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An automated assay to measure phagocytosis of *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans is an opportunistic fungal pathogen, which causes meningoencephalitis that kills 200,000 individuals worldwide each year. It is ubiquitous in the environment and is first inhaled into the lungs of the host, where it is taken up by phagocytes. The interaction of these fungal cells with host phagocytes, therefore, is a critical step in the pathogenesis of this disease. One characteristic of this initial step in host: pathogen interactions is the avidity with which fungal cells are taken up by phagocytes, described by the phagocytic index. In this chapter, we detail a high-throughput method of directly assessing the phagocytic index of fungal cells using an imaging-based paradigm. By automating image collection and processing, this method permits rapid assessment of this critical host interaction.

Keywords

Cryptococcus neoformans; phagocytosis; automation; phagocytic index

INTRODUCTION

Cryptococcus neoformans is an opportunistic fungal pathogen that is ubiquitous in the environment and causes a deadly meningitis. It is generally acquired by inhalation, to which the innate immune system in the lung initially responds by phagocytosing the fungi. This does not serve to clear the infection, however, because *C. neoformans* is capable of surviving intracellularly. The outcome of cryptococcal infection is thus heavily influenced by the interaction of *C. neoformans* with phagocytes, as reviewed in Wager, 2016; Zhang, 2015; Coelho, 2014; Johnston, 2013; McQuiston, 2012; and Garcia-Rodas, 2011. Current methods for assessing such interactions, however, are frequently limited by the labor involved, throughput, or inability to identify whether fungal cells are adherent or internalized (see below). The method presented here allows quantitation of the initial engulfment of *C. neoformans*. By using an automated assay to measure phagocytosis (Srikanta, 2011), we can

efficiently study fungal (Santiago-Tirado, 2015), host (Srikanta, 2017), or environmental factors that influence the avidity of uptake.

Note: *Cryptococcus neoformans* is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See UNIT 1A.1 and other pertinent resources (APPENDIX 1B) for more information.

BASIC PROTOCOL 1: An automated assay to measure phagocytosis of *Cryptococcus neoformans*

This method uses inexpensive dyes to distinguish internalized from non-internalized fungal cells. All fungi are labeled before exposure to host cells, while a counter-stain applied after host interactions labels only external fungi, so that they become doubly labeled (See Fig. 1). Since the assay uses dye-labeled fungal cells, rather than specific fungal strains, for example that express a genetically-encoded fluorophore, mutant libraries can be easily screened. This assay is also highly adaptable in that any adherent host cells, either primary cells or immortalized cell lines, may be used. Below we use the THP-1 cell line as an example (Tsuchiya, 1980).

This image-based assay requires high-quality, focused images with good signal and low background. The latter is generally more important than signal strength, as variable background will confound downstream automated image analysis while sensitive instruments can detect even low signals. Below we outline automated analysis that uses the Biotek line of Cytation Multi-Mode Automated Imagers, but these protocols may be adapted for other imaging equipment.

Materials

THP-1 cell line, ATCC TIB-202

THP-1 growth medium: see recipe below

Phorbol 12-myristate 13-acetate (PMA)

Yeast extract peptone dextrose (YPD) medium: see recipe below

Phosphate buffered saline

McIlvaine buffer: see recipe below

Lucifer Yellow dye

Human serum

RPMI 1640 medium

Calcofluor White solution: see recipe below

Human serum

Formaldehyde

Saponin

Propidium iodide

Sodium azide

70% ethanol

Aluminum foil sealing film

96-well plate, Corning #3904

Biotek Cytation 3 multi-mode reader with accompanying software, or equivalent automated widefield microscope

1. Undifferentiated THP-1 cells are non-adherent. Harvest a T75 flask of cells by swirling the flask and use a sterile 25-mL pipette to transfer the cells to a 50-mL conical tube.

Note: Primary cells or other adherent phagocytic cell lines (e.g. RAW or J774) may be used but ensure that the serum used for opsonization matches the species of host cells used.

