Chapter 14

Labelling of *Candida auris* cell walls to examine levels of PAMP exposure by flow cytometry and fluorescence microscopy

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Abstract Pathogen-associated molecular patterns (PAMPs) of the fungal cell wall are primary targets for the innate immune system of animals. Therefore, characterising PAMP exposure of fungal pathogens helps to elucidate how they interact with their hosts at a molecular level. Fluorescent labelling can be used to monitor exposure of multiple fungal cell wall PAMPs in a single experiment. Here, we describe a protocol to simultaneously label chitin, mannan, and β-1,3-glucan in *Candida auris* to study these PAMPs by fluorescence microscopy and allow high-throughput examination of their exposure by flow cytometry.

Key words: *Candida auris*, flow cytometry, fluorescence microscopy, PAMPs, cell wall, chitin, mannan, β-1,3-glucan.

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1. Introduction

Candida auris was first isolated from an ear infection in 2009 [1]. Since then, it has been responsible for life-threatening candidemia and outbreaks in intensive care units, with high levels of antifungal resistance (often against multiple drugs) being a hallmark of this pathogen [2]. Yet the infection process is still largely uncharacterised. To help understand the cellular features of infection progression, the fungal cell wall can be examined for the presence of pathogen-associated molecular patterns (PAMPs), which are recognised by pattern-recognition receptors (PRR) on host immune cells during infection [3]. The cell wall of *C. auris* has the same basic components as that of many other fungal species, including other Candida species [4–6]. To better understand the host-pathogen interaction of *C. auris* exposure of PAMPs on the surface of the cell wall can be studied.

One method to study exposed PAMPs on the cell surface is to use flow cytometry. It is an excellent high-throughput tool for analysis of multiple molecules of interest. The technique uses a fluidic system to analyse cell populations by measuring light scatter and fluorescence of single cells passing a laser and a detector [7]. Generally, measurements of a molecule of interest are produced by conjugating selected fluorophores to agents that bind the molecule of interest, such as lectins or pattern-recognition receptors, and detecting this fluorescence. The median fluorescent intensity for a cell population can be calculated and used to compare one experimental condition to another due to fluorescent intensity correlating with level of labelling of the target [8]. Flow cytometry is a technique that has been utilised effectively in immunophenotyping, cell cycle studies, proliferation assays, and many more applications [7]. While flow cytometry allows the cell population to be examined a complementary technique is to image individual cells with fluorescence microscopy [9]. Using flow cytometry avoids sample size biases that must be considered for microscopy, while fluorescence

microscopy allows visualisation of exposure at the single-cell level to distinguish between bud scaring and changes in overall PAMP exposure. Fluorescence microscopy has already been used to look at localisation of cell wall constituents and used to examine remodelling of the cell wall [10, 11].

For fungal cells, such as *Candida albicans*, flow cytometry has been used to analyse exposed cell wall PAMPs, notably β-1,3-glucan a key cell wall structure [12, 13]. Other cell wall PAMPs that influence immune cell recognition of fungal pathogens, such as *Candida* species, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, include mannan and chitin [3]. With changes in PAMP exposure and cell wall architecture documented in response to infection conditions, cellular stress induced by the host, and antifungal treatment, it is advantageous to be able to examine and quantify these changes [3, 12–15].

The labelling protocol presented here, measures the exposure of β-1,3-glucan, mannan and chitin on the cell surface of *C. auris*, e.g., this can be used to characterise how differential PAMP exposure could change interactions of the fungal cell with the immune system of a host. β-1,3-glucan exposure is detected using a recombinant protein of the C-terminal extracellular domain of the human pattern-recognition receptor Dectin-1 fused to an Fc domain allowing a secondary antibody conjugated to a fluorophore to bind. Exposed chitin is recognised by a lectin called wheat germ agglutinin. In a similar approach, exposed mannans can be detected by the lectin concanavalin A. Labelling of these PAMPs with pattern-recognition receptors and lectins enables the study of changes on the cell surface, between populations. *C. auris* cells labelled for these PAMPs can then be analysed by fluorescence microscopy or flow cytometry.

2. Materials

All solutions should be prepared with sterile, ultrapure, deionised water (ddH2O) unless stated otherwise and analytical grade reagents. Follow local regulations for disposal of liquid and solid waste.

