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USING FLOW CYTOMETRY TO ANALYSE *CRYPTOCOCCUS* INFECTION OF MACROPHAGES

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CRYPTOCOCCUS MACROPHAGE INFECTION FLOW CYTOMETRY

Summary

Flow cytometry is a powerful analytical technique, which is increasingly being used to study the interaction between host cells and intracellular pathogens. Flow cytometry is capable of measuring a greater number of infected cells within a sample compared to alternative techniques such as fluorescence microscopy. This means that robust quantification of rare events during infection is possible. Our lab and others have developed flow cytometry methods to study interactions between host cells and intracellular pathogens, such as *Cryptococcus neoformans,* to quantify phagocytosis, intracellular replication and non-lytic expulsion or 'vomocytosis' from the phagosome. Herein we describe these methods and how they can be applied to the study of *C. neoformans* as well as other similar intracellular pathogens.

Key words

Cryptococcus neoformans

Cryptococcus gattii

Flow cytometry

Macrophage

Infection

Mycology

1. Introduction

1.1 *Cryptococcus neoformans* and *Cryptococcus gattii*

The *Cryptococcus* genus is part of the basidiomycete phylum of the fungal kingdom of life. The genus contains over 50 described species, however almost all human and veterinary cases of cryptococcal infection are caused by just two species – *Cryptococcus neoformans* and *Cryptococcus gattii*. Although these two species are closely related they present with different pathologies during infection. *C. neoformans* is an opportunistic pathogen of individuals with existing immune deficiencies whereas *C. gattii* can infect immune competent hosts. During infection both fungal species interact with host phagocytes in the lungs and phagocytosis by host alveolar macrophages provides an intracellular niche for *Cryptococcus* to replicate ***(4)***. However, occasionally cryptococci can escape from the macrophage via a process known as non-lytic expulsion or 'vomocytosis' ***(3, 5, 6)***. The central role of macrophages during cryptococcal infection makes this host pathogen interaction a key area of *Cryptococcus* research. *In vitro* cell culture is often used to study this interaction as in many cases, the interaction between *Cryptococcus* species and macrophages cultured *in vitro* can be used as a reliable indicator of virulence *in vivo* ***(2, 7)***.

Methods to quantify cryptococcal parasitism of macrophages with flow cytometry have been previously published by our lab and others ***(1-3)***. Typically, such methods exploit *Cryptococcus* strains with a genomic fluorescent tag ***(1)***, or a combination of antibody and cell dye stains ***(2, 3)***. Herein we will describe our previously published method for flow cytometry analysis of cryptococcal phagocytosis by macrophages and subsequent *Cryptococcus* replication within the phagosome ***(1)***.

1.2 Creating fluorescently tagged *Cryptococcus* strains

Our protocol relies on the use of *Cryptococcus* strains that have been genetically modified to express a fluorescent marker protein such as green fluorescent protein (GFP). During the development of this assay we created two fluorescent strains in the H99 (*C. neoformans var. grubii* serotype A, genotype VNI) and R265 (*C. gattii* serotype B, genotype VGII) genetic backgrounds. These two strains are both common 'wild type' reference strains used by many *Cryptococcus* researchers.

The step by step generation the H99-GFP and R265-GFP used in this study can be found in our previous publication ***(1)*** but, in brief, cryptococci were transformed via biolistic delivery with an insertion cassette containing the GFP gene flanked upstream by the *Cryptococcus* JEC21 actin promoter *act1* and downstream by the JEC21 tryptophan terminator *trp1*.

For an investigator seeking to make their own fluorescently tagged strains a number of factors must be considered once stable transformants have been produced to ensure valid results in the flow cytometry assay. It must be ensured that the insertion of the transgene and/or its expression has not significantly altered the physiology of the fluorescent mutant in comparison to its wild type parent. This process is important as it helps to ensure the validity of all future findings using the transformed strain (**see Note 1**).