2. Sediment the cells by centrifugation (5 minutes, 250 x *g*, RT).
3. Remove the supernatant fraction and resuspend the cells in 4 mL of THP-1 growth medium.
4. Dilute the cells to 1E5 cells/mL in THP-1 growth medium. For example, a full 96-well plate will require 11 mL of cells at this density.
5. To begin cell differentiation, add PMA to the diluted THP-1 cells for a final concentration of 0.32 μ M (2.2 μ L PMA per 11 mL cells).
6. Promptly add 100 μ L of the cells mixed with PMA to the selected wells of a Corning #3904 plate, using a multichannel pipette. This will be 10,000 cells/well. Incubate at 37 °C in 5% CO₂ for 3 days.

Note: In choosing a layout for experiments, we find that technical replicates of 3 wells per sample work well. Depending on your imaging equipment and the susceptibility of your protocol to edge effects it may also be advisable to avoid wells on the edges of the plates. Pipette cells along the edge of the well to avoid them settling in the middle of the well.

7. On day 2 after plating the THP-1 cells, start an overnight culture of *C. neoformans* by picking a single colony with a sterile loop and using it to inoculate 5 mL of YPD medium in a 14-mL culture tube. Incubate in a 30 °C shaker at 240 rpm overnight (Jung, 2018).

Note: Any desired cryptococcal strains or mutants can be used.

8. On the following morning, harvest the *C. neoformans* cells by centrifugation (5 minutes, 1000 x *g*, RT).
9. Aspirate the supernatant fraction and wash the cells by resuspending the pellet in 5 mL of sterile PBS. Centrifuge as in step 2. Repeat this step for a total of 2 washes.
10. Aspirate the supernatant fraction and resuspend the washed cells in 5 mL of McIlvaine buffer.
11. Measure the cell density using a hemocytometer or automated counting device and centrifuge 1E8 cells as in step 2.
12. Aspirate the supernatant fraction and resuspend in 1 mL of McIlvaine buffer. Transfer to a 1.5 mL microtube and add 90 µL of Lucifer Yellow (Fig. 1, LY) dye for a final concentration of 450 µg/mL to stain the fungal cell walls. Wrap the tubes in foil and rotate end-over-end for 30 minutes at RT.
13. Collect the stained cells by centrifugation (3 minutes, 1000 x *g*, RT) and aspirate the supernatant fraction.
14. Resuspend the pellet in 1 mL sterile PBS, and centrifuge as in step 13.
15. Aspirate the supernatant fraction and resuspend in 1 mL of sterile PBS.
16. Transfer 400 µL of the cell suspension into a new microtube and place on ice. This is the unopsonized sample. The remaining 600 µL will be the serum-opsonized sample.
17. Add 400 µL of human serum to the serum-opsonized sample. Then vortex both the unopsonized and opsonized samples briefly, wrap them in foil, and rotate them end-over-end for 30 minutes at 37 °C.
18. Subject both samples to centrifugation as in step 13, aspirate the supernatant fraction, and resuspend the cells in 1 mL of sterile PBS.
19. Repeat the previous step but resuspend in 1 mL of pre-warmed RPMI 1640.
20. Measure cell density using a hemocytometer and dilute the cells to a density of 1.67E6 cells/mL in pre-warmed RPMI 1640.
21. Remove non-adherent THP-1 cells from the plates prepared in step 6 by aspirating the medium from the plate. Rinse the plate twice by gently adding 100 µL of pre-warmed RPMI to each well and then aspirating.
22. Add 100 µL of *C. neoformans* suspension to the designated wells, for a total of 1.67E5 cells per well. Incubate the plate at 37 °C in 5% CO₂ for 60 minutes.

Note: At this point of the assay, fungi in the wells may be engulfed by the host cells, adherent to them (externally associated), or free in the medium.