1. 100 mM thimerosal: Dissolve 2 g in in 50 mL of ddH2O (see Note 1).
2. FACS wash: 900 mL of 1× phosphate buffered saline (PBS), add 100 mL of foetal bovine serum (FBS), add 0.146 g ethylenediaminetetraacetic acid (EDTA).
3. Fc-fusion protein of the human Dectin-1 pattern recognition receptor to human IgG Fc domain (see Note 2).
4. Suitable anti-Human IgG Fc secondary antibody conjugated to a fluorophore of choice (see Note 3).
5. Wheat Germ Agglutinin conjugated to a suitable fluorophore.
6. Concanavalin A conjugated to a suitable fluorophore.
7. Vortex mixer.
8. Haemocytometer (type Neubauer-improved).
9. Fluorescence microscope equipped with filter sets enabling the imaging of the chosen fluorophores and a 100× objective lens.
10. Anti-fade mounting medium.
11. Clear nail polish.
12. Immersion oil for microscopy.
13. Poly-L-Lysine coated microscopy slides.
14. Cover glasses 22 mm × 50 mm.
15. Flow cytometer capable of detecting the chosen fluorophores.
16. Flow cytometry analysis software.

3. Methods

3.1 Labelling cells

1. Grow cells in the desired condition(s).
2. Harvest cells by centrifugation 3,800 ×g for 5 minutes.
3. Retain cell pellet and approximately 200 µL of residual media.

4. Resuspended cells in an equal amounts of 100 mM thimerosal, giving a final thimerosal concentration of 50 mM (see Note 3).
5. Incubate for at least 16 hours in the dark at room temperature (see Note 4).
6. Centrifuge cells for 5 minutes at 2,400 ×g.
7. Discard the supernatant containing the thimerosal (follow local waste regulations).
8. Resuspend cells in 500 µL of 1× PBS by using a vortex mixer.
9. Centrifuge cells for 5 minutes at 2,400 ×g.
10. Retain cell pellet and discard supernatant.
11. Repeat steps 8-10 above, two more times to ensure that no residual thimerosal is present.
12. Resuspend cell pellet in 500 µL of 1× PBS using a vortex mixer (see Note 6).
13. Determine cell concentration using a haemocytometer.
14. Add 5×106 cells to a 1.5-mL reaction tube.
15. Centrifuge cells at 2,400 ×g for 5 minutes, remove excess 1× PBS.
16. Resuspend cells in 200 µL of ice-cold FACS wash using a vortex mixer.
17. Pellet cells by centrifugation at 2,400 ×g for 5 minutes and remove FACS wash.
18. Add to the pellet 100 µL of ice-cold FACS wash with human Fc-Dectin fusion protein (see Note 7).
19. Resuspend cells using a vortex mixer.
20. Incubate on ice for 45 minutes (see Note 8).
21. After incubation, pellet cells by centrifugation at 2,400 ×g for 5 minutes (see Note 9).
22. Discard supernatant and resuspend pellet in 200 µL of ice-cold FACS wash using vortex mixer.
23. Pellet cells by centrifugation at 2,400 ×g for 5 minutes, discard supernatant.
24. Repeat steps 22-23 above twice.
25. Resuspend cells in 100 µL ice-cold FACS wash with 1:200 secondary antibody conjugated to a fluorophore, 50 µg/mL Wheat Germ Agglutinin fluorophore-conjugate, 25 µg/mL of Concanavalin A fluorophore-conjugate (see Note 10).
26. Incubate on ice in the dark for 30 minutes.
27. Repeat steps 21-24 above.
28. Cells are ready to be prepared for microscopy (section 3.2) or flow cytometry (section 3.3).