2. Materials

2.1 Cells and strains

1. J774.A1 cells were acquired from the European Collection of Authenticated Cell Cultures (ECACC).
2. The genetic background for the fluorescent *Cryptococcus neoformans* mutants used was H99 *C. neoformans var grubii*. serotype A.

2.2 Specialist equipment

1. Class II laminar flow hood: *Cryptococcus neoformans* is a class II organism and as such should be worked on inside a class II safety hood to protect the user. Additionally, cells in culture are easily susceptible to outside contamination; therefore, any work with uninfected cells should be performed within a laminar flow hood.
2. Tissue culture incubator: mammalian cells such a J774 cells should be grown in a specialist incubator that can control heat, humidity and CO_2 levels. J774 cells are grown at 37 ^{o}C with 5% CO_2 and 95 % relative humidity.
3. Haemocytometer: to determine the correct concentration of *Cryptococcus neoformans* cells for infection a haemocytometer counting chamber should be used. A BS 748 standard haemocytometer with a chamber depth of 0.1 mm is recommended.
4. Flow cytometer – this protocol requires a flow cytometer that is capable of exciting and measuring GFP (excitation wavelength 395 nm, and detection wavelength 509 nm).

2.3 Media (Macrophage)

1. Serum supplemented DMEM: Dulbecco's Modified Eagle medium, low glucose, 10 % v/v Fetal bovine serum (FBS), 1 % v/v 10,000 units Penicillin & 10 mg/ml streptomycin, 1 % v/v 200 mM L-glutamine. Keep sterile store at 4°C.

2. Serum free DMEM: Dulbecco's Modified Eagle medium, low glucose, 1 % v/v 10,000 units Penicillin & 10 mg/ml streptomycin, 1 % v/v 200 mM L-glutamine. Keep sterile and store at 4°C.

2.4 Media (*Cryptococcus*)

- YPD (Yeast, peptone, dextrose) liquid growth media: 1 % w/v peptone, 1 % w/v yeast extract and 2 % w/v D-(+)-glucose. Autoclave to sterilise.
- YPD agar: Liquid YPD growth media + 2 % w/v agar. Autoclave to sterilise, pour into 9 cm Petri dishes.

2.5 Miscellaneous reagents

- Phorbol 12-myristate 13-acetate (PMA): 1 mg/ml PMA in dimethyl sulfoxide (DMSO) store at -20 ^{o}C aliquoted.
- Phosphate buffered saline (1x PBS): 8 g/L sodium chloride, 0.2 g/L potassium chloride, 1.15 g/L disodium hydrogen phosphate, 0.2 g/L potassium dihydrogen phosphate, in deionised H_2O pH 7.3, autoclave to sterilise and store at room temperature.
- 18B7 antibody (: 10 mg/ml in 100 % glycerol (a kind gift from Arturo Casadevall, Johns Hopkins Bloomberg School of Public Health, Maryland USA), mouse IgG against *Cryptococcus* capsule polysaccharide Glucuronoxylomannan store at -20 ^{o}C aliquoted.
- Accutase: use at concentration suggested by manufacturer, store at -20°C aliquoted.
- Fixing media: 2 % w/v formaldehyde, 2 % v/v FBS in 1x PBS, store at -20 ^{o}C aliquoted, once thawed discard within 2 weeks.

3. Protocol

3.1 Experimental design

1. *C. neoformans* samples to be analysed by this protocol need to be fluorescently tagged. Additionally, a sample that contains macrophages infected with a non-fluorescent *C. neoformans* strain is also need as a control for the flow cytometer (see section 3.4). Ideally this non fluorescent strain should be the same genetic background as the fluorescent *Cryptococcus* strain.
2. For each condition, 4 duplicate infections need to be prepared (e.g. four separate wells) for measurement at each time point. The following time points are recommended for the calculation of intracellular proliferation - 0 hours (2 hours post infection, immediately after washing away extracellular yeast), 18 hours (20 hours post infection), 24 hours (26 hours post infection) and 48 hours (50 hours post infection) (**see Note 2**).

