells co-cultured with amoeba. The reading of relative fluorescence units allows for the interpretation and comparison of the efficiency of amoebae to internalize *C. neoformans* UOFS Y-1378 and *C. neoformans* LMPE 046. The error bars represent the calculated standard errors based on three biological replicates. This figure has been modified from Madu et al.[15].

Figure 3: Transmission electron micrographs showing amoeba-*Cryptococcus* interactions. TEM micrographs (**A**, **B**) confirm the observations in **Figure 1C,D**. (**A**) Shown is a *C. neoformans* UOFS Y-1378 cell trapped inside the amoeba food vacuole, while (**B**) is a close-up view of **Figure 3A**. This figure has been modified from Madu et al.[15]. A = amoeba cell; C = *C. neoformans* cell. The red arrow points at a capsular protuberance.

Figure 4: A transmission electron micrograph showing *C. neoformans* UOFS Y-1378 cells. The cells are damaged and thus cannot provide meaningful data. Red arrows indicate points where the section is torn.

DISCUSSION:

In the paper, different techniques were successfully employed to reveal the possible outcome that may arise when amoeba interact with cryptococcal cells. Also, we were interested to show the effects of 3-hydroxy fatty acids on the outcome of *Cryptococcus*-amoeba interactions.

The first technique used was confocal microscopy, which rendered still images. The major drawback of this technique here was that it only gave us information that is limited to a

particular timepoint. Any conclusion that can be drawn based on the results lends itself to inductive reasoning, wherein one can arrive at a conclusion based on a set of observations[32]. However, just because one observes several situations in which a pattern exists does not mean that that pattern is true for all situations. Thus, in the study, it is shown and possibly cautioned how such limited information may lead to unfounded conclusions. To the point, in the absence of contradictory or supportive, complementary evidence, it may be concluded that internalization may have led to the phagocytosis of cells.

The pace of development in imaging brings new opportunities to make scientific discoveries, as was the case with the uncovering of vomocytosis[29,30]. To illustrate this point without use of a microscope that can record time-lapse videos, this discovery would have not been possible. Therefore, a lack of access to such high-end instrumentation will always be an obstacle in resource poor-settings that are not at the forefront of uncovering such processes. One way to overcome this is to seek out new collaborations or discover innovative ways to address research questions. One welcome development has been the introduction and application of specialized stains such as the phagocytic stain used here[21,22]. This stain is pH-sensitive and fluoresces only in acid environments such as in the lumen of amoeba food vacuole[15]. It is worthwhile to point out that the stain only gives information related to the internalization of cells. Determination if cells are eventually phagocytized in additional experiments may be required.

Importantly, such a stain also proved to be useful in the measurement of fluorescence. The latter allowed integration of quantitative data in an attempt to explain what happens biologically at one specific timepoint. Here, fate of cells was discerned (i.e., it was determined whether the presence of 3-hydroxy fatty acids impaired or promoted the internalization of cells) by extrapolating meaning from the readings of relative fluorescence units.

Unlike in this study, researchers may also opt to measure the fluorescence of cells over a time period. The obtained information is useful in determining the number of cells that are internalized at one timepoint and following how the amount changes over the period. Likewise, images can also be taken at corresponding timepoints.

This study shows the power of combining a number of methods to reach a reasoned conclusion. The approach of combining multiple approaches to monitor phagocytosis either to compare or complement an initial technique is not new. For example, Meindl and co-workers[33] compared three techniques (image analysis, fluorescence, and flow cytometry readings) to investigate how fluorescence-labelled particle size affects macrophage phagocytosis. The study proved that of the three techniques, plate reading may be the best option to monitor phagocytosis[33].

