Rapid Quantification of Pathogenic Fungi by Cellometer Image-Based Cytometry

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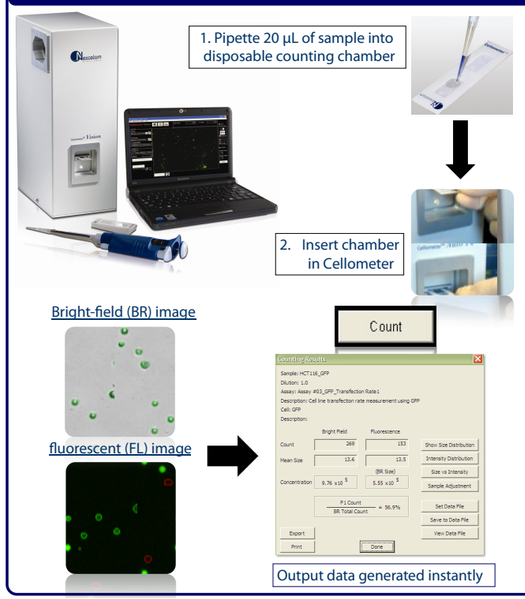
1. ABSTRACT

The objective of this study was to develop an image-based cytometric methodology for the quantification of viable pathogenic yeasts, which can offer increased sensitivity and efficiency when compared to the traditional colony forming unit (CFU) assay. Live/dead yeast quantification by flow cytometry has been previously demonstrated, however, adoption of flow cytometric detection of pathogenic yeasts has been limited for a number of practical reasons including its high cost and biosafety considerations. 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. Here, we quantitatively assess the growth of H. capsulatum yeasts within RAW 264.7 macrophages using acridine orange/propidium iodide staining in combination with Cellometer image-based cytometry; this method faithfully recapitulates growth trends as measured by traditional CFU enumeration, but with significantly increased sensitivity. Additionally, we directly assess infection of bone marrow-derived macrophages with a GFP-expressing strain of C. albicans. To demonstrate that image-based cytometry can be used as a tool to assess the susceptibility of fungi to antifungal drugs, we perform dose response experiments with the antifungal drugs amphotericin B and itraconazole and show that image cytometry allows rapid assessment of the kinetics of cytotoxicity induced by these antifungals. Our methodology offers a rapid, accurate, and economical means for detection and quantification of important human fungal pathogens, either alone or in association with host cells.

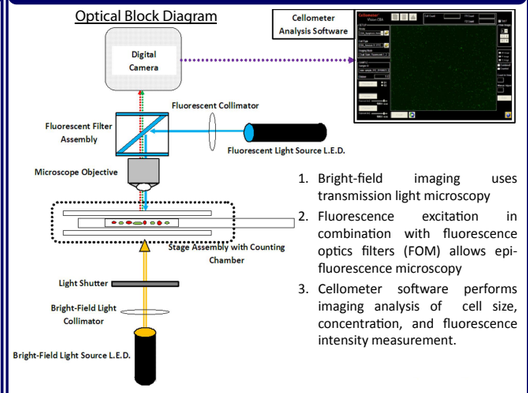
2. INTRODUCTION

In fungal pathogenesis studies the number of viable pathogenic fungi (including Candida spp., Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Cryptococcus neoformans, and Coccidioides immitis), has traditionally been monitored by enumeration of colony forming units (CFU). While CFU enumeration has been the standard in the field, this technique has many significant disadvantages and limitations, including sub-optimal plating efficiency and slow growth. Image cytometry provides a simple and user-friendly method for quantifying fungal cells in a variety of contexts.

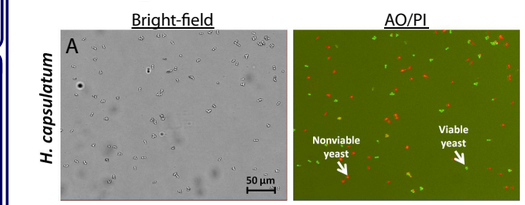
3. CELLOMETER® VISION IMAGE -BASED CYTOMETRY



4. IMAGE-BASED C YTOMETRIC METHOD OF YEAST

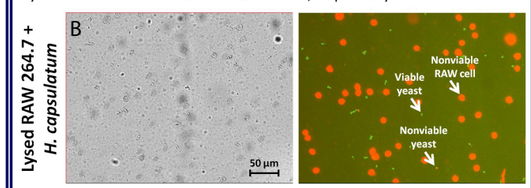


1. Bright-field imaging uses transmission light microscopy
2. Fluorescence excitation in combination with fluorescence optics filters (FOM) allows epi-fluorescence microscopy
3. Cellometer software performs imaging analysis of cell size, concentration, and fluorescence intensity measurement.