3.2 *Cryptococcus* preparation

Long term, *Cryptococcus* strains can be stored at – 80 ^{o}C in glycerol stocks or using Biobank storage beads. For experimentation *Cryptococcus* strains are grown on YPD agar at 25 ^{o}C and stored at 4 ^{o}C.

1. Prepare overnight cultures of the fluorescently tagged *Cryptococcus* strain from YPD agar stock plates 24 hours prior to the start of the assay. Grow overnight cultures in 2 ml YPD broth with constant rotational movement to prevent sedimentation.
2. On the day of experiment, transfer 1 ml of the overnight culture into a sterile 1.5 ml microcentrifuge tube and wash 3 times with sterile PBS.
3. Once washed, count the overnight culture with a haemocytometer (a 1:20 dilution of the overnight is usually sufficient for accurate counting) and dilute to a concentration of $1x10^{7}$ *Cryptococcus* cells per 1ml in PBS in 1.5 ml microcentrifuge tubes.
4. Opsonise *Cryptococcus* for 1 hour with 10 µg/ml anti capsular 18B7 antibody (a kind gift from Arturo Casadevall, Albert Einstein College of Medicine, New York USA) for best results put microcentrifuge tubes on a rotator. Alternatively, *C. neoformans* can be opsonised with pooled human serum (separated from donor blood).

3.3 Macrophage infection

The J774 murine macrophage cell line is used here as an example, however this protocol could easily be adapted for other *in vitro* cell lines or *in vitro* cultured primary monocytes/macrophages. While the basic principles remain the same, when using this protocol for other cell types it will be important to adjust the media and growing conditions to best suit the cells used.

1. Before experimentation maintain J774 macrophages in serum supplemented (10 % FBS) DMEM media for at least 4 passages (from liquid nitrogen storage) before use, only use J774 cells between passages 4 and 15. Maintain J774 cells in T75 tissue culture flasks and incubate at 37 °C with 5 % CO_2 (**see Note 2**).
2. 24 hours prior to the start of the experiment, take a confluent T75 flask of J774 cells and seed into a 24-well plate at a concentration of $1x10^5$ cells per well in 1 ml serum supplemented DMEM. Incubate the plate for 24 hours at 37 °C, 5 % CO_2.
3. 1 hour before the start of the experiment, activate the seeded macrophages by removing the media and replacing with 1 ml serum free DMEM media supplemented with 150 ng/ml phorbol 12-myristate 13-acetate (PMA) per well. Incubate for 1 hour at 37 °C, 5 % CO_2
4. After 1 hour incubation, remove the media from each well and replace with 1 ml serum-free DMEM.
5. Infect the macrophage monolayer by adding 100 µl washed and opsonised fluorescently tagged *C. neoformans* ($1x10^7$ *Cryptococcus* per ml, see step 3 in '3.1 *Cryptococcus* preparation'). Incubate for 2 hours at 37 °C, 5 % CO_2.
6. After 2 hours incubation, aspirate the media from each well and wash gently with 37°C PBS to remove extracellular, non phagocytosed cryptococci. Repeat this wash step, checking periodically under a tissue culture microscope to check for remaining extracellular *Cryptococcus* cells (**see Notes 3 and 4**).
7. Once extracellular cryptococci cells have been washed away, add 250 µl accutase to each well and incubated for 15 minutes at 37 °C. After this incubation, gently pipette the accutase to disassociate infected macrophages from the growing surface and to create a single cell suspension.

3.4 Flow cytometer setup

The setup for each flow cytometer is different. This assay was developed using a FACSCaliber (BD Biosciences), it should be possible to perform this assay using any flow cytometer that can detect GFP.

1. Take the cell suspensions from the previous step. Fix the samples by adding an equal volume of fixing media (2% formaldehyde, 2 % fetal bovine calf serum in PBS) to the accutase samples (**see Note 5**).
2. To setup the flow cytometer, first calibrate the instrument using a non-fluorescent control – (macrophages infected with a non fluorescent *C. neoformans* strain). Using the dot plot output adjust the forward scatter (FSC-H) and side scatter (SSC-H) parameters to ensure that all populations of interest are visible. Using the same non-

fluorescent control also adjust the GFP detection channel (in this case FL1-H) using a histogram output to set the negative fluorescent signal to a baseline value (on most instruments this is three $\log_{10}$ from the detection maximum).