TEM is particularly a powerful tool, as it provides a bird's eye view into the lumen of the food vacuole. Often, this level of detail is frequently missed by confocal microscopy in the form of still images, including time-lapse videos. To this point of the TEM, it was interesting to visualize protuberances on the surfaces of the cryptococcal capsule. It was previously hypothesized that these cell surface structures are used as a channel to release 3-hydroxy fatty acids into the

surrounding environment to possibly promote cell survival[9,10,15,31]. The detail on the TEM micrograph further reveals that protuberances on the internalized cell are not distorted and have maintained their integrity. Thus (given the integrity of the protuberances), it is possible that they may deliver 3-hydroxy fatty acids into the food vacuole environment and alter internal conditions, leading to cell survival as reported by Madu et al.[15,31]. A major limitation of using the electron microscope is that sample preparation is very laborious. Moreover, to avert destroying the samples as seen in **Figure 4**, the experimenter should be well-trained to manually operate the ultramicrotome and microscope.

In conclusion, it is envisaged that researchers will be encouraged by the prospect of studying phagocytosis simply by combining still fluorescent images with quantitative data. It is trusted that researchers can obtain enough information from this protocol and optimize it in their own studies. This may include the development of antibodies against targeted metabolites and applying this to immunofluorescence studies, including immuno-gold labelling during TEM examination.

ACKNOWLEDGMENTS:

The work was supported by a grant from the National Research Foundation of South Africa (grant number: UID 87903) and the University of the Free State. We are also grateful to services and assistance offered by Pieter van Wyk and Hanlie Grobler during our microscopy studies.

DISCLOSURES:

The authors declare that they have no competing financial interests.

REFERENCES:

1. Barton, L. L., Northup, D. E. *Microbial Ecology*. Wiley-Blackwell. New Jersey (2011).
2. Hunter, P. Entente cordiale: multiple symbiosis illustrates the intricate interconnectivity of nature. *EMBO Reports.* **7**, 861-864 (2006).
3. Comolli, L. R. Intra- and inter-species interactions in microbial communities. *Frontiers in Microbiology.* e629 (2014).
4. Siddiqui, R., Khan, N. A. *Acanthamoeba* is an evolutionary ancestor of macrophages: a myth or reality? *Experimental Parasitology.* **130**, 95-97 (2012).
5. Khan, N. A. *Acanthamoeba: Biology and Pathogenesis.* Caister Academic Press. Nottingham (2014).
6. Steenbergen, J. N., Casadevall, A. The origin and maintenance of virulence for the human pathogenic fungus *Cryptococcus neoformans*. *Microbes and Infection.* **5**, 667-675 (2003).
7. Casadevall, A., Perfect, J. R. *Cryptococcus Neoformans*. ASM Press. Washington D.C. (1998).
8. Axelsson-Olsson, D., Olofsson, J., Ellstrom, P., Waldenstrom, J., Olsen, B. A simple method for long term storage of *Acanthamoeba* species. *Parasitology Research*. **104**, 935-937 (2009).
9. Sebolai, O. M. et al. 3-Hydroxy fatty acids found in capsules of *Cryptococcus neoformans. Canadian Journal of Microbiology.* **53**, 809-812 (2007).
10. Sebolai, O. M., Pohl, C. H., Botes, P. J., van Wyk, P. W. J., Kock, J. L. F. The influence of acetylsalicylic acid on oxylipin migration in *Cryptococcus neoformans* var. *neoformans* UOFS Y-1378. *Canadian Journal of Microbiology.* **54**, 91-96 (2008).