23. To remove free fungal cells, wash the plate four times with sterile PBS, using a plate washer or squeeze bottle and aspirator.

- 24.** To specifically label externally associated fungal cells, add 150 μ L of the Calcofluor White (Fig 1, CFW) stock solution to each well after the last wash and incubate at 4 °C for 20 minutes.

Note: Calcofluor White does not permeate the host cells, so that this step specifically labels fungi that are adherent to the host cells, but not engulfed.

- 25.** Wash the plate twice with sterile PBS as in step 23.
- 26.** To fix the samples, add 150 μ L of ice-cold 4% formaldehyde to each well, incubate in the dark at RT for 10 minutes, and wash as in step 23.
- 27.** To permeabilize the THP-1 cells add 150 μ L of 0.1% saponin to each well, incubate in the dark at RT for 20 minutes, and wash the plate twice as in step 23.
- 28.** To stain nuclei, add 150 μ L of propidium iodide (Fig 1, PI) to each well, incubate in the dark at RT for 20 minutes, and wash the plate twice as in step 23.
- 29.** Add 150 μ L 10mM NaN_3 to each well, seal the plate with aluminum foil sealing film, and store it at 4 °C until ready to image.
- 30.** Before imaging, gently clean the optical surface of the Corning plate with 70% ethanol using a lint-free wipe.
- 31.** Select the 10x objective lens (or equivalent) for the Cytation imager (or equivalent).
- 32.** Install the appropriate filters (excitation, emission) for imaging Lucifer Yellow (465 nm, 510 nm), Calcofluor White (365 nm, 435 nm), and PI (531 nm, 647 nm). For the Cytation instrument, these are the GFP, DAPI, and propidium iodide filter cubes, respectively.
- 33.** Create an automated imaging protocol using each of the above three channels, in the order mentioned above.
- 34.** Manually select the exposure settings (LED intensity, integration time, and gain) using the adjust interface.

Note: The Biotek Cytation analysis software is sensitive enough to generally detect objects accurately if there is low background. To manually adjust exposure for optimal analysis later, LED intensity should first be maximized before adjusting the integration time or gain. Integration time and gain should be increased in a balanced manner until images appear sharp (but not necessarily extremely bright).

- 35.** Select autofocus for the GFP channel.
- 36.** Select autofocus based on the GFP channel for the DAPI and propidium iodide channels.

Note: Autofocus for the DAPI channel should be set to the same focal length as GFP. You may need to enter manual mode to determine the proper autofocus depth for the propidium iodide channel; this typically requires an 8–10 μ m offset.

37. Select image acquisition settings (at least a 4 by 4 grid is recommended, centered in each well, spaced 100 μm apart).
38. Select the wells that contain samples for assessment.
39. Begin the imaging process (it generally takes about one hour for a full plate for the Cytation).
40. Enter analysis mode and identify 3 categories of objects – one from each of the DAPI, GFP, and propidium iodide channels. Adjust parameters to minimize false detection of non-cell objects as described in the following steps.
41. Select object parameters for the DAPI and GFP channels. These parameters should include appropriate size sieves and roundness requirements for discerning fungal cells versus debris. Instruct the analysis to return the sum of objects within the field.
42. Select object parameters for the propidium iodide channel. These parameters should include size sieves for discerning host nuclei versus debris. Instruct the analysis to return the sum of objects within the field.
43. For each field, subtract the sum of DAPI objects from the sum of GFP objects. This returns the number of internalized fungal cells per field.
44. Divide the internalized fungal cells per field by the sum of propidium iodide objects and multiply by 100. This returns the phagocytic index.

REAGENTS AND SOLUTIONS

THP-1 growth medium:

RPMI-1640 (Sigma R8758), 10% (v/v) heat-inactivated (56 °C for 30 minutes) fetal bovine serum (Gibco 26140–079), 1 mM sodium pyruvate (CellGro 25–000–Cl), 100 μM penicillin and 100 U/mL streptomycin (Pen Strep) (Gibco 15140–122), 48 μM beta-mercaptoethanol (Sigma M3148), store up to 1 month at 4 °C.