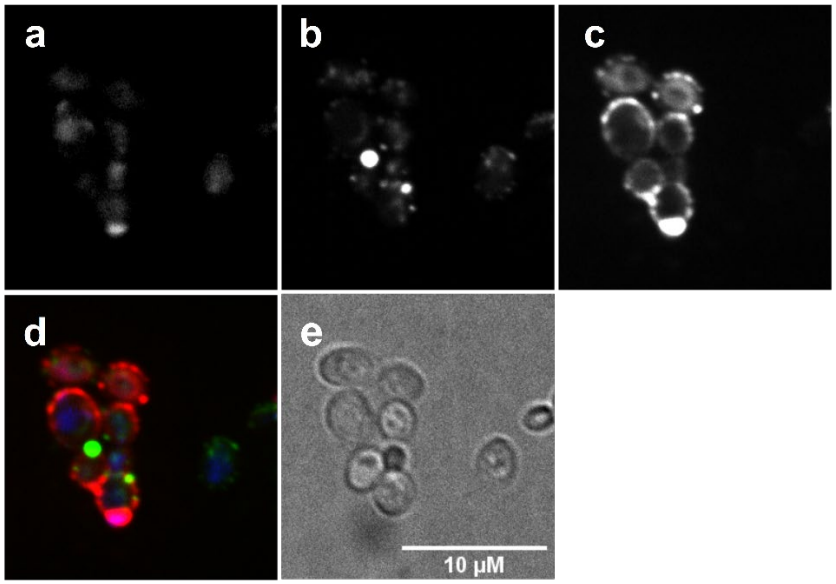

Fig. 1 Labelling of exposed *C. auris* cell wall PAMPs imaged by confocal microscopy. (**a-c**) Individual images of the fluorescent labelling of the given PAMP: (**a**) Chitin labelled with Wheat Germ Agglutinin Alexa Fluor 350-conjugate, (**b**) β -1,3-glucan labelled with Fc-hDectin-1a pattern-recognition receptor and F(ab')2-goat anti-human IgG Fc secondary antibody conjugated to Alexa Fluor 488, (**c**) mannans labelled with red Concanavalin A, Texas Red-conjugate. (**d**) Pseudo-coloured merged image of (a-c) with chitin in blue, β-1,3-glucan in green and mannans in red. (**e**) Differential interference contrast (DIC) image of the cells in (**a-c**).

3.2 Microscopy

1. Cells should be resuspended by pipetting 10 µL FACS wash up and down gently (see **Note 11**).
2. Add 10 µL of anti-fade mix by pipetting up and down slowly to mix cells.
3. Add 10 µL to a poly-L-lysine slide.
4. Place cover glass and gently press down to remove air bubbles.
5. Seal cover glass in place with clear nail polish.
6. Image straight away or store at 4 °C in the dark.
7. Image cells using a 100× objective lens with oil immersion (see **Note 12**).
8. Resulting images show where exposure on the cell wall is occurring (Fig. 1).

3.3 Flow Cytometry

1. Resuspend cells in 300 µL of ice-cold FACS wash and transfer to flow cytometry tube (see Note 13).
2. Cells are then ready to be run on a flow cytometer (see Note 14).
3. Using appropriate controls, set up laser power and threshold for cells (see Note 15).
4. Measure 50,000 events.
5. Transfer data to preferred software of analysis.
6. Gate for singlets and proceed with this single-cell population, this is done by analysis forward scatter height against forward scatter area (Fig. 2a).
7. A histogram showing a shift in median fluorescence intensity (Fig. 2b). The median fluorescence intensity of gated single-cell populations can then be directly compared graphically (Fig. 2 c-f).