11.Yap, A. S., Michael, M., Parton, R. G. Seeing and believing: recent advances in imaging cell-cell interactions. *F1000 Research.* e6435.1 (2015).
12. Follain, G., Mercier, L., Osmani, N., Harlepp, S., Goetz, J.G. Seeing is believing – multiscale spatio-temporal imaging towards *in vivo* cell biology. *Journal of Cell Science*. **130**, 23-38 (2017).
13. Lewis, L. E., Bain, J. M., Okai, B., Gow, N. A. R., Erwig, L.P. Live-cell video microscopy of fungal pathogen phagocytosis. *Journal of Visualized Experiments*. e50196 (2013).
14. Duane, E. G. A practical guide to implementing a BLS-2+ biosafety program in a research laboratory. *Applied Biosafety.* **18**, 30-36 (2013).
15. Madu, U. L. et al. Cryptococcal 3-hydroxy fatty acids protect cells against amoebal phagocytosis. *Frontiers in Microbiology.* e1351 (2015).
16. Smith, D., Onions, A. H. S. *The Preservation and Maintenance of Living Fungi*: *IMI Technical Handbooks No. 2*. CAB International. Wallington (1994).
17. Schuster, F. L. Cultivation of pathogenic and opportunistic free-living amebas. *Clinical Microbiology Reviews*. **15**, 342-344 (2002).
18. Strober, W. Trypan blue exclusion test of cell viability. *Current Protocols in Immunology*. **21**, 1-2 (2001).
19. Beisker, W., Bolbeare, F., Gray, J. W. An improved immunocytochemical procedure for high sensitivity dectection of incorporated bromodeoxyuridine. *Cytometry.* **8**, 235-239 (1987).
20. Clancy, B., Cauller, L. J. Reduction of background autofluorescence in brain sections following immersion in sodium borohydride. *Journal of Neuroscience Methods*. **83**, 97-102 (1998).
21. Simons, E. R. Measurement of phagocytosis and of phagosomal environment in polymorphonuclear phagocytes by flow cytometry. *Current Protocols in Cytometry.* Unit 9, 31 (2010).
22. Grillo-Hill, B. K., Webb, B. A., Barber, D. L. Ratiometric Imaging of pH Probes. *Methods in Cell Biology*. **123**, 429-448 (2014).
23. van Wyk, P. W. J., Wingfield, M. J. Ascospores ultrastructure and development in *Ophiostoma cucullatum. Mycologia.* **83,** 698-707 (1991).
24. Spur, A. R. A low viscosity epoxy resin embedding medium for electron microscopy. *Ultrastructural Research*. **26**, 31-43 (1969).
25. Department of Minerals and Energy. *Radioactive Waste Management Policy and Strategy for the Republic of South Africa*. Department of Minerals and Energy. Pretoria (2005).
26. Reynolds, E. S. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Cell Biology*. **17**, 208-212 (1963).
27. Monici, M. Cell and tissue autofluorescence research and diagnostic applications. *Biotechnology Annual Review.* **11**, 227-256 (2005).
28. Janeway, C. A., Travers, P., Walport, M., Shlomchik, M. *Immunobiology: The Immune System in Health and Disease, 5th edition*. Garland Science. New York (2001).
29. Ma, H., Croudace, J. E. Lammas, D. A., May, R. C. Expulsion of live pathogenic yeast by macrophages. *Current Biology*. **16**, 2156-2160 (2006).
30. Alvarez, M., Casadevall, A. Phagosome extrusion and host-cell survival after *Cryptococcus neoformans* phagocytosis by macrophages. *Current Biology*. **16**, 2161-2165 (2006).

610 31. Madu, U. L., Ogundeji, A. O., Pohl, C. H., Albertyn, J., Sebolai, O. M. Elucidation of the role of
611 3-hydroxy fatty acids in *Cryptococcus*-amoeba interactions. *Frontiers in Microbiology.* e765
612 (2017).
613 32. Hayes, B. K., Heit, E. Inductive reasoning 2.0. *Wiley Interdisciplinary Review: Cognitive*
614 *Science*. e1459 (2018).
615 33. Meindl, C., Öhlinger, K., Ober, J., Roblegg, E., Fröhlich, E. Comparison of fluorescence-based
616 methods to determine nanoparticle uptake by phagocytes and non-phagocytic cells *in vitro*.
617 *Toxicology.* **378**, 25-36 (2017).

Figure 1

Click here to access/download;Figure;Figure 1.ai

Figure 2

Click here to access/download;Figure;Figure 2.ai

Figure 3

Click here to access/download;Figure;Figure 3.ai

Figure 4

Click here to access/download;Figure;Figure 4.ai

Table 1. Ingredients for making YPD agar.

Ingredient	Quantity
bacteriological peptone	20 g/L
yeast extract	10 g/L
glucose	20 g/L
agar	15 g/L

Table 2. Ingredients for making YNB broth.