Yeast extract peptone dextrose (YPD) medium:

Bacto yeast extract (10 g/L), Bacto peptone (20 g/L), D-glucose (20 g/L), in H_2O , store up to 6 months at room temperature.

McIlvaine buffer:

combine 63 mL of 200 mM Na_2HPO_4 and 37 mL of 100 mM citric acid, adjust to pH 6 and filter-sterilize, store up to 6 months at room temperature.

Calcofluor White solution:

dissolve 1 g Calcofluor White (Sigma F3543) in 100 ml PBS, and dilute 1000x fold in 100 ml PBS for a stock 10 $\mu\text{g}/\text{mL}$ concentration, store up to 6 months at –20 °C.

COMMENTARY

Background Information

The interaction of *Cryptococcus* and phagocytes is crucial to the outcome of infection. In line with this, multiple assays for measuring these interactions have been reported, each with its own strengths and weaknesses. One approach which requires minimal equipment is assaying uptake by counting colony forming units (CFUs) (Sabiiti, 2014). In this assay, phagocytic cells are first incubated with opsonized fungal cells. After a set time, the free yeast are washed away, the phagocytic cells are lysed, and the resulting lysate is plated to quantify yeast CFUs. While this method is simple, requires no specialized equipment, and counts only viable cells, the disadvantage is that it cannot discriminate between adherent and internalized yeast. Furthermore, the method depends on growth after plating, which may skew results.

The most commonly utilized method to assay phagocytosis is direct microscopy (Syme, 2002; Zaragoza, 2003; Geunes-Boyer, 2009; Lim, 2018). Phagocytes are grown in imaging plates or well slides, incubated with the yeast, washed to remove free yeast, and fixed. After fixation, the cells can be labeled with a variety of stains or antibodies for imaging. Histological stains such as Giemsa are often used to assay uptake and cell morphology (Geunes-Boyer, 2009; Lim, 2018). This assay allows manual quantification of the number of phagocytes with intracellular yeast, as well as the number of yeasts in each cell. The disadvantage is that it is labor-intensive, and it cannot always discriminate between adherent and internalized yeast. Fluorescent microscopy can overcome this problem by the use of counterstains (Lim, 2018), but the assay remains labor-intensive. Some adaptations of this assay have been applied to multi-well plates using fluorescence readers (Walenkamp, 2000).

Another group of assays, that has been used to assess interactions between *C. neoformans* and host cells on a larger scale, is based on flow cytometry (Chaka, 1995; Voelz, 2010; Alanio, 2011; Nicola, 2011). These methods use a variety of reagents to stain fungal and host cells but may be limited by inability to discriminate fungal cells that are engulfed, adherent, or free; depending on the available flow cytometry equipment they may also be limited in speed and throughput.

The method presented in this protocol is based on automated imaging. It reduces labor and increases speed and throughput by taking advantage of recent advances in laboratory-scale automation of imaging and image annotation. It also uses fluorescent counterstains to resolve adherent and engulfed fungi (free fungi are removed by washing) and uses inexpensive reagents. This method may be applied to screens of the interactions between *C. neoformans* and host cells, including screens of conditions, fungal mutants, systematically perturbed host cells, and exogenous compounds. This highly flexible protocol may also be adapted to investigate other microbe:host interactions, such as those involving other fungal species.

Critical Parameters & Troubleshooting

As this assay is based on imaging, the accuracy of the data depends on the quality of the images taken by the automated microscope, including both sufficient resolution (determined

by the available equipment) and an adequate ratio of signal to noise. For optimizing the signal-to-noise ratio, titration of stain concentrations may be required, or additional washes of the stained sample to lower background signal. In addition, part of the image quality is dependent on the cell densities.