3. Analyse each sample, making sure to keep the same calibration settings throughout the experiment. To enable reliable comparison between samples, collect events for each sample over a fixed period. Ideally 10,000 events should be collected for each sample to provide reliable data.

3.5 Data analysis

1. **Phagocytosis –** Display the collected data on a dot plot with FSC-H on the 'X' axis and FL1-H on the 'Y' axis (as in figure 2). The rate of phagocytosis can be calculated by comparing the number of macrophages with intracellular yeast (Figure 2 'Region2') to the number of macrophages without intracellular yeast (Figure 2 'Region3').
2. **Intracellular proliferation –** For each strain, collect data for a series of time points post infection – 0 hours (2 hours post infection, immediately after washing away extracellular yeast), 18 hours (20 hours post infection), 24 hours (26 hours post infection) and 48 hours (50 hours post infection). Display the collected data for each time point on a dot plot with FSC-H on the 'X' axis and FL1-H on the 'Y' axis (as in figure 2). Draw a gate around the region of the plot containing macrophages with intracellular yeast (Figure 2 'Region2'). For each time point calculate the geometric mean fluorescent intensity of events within this gate. To calculate the intracellular proliferation rate (IPR) take the time point with the highest mean fluorescent intensity (usually 18 hours or 24 hours) , divide this mean fluorescent intensity by the mean fluorescent intensity of the events at the 0 hour time point.

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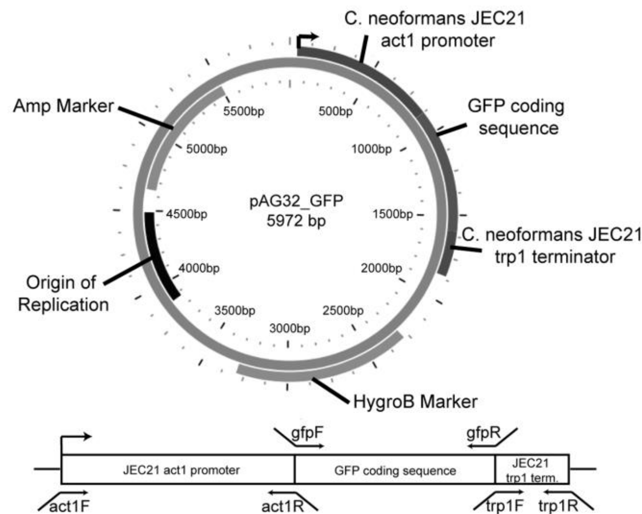

Figure 1 – Schematic layout of pAG32_GFP plasmid used to create H99-GFP and R265-GFP *(1)*.]

