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Video Article

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Abstract

Studies of the cellular pathogenesis mechanisms of pathogenic yeasts such as *Candida albicans*, *Histoplasma capsulatum*, and *Cryptococcus neoformans* commonly employ infection of mammalian hosts or host cells (*i.e.* macrophages) followed by yeast quantification using colony forming unit analysis or flow cytometry. While colony forming unit enumeration has been the most commonly used method in the field, this technique has disadvantages and limitations, including slow growth of some fungal species on solid media and low and/or variable plating efficiencies, which is of particular concern when comparing growth of wild-type and mutant strains. Flow cytometry can provide rapid quantitative information regarding yeast viability, however, adoption of flow cytometric detection for pathogenic yeasts has been limited for a number of practical reasons including its high cost and biosafety considerations. Here, we demonstrate an image-based cytometric methodology using the Cellometer Vision (Nexcelom Bioscience, LLC) for the quantification of viable pathogenic yeasts in co-culture with macrophages. Our studies focus on detection of two human fungal pathogens: *Histoplasma capsulatum* and *Candida albicans*. *H. capsulatum* colonizes alveolar macrophages by replicating within the macrophage phagosome, and here, we quantitatively assess the growth of *H. capsulatum* yeasts in RAW 264.7 macrophages using acridine orange/propidium iodide staining in combination with image cytometry. Our method faithfully recapitulates growth trends as measured by traditional colony forming unit enumeration, but with significantly increased sensitivity. Additionally, we directly assess infection of live macrophages with a GFP-expressing strain of *C. albicans*. Our methodology offers a rapid, accurate, and economical means for detection and quantification of important human fungal pathogens in association with host cells.

Video Link

The video component of this article can be found at http://www.jove.com/video/50599/

Introduction

Studies of pathogenic fungi in association with their hosts and/or host cells often require quantification of viable fungal cells over a time course or under different infection conditions. Enumeration of colony forming units (CFU) is the standard method by which the number of viable fungal cells has been measured, however, this technique has several drawbacks and limitations. First, many fungal species are slow-growing. Growth of visible colonies on solid media can take 1-2 weeks, significantly slowing the pace of research. Second, manipulation of samples during CFU plating is a laborious process, since several dilutions must be plated to ensure a countable number of colonies. Third, the number of CFU is typically lower than the number of viable organisms plated because the plating efficiency is well below 100%. For example, plating efficiencies for the dimorphic fungal pathogen *Histoplasma capsulatum* can be as high as 90%, but are routinely as low as 30% and are even lower (10%) for the related fungal dimorph *Paracoccidioides brasiliensis* [1, 2]. Plating efficiency for *Candida albicans* is also subject to variability [3]. Finally, CFU analysis accounts for only live and actively dividing cells capable of establishing growth on solid media, whereas in many situations, it would be useful to determine the presence and concentration of dead and/or metabolically inactive cells.

Previously, flow cytometric methods for quantification of several pathogenic fungal species has been described [4-6]. However, due to biosafety containment issues involved with using biosafety level 2 (BSL2) or BSL3-level pathogens on shared flow cytometers, adoption of this technique has been limited. Like flow cytometry, image cytometry is a sensitive and rapid method of cell quantification. However, image cytometry can be performed at a fraction of the cost with comparable results [7-11]. Here, we describe methods for performing image cytometry of pathogenic fungi in association with host cells. We demonstrate our methods using two human fungal pathogens: *Histoplasma capsulatum* and *Candida albicans*. *H. capsulatum* is a dimorphic fungal pathogen that causes respiratory disease; in humans, it grows as budding yeast and replicates within alveolar macrophages. *Candida albicans* is a human commensal species that occasionally causes candidiasis. We show that image cytometry allows rapid quantification of these yeasts, together with visualization capability.