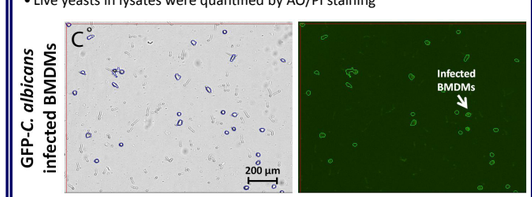
A. Identification of viable yeast by acridine orange/propidium iodide (AO/PI)

- H. capsulatum G217B ura5 yeasts were subjected to heat killing and mixed with non heat-treated yeasts at a ratio of 1:1. Yeasts were stained with AO/PI
- Images were captured under 10X magnification and viable and non-viable yeasts identified in the FL1 and FL2 channels, respectively



B. Measurement of H. capsulatum Proliferation within Macrophages

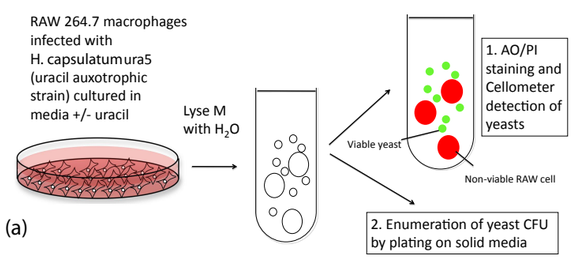
- RAW 264.7 macrophages were infected with H. capsulatum yeasts at MOI=0.2
- Macrophages were lysed at regular intervals to release intracellular yeasts
- Live yeasts in lysates were quantified by AO/PI staining



C. Measurement of C. albicans in Association with Live Macrophages

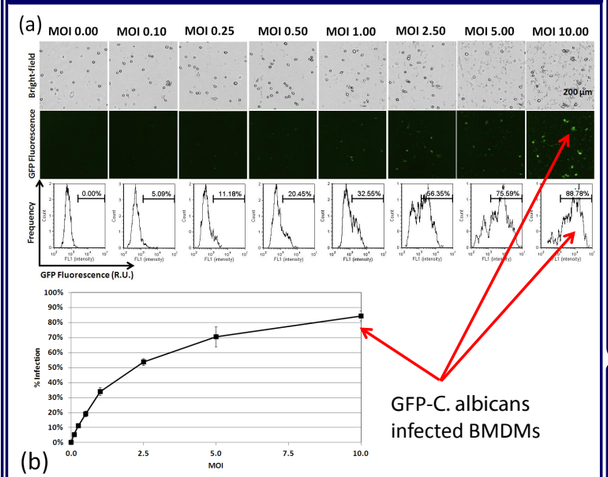
- Bone marrow derived macrophages (BMDM) were infected with GFP-C. albicans at a range of MOI
- GFP fluorescence signals were quantified in live BMDM

5. COMPARISON OF IMAGE CYTOMETRY AND CFU ANALYSIS



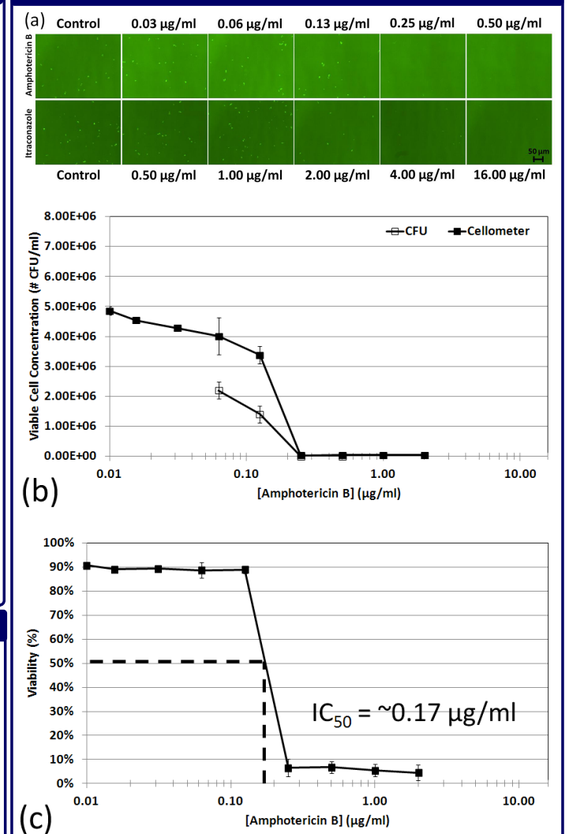
- RAW 264.7 macrophages were infected with H. capsulatum ura5 yeasts at an MOI of 0.2 with/without uracil supplementation
  - At 24-hour, macrophages were lysed and analyzed with AO/PI
- Comparison of yeast proliferation assessed by AO/PI and CFU, which showed increase with uracil supplementation
- Viability assessed by AO/PI showed decrease in viability of yeasts without uracil supplementation

6. MEASUREMENT OF C. ALBICANS WITHIN LIVE MACROPHAGES



- Bright-field images showed increase in GFP-C. albicans concentrations
- Fluorescent images showed increase in fluorescence signals of GFP-C. albicans infected BMDMs
- Fluorescent histograms showed distinct infected population with increased fluorescence
- The percentages of infection were directly correlated to MOIs, which were measured using FCS Express

7. DOSE RESPONSE OF ANTIFUNGAL-INDUCED CYTOTOXICITY



- Fluorescent images showed decrease in number of H. capsulatum incubated with Amphotericin B and Itraconazole
- Comparison of yeast concentration assessed by AO/PI and CFU, which showed indirect correlation to compound concentrations
- Viability assessed by AO/PI was used to determine IC50 of Amphotericin B (0.17 µg/ml) and Itraconazole (2.61 µg/ml)

8. CONCLUSIONS

- Image-based cytometry is a rapid and facile method for quantifying the pathogenic fungi C. albicans and H. capsulatum in a variety of contexts including in vitro macrophage infection and antifungal dose response studies
- AO/PI staining in conjunction with Cellometer quantification of H. capsulatum closely mimics trends in intracellular yeast growth detected by CFU enumeration
- Image based cytometry offers several advantages over CFU enumeration, including increased speed and higher sensitivity at low cellular concentrations
- Cellometer Vision image-based cytometer can be used to screen a variety of chemical compounds for potential antifungal drugs

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