Ingredient	Quantity
ammonium sulfate	5 g/L
biotin	2 µg/L
calcium pantothenate	400 µg/L
folic acid	2 µg/L
inositol	2000 µg/L
niacin	400 µg/L
p-aminobenzoic acid	200 µg/L
pyridoxine hydrochloride	400 µg/L
riboflavin	200 µg/L
thiamine hydrochloride	400 µg/L
boric acid	500 µg/L
copper sulfate	40 µg/L
potassium iodide	100 µg/L
ferric chloride	200 µg/L
manganese sulfate	400 µg/L
sodium molybdate	200 µg/L
zinc sulfate	400 µg/L
monopotassium phosphate	1 g/L
magnesium sulfate	0.5 g/L
sodium chloride	0.1 g/L
calcium chloride	0.1 g/L

Table 3. Ingredients for making ATCC medium 712.

Part I: Basal medium.

Ingredient	Quantity
proteose peptone	20 g/L
yeast extract	1 g/L
agar (if needed)	20 g/L

Part II: Supplements.

Ingredient (stock solutions)	Quantity
0.05 M $CaCl_2$	8 mL
0.4 M $MgSO_4$ x $7H_2O$	10 ml
0.25 M Na_2HPO_4 x $7H_2O$	10 mlL
0.25 M KH_2PO_4	10 mL
Na Citrate x $2H_2O$	1 g
0.005 M $Fe(NH_4)2(SO_4)2$ x $6H_2O$	10 mL

Table of Materials

Name of Material/Equipment	Company/Manufacturer	Catalog Number	Comments/Description
1,4-Diazabicyclo-[2.2.2]-octane	Sigma-Aldrich	D27802	-
1.5-mL plastic tube	Thermo Fisher Scientific	69715	-
15-mL Centrifuge tube	Thermo Fisher Scientific	7252018	-
50-mL Centrifuge tube	Thermo Fisher Scientific	1132017	-
8-Well chamber slide	Thermo Fisher Scientific	1109650	-
Acetone	Merck	SAAR1022040LC	-
Amoeba strain	ATCC®	30234™	-
ATCC medium 712	ATCC®	712™	Amoeba medium
Black 96-well microtiter plate	Thermo Fisher Scientific	152089	-
Centrifuge	Hermle	-	-
Chloroform	Sigma-Aldrich	C2432	-
Confocal microscope	Nikon	Nikon TE 2000	-
Epoxy resin:			
[1] NSA	[1] ALS	[1] R1054	-
[2] DER 736	[2] ALS	[2] R1073	-
[3] ERL Y221 resin	[3] ALS	[3] R1047R	-
[4] S1 (2-dimethylaminoethanol)	[4] ALS	[4] R1067	-
Fluorescein isothiocyanate	Sigma-Aldrich	F4274	-
Formic Acid	Sigma-Aldrich	489441	-
Fluoroskan Ascent FL	Thermo Fisher Scientific	374-91038C	Microplate reader
Glucose	Sigma-Aldrich	G8270	-
Glutaraldehyde	ALS	R1009	-
Hemocytometer	Boeco	-	-
Lead citrate	ALS	R1209	-
Liquid Chromatography Mass Spectrometer	Thermo Fisher Scientific		-
Methanol	Sigma-Aldrich	R 34,860	-
Orbital shaker	Lasec	-	-
Osmium tetroxide	ALS	R1015	-
pHrodo Green Zymosan A BioParticles	Life Technologies	P35365	This is the pH-sensitive dye
Physiological buffer solution	Sigma-Aldrich	P4417-50TAB	-
Rotary shaker	Labcon	-	-
Sodium phosphate buffer:			
[1] di-sodium hydrogen orthophosphate dihydrate	[1] Merck	[1] 106580	-
[2] sodium di-hydrogen orthophosphate dihydrate	[2] Merck	[2] 106345	
Transmission electron microscope	Philips	Philips EM 100	-
Trypan blue	Sigma-Aldrich	T8154	-
Ultramicrotome	Leica	EM UC7	-
Uranyl acetate	ALS	R1260A	-
Vacuum dessicator	Lasec	-	-
Vial	Sigma-Aldrich	29651-U	-
YNB	Lasec	239210	-
YPD agar	Sigma-Aldrich	Y-1500	-