Protocol

1. Infection of Macrophages with *H. capsulatum, C. albicans*

1. 16 hr prior to infection, seed macrophages at desired density in 24-well plates. In this protocol, a density of 3.0 x 10^5 cells/well was used.
2. Add fungal cells in log-phase growth at a desired multiplicity of infection (MOI). This protocol can accommodate a range of MOI (0.2 - 5.0). For the infection of RAW264.7 macrophages with *H. capsulatum*, we used an MOI of 0.2.
3. Following 1.5 hr to allow for phagocytosis, wash macrophages three times with PBS to remove extracellular fungi.
4. Incubate for desired number of hours prior to sample analysis. At low MOI (0.2 in our experiment), infected macrophage cells remain viable for several days, and samples can be analyzed approximately every 12-24 hr.

2. Macrophage Lysis

1. To liberate fungi from macrophage cells, remove media, wash 3 times with PBS, and add 0.5 ml sterile water. Under these conditions, macrophages will lyse and fungal cells will remain intact.
2. Incubate for 5 min at room temperature.
3. Transfer lysate to sterile tube, keep on ice.
4. Transfer 20 µl lysate to a separate tube, then add 20 µl AO/PI solution. Proceed directly to step 5: "Sample Preparation for Image Cytometric Analysis"

3. CFU Plating

1. Perform ten-fold dilution of lysates (from step 2.3) in media.
2. Plate 100 µl of each dilution, in duplicate, on HMM-agarose plates. Incubate plates in a humidified chamber at 37 °C with 5% CO_2 for 7-8 days.
3. Manually count colonies on plates displaying a minimum of 100 and a maximum of 1,000 distinct colonies.

4. Visualization of Fungi within Live Macrophages

1. To collect live macrophages, wash cells 3 times with PBS. Add 0.5 ml PBS and incubate for 30 min at 4 °C.
2. To remove macrophages from tissue culture wells, gently pipette up and down several times. Transfer liquid to sterile tube, keep on ice.
3. Transfer 20 µl sample to a separate tube, then add 20 µl AO/PI solution. Proceed directly to step 5: "Sample Preparation for Image Cytometric Analysis".

5. Sample Preparation for Image Cytometric Analysis

1. Pipette the target sample thoroughly and then transfer 20 µl of sample into the disposable cell counting chamber.
2. Allow the cells to settle in the chamber for 30 sec.
3. Insert counting chamber into the image cytometer.

6. Cellometer Instrument Setup

1. Insert the Fluorescence Optics Modules: VB-535-402 and VB-660-502 into the system and make sure they are locked in place.
    1. VB-535-402 (excitation at 475 nm, emission at 535 nm) is used for acridine orange and GFP detection.
    2. VB-660-502 (excitation at 540 nm, emission at 660 nm) is used for propidium iodide detection.
2. Turn on the image cytometer and open the accompanying software.

7. Cellometer Software Setup

1. Select the preset "Assay Type" and "Cell Type" in the Assay Dropdown Menu.
    1. For viability, the assay is optimized for acridine orange and propidium iodide detection.
    2. For detection of *Candida albicans* infection, the assay is optimized for GFP detection.
2. Select "Options" on the top and click on "Take Background Image", and allow the operation to complete.
3. Click on "Preview Bright-Field Image".

8. Image Acquisition Procedure

1. Insert the prepared sample chamber into the image cytometer.
2. Use the focus knob and adjust the focus.
3. Once in focus, click on "Count", and allow the image acquisition operation to complete.

4. Remove the disposable counting chamber and dispose of appropriately.

9. Image Data Analysis

1. Concentration and viability measurement
    1. Once the counting is completed, the concentration and viability of the target cells are displayed in the result page.
    2. Click on "Export" to export the data into FCS Express 4 for cell population analysis of GFP in the *Candida albicans* infection experiment.

2. FCS Express analysis of *Candida albicans*-infected cells
    1. Import the ".NXDAT" file into FCS Express 4 and plot the results in a fluorescence histogram.
    2. Apply cell population gate to the histogram to determine the population percentages of *Candida albicans*-infected cells.