It is also essential that the annotation software be able to accurately define and quantify objects. In general, software annotation of objects is far more sensitive than the human eye. Exposure should thus generally be adjusted until objects are only faintly visible, because imaging at too high of an exposure risks loss of sensitivity. Software settings may also need adjustment to determine the best parameters for quantitation; during methods development it is imperative to confirm annotation by manual examination, to be sure the desired characteristics are being accurately annotated and counted.

Additional trouble-shooting will depend on specific aspects of the assay being performed. For example, the phagocytic index is sensitive to the passage number of immortalized host cells, so this should be noted, and activity confirmed with appropriate controls. Depending on the cell type chosen, the incubation time may be varied; time course studies may also be informative. If there are too many host cells or fungal cells, it becomes difficult to accurately quantify the phagocytic index, so in such cases the numbers should be scaled back (or the incubation time reduced if over-abundant fungi are the problem). If nuclei are hard to distinguish, because of diffuse staining of RNA by PI, samples can be treated with RNase A.

Statistical Analyses

Upon completion of automated analysis, the software should return three values for each well: the number of extracellular fungal cells, the total number of fungal cells, and the total number of host cells present. The difference between the extracellular fungal cells and total fungal cells is the intracellular cells. This is useful in calculating the phagocytic index – simply divide the intracellular fungal cells by the total number of host cells counted. Similar assessment can be performed by measuring the number of adherent cells – those that are extracellularly associated with host cells but are not engulfed. The adherence index may be obtained by dividing the number of extracellular fungal cells by the total number of host cells.

The phagocytic index should be assessed for each strain or condition in multiple wells across multiple experiments, comparing results both within and across plates to appropriate controls (e.g. wild-type fungi, unperturbed host cells, or unopsonized cells to demonstrate the low background level of interactions). The phagocytic index of each strain may be compared to that of the control strain and other strains using a one-way ANOVA and the Kruskal-Wallis test. For medium or high throughput screens the final optimized assay should also be assessed to be certain it has the required power (Ghosh, 2007; Zhang, 1999).

Anticipated Results

In general, we find that with this protocol we measure phagocytic indices in a range of 60–80% for serum-opsonized wild-type KN99α cells, incubated for 1 hour with THP-1 cells. This value may be higher and lower, depending on opsonization time, uptake time, and host

cell type and passage number. Using alternative cryptococcal strains can also affect phagocytic index. Overall, this assay, modified as needed to address specific experimental questions, should provide a reproducible and rapid method for quantitating early interactions between host and pathogen cells.

Time Considerations

The time required in advance of the assay is primarily determined by the choice of host cell; for example, it takes approximately two weeks to prepare THP-1 cells for the assay. The time needed to perform the automated phagocytosis experiment will depend on the incubation times chosen; the described protocol requires approximately 8 hours. Imaging the resultant plate requires approximately 3 hours on a Cytation instrument, and image analysis requires approximately 1 hour.

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Significance Statement

Cryptococcus neoformans is an opportunistic fungal pathogen, which causes a meningoencephalitis that kills 200,000 individuals worldwide each year. Its ability to cause this deadly disease depends on its interaction with phagocytes that it encounters upon inhalation. The uptake of *C. neoformans* by these phagocytes is thus a critical area of study in understanding the pathogenesis of this disease. In this article, we describe a protocol that can be used to assess the initial step in this phagocytic process. This method can be used to study a variety of factors that regulate phagocytosis, including fungal genes and host factors.

Figure 1:

Vertical pairs of images represent individual channels from Cytation image data, corresponding to staining with Lucifer Yellow (LY), Calcofluor White (CFW), and propidium iodide (PI); top panel of each pair, unannotated image; bottom panel of each pair, image with detected objects annotated by yellow outlines. The large panel at right shows merged channels without annotation. All fungi are LY-stained, with examples of extracellular fungi (CFW-positive) indicated by open arrowheads and intracellular fungi (CFW-negative) indicated with solid arrowheads. Scale bar, 50 μ m.