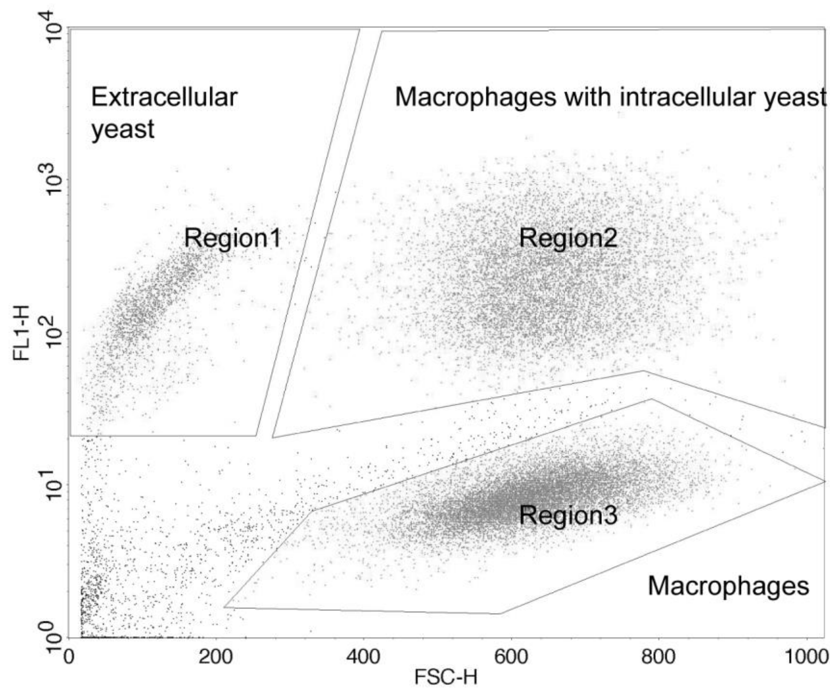

Figure 2 – Dot plot (FL1-H vs FSC-H) generated from flow cytometry data collected from a sample of macrophages infected with a GFP tagged *Cryptococcus* strain. Three cell populations have been 'gated' – Region1 Extracellular yeast (GFP high, FSC low), Region2 Macrophages with intracellular yeast (GFP high, FSC high) and Region3 macrophages that do not contain intracellular yeast (GFP low, FSC high).

4. Notes

1. To calculate the intracellular proliferation rate, the *Cryptococcus* count at the maximal time point (e.g. the time point when *Cryptococcus* count is highest) is divided by the count at the baseline (0hr) time point. Generally, the maximal time point is either 18 hr or 24 hr. At later time points the fungal burden within macrophages decrease due to macrophage lysis.
2. To confirm a newly transformed strain behaves like the original during infection, a number of tests should be performed. Firstly, the location of the insertion should be determined – e.g. Southern blotting, genome sequencing of flanking regions. Ensure that the transformed fragment has not inserted into a (known) gene. Additionally check how many copies of the fragment have inserted into the strain (multiple insertions are possible), multiple insertions help to increase a strain's fluorescent signal however excessive expression of transgenes can lead to sub-optimal growth due to increased cellular stress. Secondly the transformed strain should be tested in a number of culture conditions and in cell culture (preferably in the same cell line which will be used for the flow cytometry assay) to check for differences to the wild type, which again could have been caused due to insertion and/ or expression of the transgene. To see the full range of tests we performed on our H99-GFP and R265-GFP strains please refer to our previous publication ***(1)***. Finally when using the strain for flow cytometry analysis it must be confirmed that the fluorescent signal of the strain is resolvable from the background auto-fluorescence which all cells display. In the case of our strains the fluorescence signal was strong and easily discernible from non-transformed strains using spectral confocal microscopy ***(1)***.
3. For best results never split the cells less than 1/8, while passaging as sparse cell numbers affects cellular viability and potentially also immunological responses.
4. Three to four washes are usually sufficient to remove extracellular cryptococci, gentle tapping of the plate can be performed between washes to encourage cryptococci cells to unstuck from the plate surface.
5. While washing, care must be taken to avoid washing away macrophages – avoid pipetting liquid directly onto the growing surface, always pipette liquid down the edge of the well.
6. The protocol we describe herein is a one colour assay – needing only GFP excitation (395 nm) and emission (509 nm) filters. The filter set used for GFP is the same used for fluorescein isothiocyanate (FITC) – one of the most ubiquitous fluorophores in flow cytometry. Thus, almost all flow cytometers should be capable of analysing samples produced using the below protocol. Additional colours can be added to the protocol to allow the measurement of additional parameters such as phagocytosed / non phagocytosed cells or to measure macrophage or *Cryptococcus* phenotypes such as phagosomal acidification, cell viability etc.

7. When preparing macrophage samples for flow cytometry, different volumes of accutase and fixing solution can be used to optimise sample concentration. For flow cytometry the sample concentration should be adjusted such that the number of events per second detected by the instrument is in its optimal range, this range differs between instruments. Always ensure that equal volumes of accutase and fixing media are used however.