Representative Results

We used the Cellometer Vision image cytometer to monitor growth of *H. capsulatum* in macrophage cells. RAW 264.7 cells were infected with *H. capsulatum* yeast cells and at various time points, samples were subjected to AO/PI staining followed by image-based cytometric analysis. In parallel, samples were analyzed by traditional CFU enumeration. At each time point, samples were incubated in water to lyse macrophages, and the liberated yeast cells were identified by the Cellometer Vision software (**Figures 1a** and **1b**). We observe highly comparable trends in the concentration of viable yeasts as detected by image-based cytometric analysis and CFU enumeration at each time point (**Figure 1c**). As expected, the absolute number of live yeasts detected by AO/PI staining was consistently higher than the number of yeasts capable of colony formation on solid medium as assessed by CFU analysis, highlighting significant numbers of viable but non-cultivatable cells.

Visualization of fungal cells within live macrophages can be accomplished using GFP-expressing yeast strains. Bone marrow-derived macrophages (BMDM) were infected with *C. albicans* yeast cells expressing green fluorescent protein (GFP) under control of the ADH1 promoter [12] (**Figure 2a**). Infection of BMDM with GFP-*C. albicans* yeasts at multiplicities of infection (MOI) ranging from 0.1-10 corresponded to incremental increases in GFP intensity within infected macrophages (**Figure 2a**) as well as total percentage of infected macrophages (**Figure 2b**).

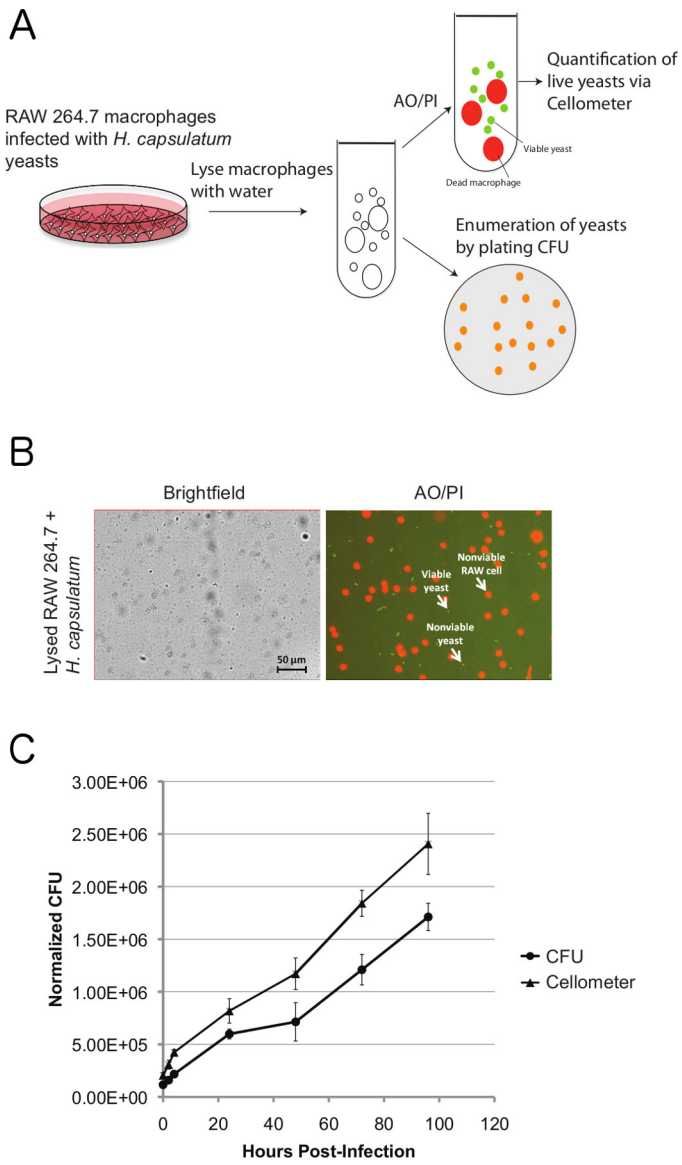

Figure 1. Comparison of image-based cytometric analysis to CFU enumeration of *H. capsulatum* during *in vitro* infection of RAW 264.7 macrophages. (a) RAW 264.7 macrophages were infected with *H. capsulatum* yeasts at an MOI of 0.2. At 24 hr intervals following infection, macrophages were lysed and viable yeasts assessed by AO/PI staining in parallel with CFU enumeration. **(b)** Representative brightfield (BR) and FL1/FL2 (green/red) combined images of AO/PI stained *H. capsulatum* released from lysed RAW 264.7 macrophages. A segmenting algorithm counted only AO and PI positive yeasts in the fluorescent images while the large PI-stained RAW 264.7 macrophages (red) are excluded. Images were captured under 10X magnification. **(c)** Direct comparison of yeast proliferation as assessed by AO/PI staining and CFU enumeration. Linear regression analysis of the growth trends as measured by CFU and Cellometer analysis yield R^2=0.9927. Click here to view larger figure.