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: THE APPLICATION OF CONFOCAL LASER SCANNING MICROSCOPY AND ELECTRON MICROSCOPY IN THE STUDY OF CRYPTOCOCCUS-AMOEBA INTERACTIONS

Author(s): UJU L. MADU AND OLUWOLE M. SEBOLAI

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove VISUALIZED JOURNAL OF EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

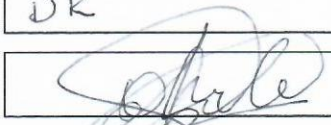
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	OLIHILE M. SEBOLAI
Department:	MICROBIAL, BIOCHEMICAL AND FOOD BIOTECHNOLOGY
Institution:	UNIVERSITY OF THE FREE STATE
Title:	DR
Signature:	
Date:	FRI, 29 JUNE 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA
UFS UV

Wednesday, 24 April 2019

Dr Jaydev Upponi, Ph.D.
Science Editor: JoVE, Immunology and Infection

Dear Dr Upponi,

We appreciate the comments made by the editorial office and the reviewers. We have prepared our responses. They are highlighted in red and follow a question/comment posed to us.

I trust you will find our responses to be sufficient. Future correspondence should be addressed to Dr. Olihile M. Sebolai (+2751 401 2004; sebolaiom@ufs.ac.za).

With sincere appreciation,

O M Sebolai

Dr Olihile M. Sebolai (Ph.D.)
Corresponding author
Senior Lecturer and Fulbright Fellow

205 Nelson Mandela Drive/Rylaan
Park West/Parkwes
Bloemfontein 9301
South Africa/Suid-Afrika

P.O. Box/Posbus 339
Bloemfontein 9300
South Africa/Suid-Afrika
T: +27(0)51 401 9111
www.ufs.ac.za

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

Responses to Editorial concerning the manuscript:

1. Journal's style.

We endeavored to retain the style.

2. Proofreading., please employ professional copyediting services.

The manuscript has been proofread.

3. Title:

We have revised the title. We have also used the same title for the video.

4. Normal consistency

We have edited out “normal consistency”. Yes, this is quantifiable. By weighing off the ingredients, one should be able to make epoxy of normal consistency.

5. We hypothesized

We have changed our text accordingly.

6. Reference

We have now included a reference.

Responses to Editorial concerning the video:

1. Title

The title of the video matches that of the manuscript.

2. Flickering

We have worked on flickering. Let us know if all is in order.

Dear Dr. Sebolai

Thank you for your email.

Under the Frontiers Terms and Conditions, authors retain the copyright to their work. Furthermore, all Frontiers articles are Open Access and distributed under the terms of the Creative Commons Attribution License (CC-BY 3,0), which permits the use, distribution and reproduction of material from published articles, provided the original authors and source are credited, and subject to any copyright notices concerning any third-party content.

You can therefore freely reuse a figure of an article published at Frontiers without having to ask for permission. More information about CC-BY can be found here: http://creativecommons.org/licenses/by/4.0/

Best regards,

Gearóid

Ethics & Integrity Manager: Gearóid Ó Faoleán, PhD

Frontiers

www.frontiersin.org
EPFL Innovation Square, Building I
Lausanne, Switzerland
Office T +44 79 34 46 47 49

For technical issues, please visit our Frontiers Help Center frontiers.zendesk.com

---

Research Integrity Manager: Gearóid Ó Faoleán, PhD

Frontiers

www.frontiersin.org
Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
Office T +44 203 514 26 98

For technical issues, please visit our Frontiers Help Center frontiers.zendesk.com

On Wed, Feb 6, 2019 at 5:41 AM Olihile Sebolai <SebolaiOM@ufs.ac.za> wrote:

Dear Sir, Dear Madam:

I hope you are doing good. I'd like to request a letter from the office wherein I am granted copyright permission to re-use a figure from my previous paper that was published with Frontiers. The article in question is: U.L. Madu, A.O. Ogundeji, B.M. Mochochoko, C.H.