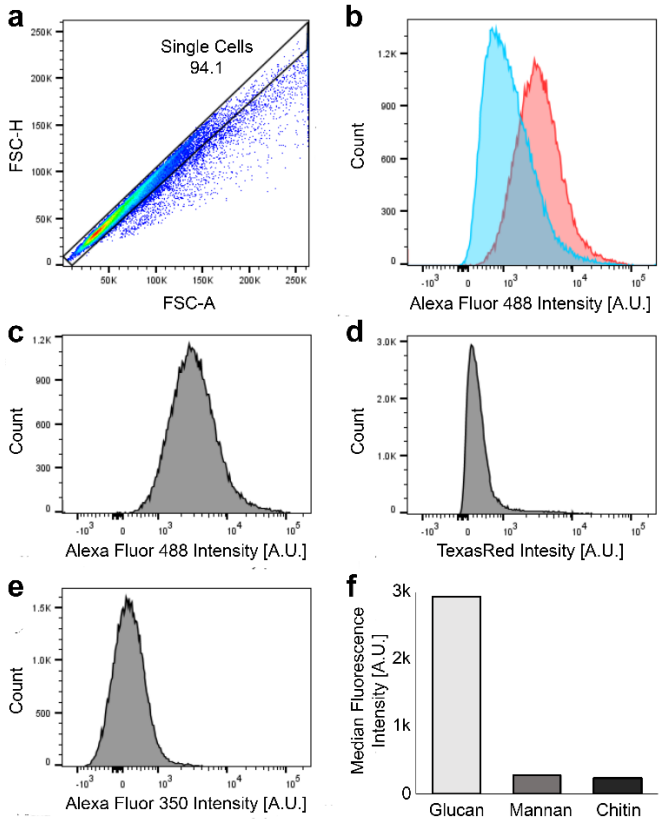

Fig. 2 Flow cytometry results. (**a**) Gating strategy used to gate single cells and exclude events that are budding cells and clumps of cells. (**b**) Histograms can be obtained to show a shift in in PAMP exposure between conditions. Here comparing one fluorescence intensity of β-1,3-glucan labelled with Fc-hDectin-1a pattern-recognition receptor and F(ab')2-goat anti-human IgG Fc secondary antibody conjugated to Alexa Fluor 488 between two experimental conditions. (**c-e**) Histograms of individual fluorophores can also be produced: (**c**), (**d**), (**e**). (**f**) Median fluorescence intensity for each PAMP can also be extracted from histograms and shown side-by-side as a bar chart. (**b-f**) Indicated fluorescent intensities are given in arbitrary units [A.U.] generated by the flow cytometry software.

4. Notes

1. Thimerosal powder must be handled in a fume hood with gloves to prevent inhalation and direct contact with the skin, also Thimerosal solution should be handled with gloves to prevent skin contact.
2. This protocol was optimised using an Fc-fusion protein from a commercial source. This was constructed by fusing the C-terminal region of human Dectin-1 PRR to a human IgG Fc domain.
3. This antibody must be able to bind the Fc domain of the protein being used to bind β-1,3-glucan. Here, a human IgG Fc domain was used and thus an anti-human secondary antibody is listed.
4. If cell suspension has a low pH, thimerosal will precipitate. If this happens, add 1 M NaOH (Dissolve 0.4 g NaOH pellets in 10 mL water) dropwise until the precipitate has redissolved.
5. Less than 16 hours might not completely kill and fix all cells. A test is recommended to ensure no viable cells are left after fixing by streaking cells on appropriate solid growth medium and incubate for 48 hours at 37 °C.
6. At this point cells can be stored in 1× PBS at 4 °C for 1 month, do not freeze.
7. A range of concentrations will need to be run prior to labelling final specimens, this is to check that binding is optimal for detection. These concentrations will also be specific to the fusion protein used.
8. The length of incubation is important here. Substantially shorter or longer incubation time can cause less than optimal binding or non-specific binding.
9. Do not use centrifugation speeds above this, as it can cause shearing of the antibody from the epitope and lead to loss of signal.
10. The concentration of secondary antibody indicated here is the manufacturer's recommendation. For the wheat germ agglutin conjugate and concanavalin A conjugate are used at concentrations above the recommended concentration to ensure cells are labelled fully.
11. Try and avoid introducing air bubbles, a short spin in a centrifuge can eliminate most but not all if they do occur.
12. Prepare a non-labelled control. This is used to make sure that the florescence signal is real and not autofluorescence, as some samples will have very little exposed chitin and β-1,3-glucan, and thus weak fluorescence. Length of exposure will depend on the light source and the sensitivity of the camera used.
13. The liquid volume that the cell pellet is re-suspended in and the receptacle might need to be adapted depending on which flow cytometer is used. Some recent machines utilise standard 1.5-mL reaction tubes, then cells can be resuspended in a smaller volume of liquid.
14. Analyse stained cells on the flow cytometer the same day or the next morning at the latest, keep samples at 4 °C in complete darkness. Otherwise fluorophores will lose brightness due to photobleaching, and this might affect results.
15. Settings and filter sets will be determined by the fluorophores and the flow cytometer used but must be kept the same between experimental replicates to ensure the comparability of results. If the threshold of the forward scatter is set too high this will result in only large cells being recorded, *C. auris* has a small cell size and events might be lost if not set up properly. Controls needed include an unstained control and a set of single stained controls. Proper set up with controls prevents false positives caused by autofluorescence (notably seen when using a 488 nm laser) and false negatives by ensuring that the fluorophore is being excited.

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