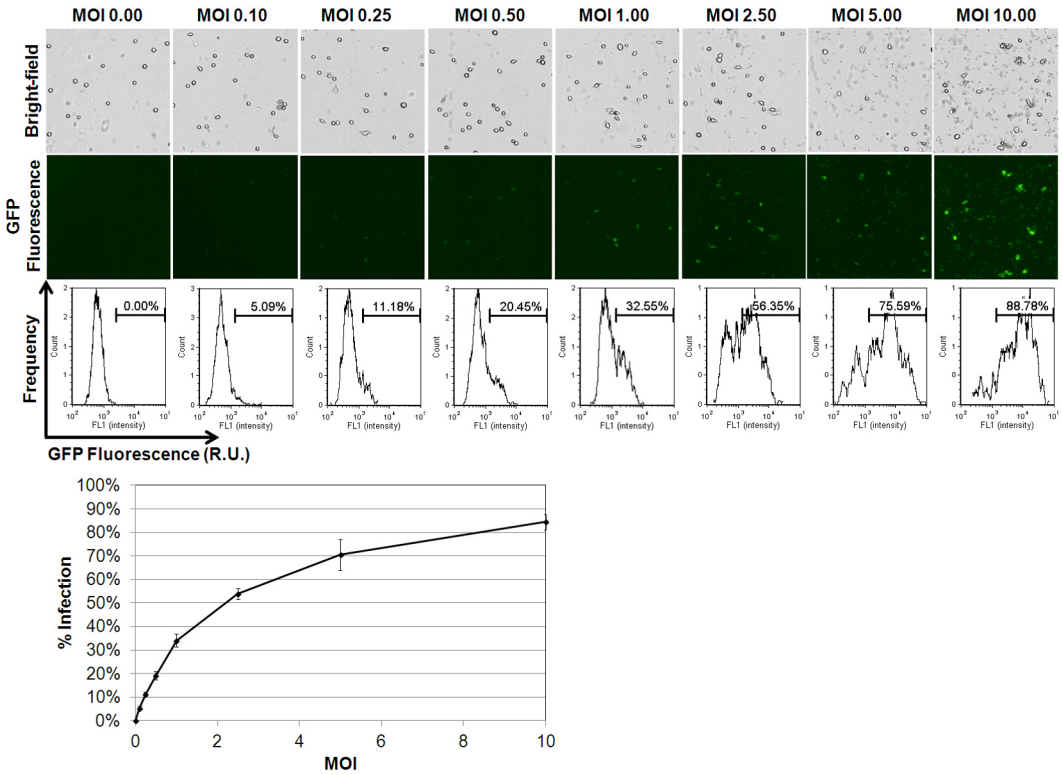

Figure 2. Quantification of BMDM infection with a GFP-expressing strain of *C. albicans*. (a) Captured brightfield (BR) (top) and fluorescent (middle) images of GFP-*C. albicans* infected BMDMs at increasing MOI. Histograms of fluorescence intensities show an increase in GFP fluorescence intensity as the MOI increases, which represents an increase in the number *C. albicans* associated with BMDM cells (bottom). The software identified infected BMDMs based on application of a linear marker at the baseline fluorescence intensity displayed by the uninfected control population (MOI = 0). **(b)** Percent infection as a function of MOI, which shows an increase in infected BMDMs as MOI increases. Click here to view larger figure.