Pohl, J. Albertyn, C.W. Swart et al. (2015). Cryptococcal 3-hydroxy fatty acids protect cells against amoebal phagocytosis. Frontiers in Microbiology 6, Article no. 1351.

I am in the process of submitting a manuscript to Journal of Visualized Experiments based on the techniques I used obtain the figure from my Frontiers paper. I trust you will find my letter to be in order and to assist me in this regard.

With sincere appreciation,

Olihile Sebolai

Senior Lecturer: Microbial Biochemical and Food Biotechnology
Senior Lektor: Mikrobiese Biochemiese en Voedselbiotegnologie
Faculty / Fakulteit: Natural and Agricultural Sciences / Natuur- en Landbouwetenskappe
PO Box / Posbus 339, Bloemfontein 9300, Republic of South Africa / Republiek van Suid-Afrika
+27 (0)51 401 2004
SebolaiOM@ufs.ac.za

The extraction and analysis of 3-hydroxy fatty acids. Modified from Madu et al.[15].

NOTE: *Cryptococcus neoformans* is regarded as biosafety level-2 (BSL-2) pathogens and thus researchers ought to take the proper precautions when working with this organism. For example, laboratory personnel should have specific training and personal protective equipment (PPE) such as lab coats, gloves and eye protection. A biological safety cabinet (level-2) should be used for procedures that can cause infection[14].

NOTE: The extraction should be carried out in a fume hood. Personnel should also use PPE during the course of the extraction procedure.

1. Prepare a 24 h old culture of *C. neoformans* UOFS Y-1378 as stated in sub-section 1 of the protocol. Information on YNB broth's ingredients can be found in Table 2.

NOTE: All the steps should be repeated for *C. neoformans* LMPE 046.

2. Use a hemocytometer to determine the cell concentration of the culture after 24 h.

3. Use the equation $C_1.V_1 = C_2.V_2$ to work out how much volume of the culture media is required to yield a final cell concentration of 1×10^7cells/mL in a final volume of 2 mL (of the same culture medium).

4. Transfer 2 mL of the culture media, containing the cells, to a 15 mL centrifuge tube.

5. Add 3% formic acid drop-wise to decrease the pH of the culture media to 4.

NOTE: To make 3% solution, add 3 mL of formic acid to 97 mL of distilled water.

6. Add a 2 mL solution of methanol-chloroform (1:1, v/v) to the culture media in the tube.

NOTE: Prepare the above cocktail solution by adding 5 mL of methanol to 2.5 mL of chloroform.

NOTE: Do not inhale the chloroform as it may cause breathing difficulty.

7. Vortex-mix the contents of the tube for 2 min.

8. Allow the tube to stand for 20 min.

9. Add 2 mL of distilled water to the mixture in the tube.

10. Vortex-mix the contents for 5 min.

11. Centrifuge at 1900 x *g* for 10 min at 4 ^{o}C.

12. Collect the chloroform fraction, which contains the 3-hydroxy fatty acids.

13. Transfer this fraction to a vial.

14. Dry the contents of the vial under a stream of nitrogen in a fume hood.

15. Reconstitute the extracts in 1 mL of methanol and transfer to a 1.5-mL plastic tube.

16. Collect 200 µL of the reconstituted extracts and transfer to an analytical vial.

17. Store the sample in the autosampler of the high-performance liquid chromatography mass spectrometer at 5 ^{o}C before injection.

18. Analyze the samples within 24 h of reconstitution in negative electrons pray ionization mode.

19. Run a blank control sample at the start and end of each run.

NOTE: This will provide a measure of the sample background and also a measure of compound carry over.

20. Run a full scan mode of between *m/z* 100–1000.

21. Obtain the relative peak area of the analyte of interest based on the retention time of the analytical standard.

NOTE: In the current study the analyte of interest was 3-hydroxy nonanoic acid i.e. 3-hydroxy C9:0.

22. Obtain the characteristic mass fragments from the mass spectrum to confirm the identity of the analyte of interest.

NOTE: These mass fragments should be similar to that of the analytical standard.