Discussion

Image cytometry allows the user to capture high quality images and, using specialized software, perform rapid quantification of cells. One potential challenge to the adoption of image cytometry in the field of microbial pathogenesis is that the microbes to be counted are present in a mixed population of cells, including mammalian host cells. Here, we demonstrate that image cytometry can be used for the quantification of viable pathogenic yeasts during *in vitro* macrophage infection. Our methodology not only faithfully recapitulates trends in fungal growth and viability observed during *in vitro* macrophage infection assays, but also displays improved sensitivity compared to the traditional CFU assay [13].

Image cytometry offers several practical advantages when compared with either CFU enumeration or flow cytometry for quantification of yeast cells. When plating CFUs, laboratory workers must plate several dilutions in order to ensure a countable number of cells on each plate, which can be very labor-intensive. Image cytometry eliminates the need for multiple dilutions. Previously, we have shown that colony formation on solid media is often variable, and at very low concentrations fungi may fail to form colonies at all, whereas image cytometry enables enumeration of cells regardless of concentration [13]. The ability to detect and count fungi at very low concentrations can be very useful during early stages of a low-dose infection, or during clearance, when numbers and viability may be at the margin of detection. Additionally, data generated by image cytometry are available instantly, whereas colonies may take several days to become visible. While many fungal species can be detected by flow cytometry, the possibility of cross-contamination is a concern in shared facilities. The counting chamber used in this study offers a significant advantage in terms of biosafety, as it is disposable and self-contained. Furthermore, image cytometry offers the practical advantages of lower price and a smaller footprint when compared with conventional flow cytometry, which can be important for smaller research laboratories [11].

In addition to its practical advantages, image cytometry of yeast cells provides information that CFU enumeration cannot. First, CFU enumeration is only able to detect fungi that are able to establish colony growth on the medium chosen, which may not accurately represent the number of viable fungi present in an infection experiment in real time. Also, the ability to establish colony growth on solid media can differ when comparing wild-type and mutant strains of fungi, which can be a source of experimental bias [14], and these hidden biases may be revealed by comparison of image cytometry and CFU quantification of wild-type and mutant strains. Once the behavior of a particular strain has been characterized by both CFU enumeration and image cytometry, we believe that image cytometry can be used as an alternative means of fungal quantification.

One limitation of image cytometry in general is that it does not allow collection of as many data points as flow cytometry. Since the image cytometer derives quantitative data from a limited number of captured images, it is difficult for the system to collect data on hundreds of thousands to millions of cells per sample. However, this limitation may be circumvented by grouping multiple samples into one set of data for analysis in order to reach similar data points as a flow cytometer. We would also like to stress that the AO/PI staining method used here

represents one way of measuring of cellular viability, and does not necessarily indicate cellular vitality and/or the ability to establish growth on solid medium in any given sample. Therefore, while our method provides a facile method of live/dead quantification of yeasts, it will also be interesting to investigate the use of image cytometry in combination with cellular metabolism indicators such as FUN-1 [15].

Image cytometry software identifies and counts cells of interest based on size and shape, and therefore, it is absolutely critical that these parameters are set carefully during each experiment. When performing image cytometry with a new yeast strain, we suggest that one first compare data generated from a pure yeast culture to data generated from counting a mixture of yeasts plus host cells to ensure that the parameters are set such that the software can distinguish between the two cell types.

In the future, this work will be the basis for further development of methods to detect and quantify microbes via image cytometry. For example, development of image cytometric methods to detect fungi within organ homogenates will be of great use in the field. Another future challenge will be to develop and refine image cytometric methods and software such that it can be used for quantification of bacterial cells. With the advancement in optical systems, digital cameras, and a wide variety of fluorescent stains in the field, the ability to image and analyze bacteria population can be implemented in image cytometers, which can provide a more rapid method for concentration and viability or vitality measurement in comparison to the CFU or optical density methods. In summary, the work presented here is a proof-of-concept showing that image cytometry is a sensitive method for quantification of pathogenic yeasts. The sophistication of image-based cytometric analysis software offers the opportunity to analyze interactions between host cells and pathogenic fungi in a highly quantitative manner. This technology may be adapted to address a range of research questions in the field of fungal pathogenesis.

Disclosures

The authors Leo Li-Ying Chan and Benjamin Paradis are employees of Nexcelom Bioscience LLC.

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