



# Quantitative Analysis of *Candida* Cell Wall Components by Flow Cytometry with Triple-Fluorescence Staining

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## Abstract

Detailed analysis of dynamic fungal cell wall components is crucial to our understanding of fungal systematics and the biology and physiology of fungal growth. In fungal pathogens this is of particular importance in examining their response to stress. However, current methodologies do not permit fast and accurate or quantitative analysis of cell wall carbohydrate components. Here, we provide a novel method permitting simultaneous quantitative analysis of the major cell wall components of *Candida* species relying on triple-staining with fluorescent labeling of chitin,  $\beta$ -glucans and mannans. Quantification is based on flow cytometry whereas qualitative analysis can be performed by direct imaging using fluorescence microscopy. We validated the method by determining the *in vitro* responses of different *Candida* species to the challenge of antifungal treatment with caspofungin. The assay facilitated rapid analysis of adaptive changes in cell wall composition upon caspofungin-induced damage. The method can be exploited for comprehensive quantitative analysis of *Candida* cell wall components, with relevant implications for clinical diagnosis of fungal infections in general.

**Keywords:** *Candida* spp.; Cell wall; Triple staining; Chitin; Glucans; Mannans; Flow cytometry

## Introduction

*Candida* species represent the most prevalent opportunistic fungal pathogens associated with mortality rates as high as 30-40% in the immunocompromised clinical setting [1-5]. Although *Candida albicans* is still the most common yeast pathogen, other *Candida* species such as *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *C. lusitaniae*, *C. guilliermondii* and *C. auris* have emerged as important pathogens. The emergence is partly attributable to prophylactic treatment strategies, and poses a challenge for effective therapy due to inherent resistance to a number of antifungal agents [1,6-21].

The fungal cell wall is the first point of contact with the host, and therefore a key player in resistance and virulence. It mediates adherence to host tissues, antigenicity and modulation of the immune response [22-24]. The *Candida* cell wall is a complex and dynamic structure composed of an inner chitin layer, adjacent to an outer glucan layer containing  $\beta$ -(1,3)-glucan and highly glycosylated mannoproteins. These are predominantly linked to the  $\beta$ -(1,3)-glucan framework via GPI-remnants connected to  $\beta$ -(1,6)-glucan linkers [22,24-34]. The fungal cell wall responds to and counteracts environmental stresses or damage by dynamic remodeling of its composition, and therefore plays a key role in immune recognition, fitness and resistance to antifungal drugs, such as echinocandins [35-39]. Several studies in murine models of candidemia using *C. albicans* showed increased representation of  $\beta$ -glucan and chitin in the cell wall during infection and drug treatment [39-45].

Echinocandins are antifungal agents displaying high efficacy against most *Candida* species [46-48]. They act by inhibiting the enzyme  $\beta$ -(1,3)-glucan synthase Fks1, thereby preventing assembly of the  $\beta$ -glucan layer. As a consequence, the cell wall is

weakened, rendering the cells more susceptible to lysis [46-49]. However, inhibition of a cell wall component can lead to defense mechanisms with compensatory enhancement of other cell wall components, repair and remodeling triggered by the Protein Kinase C (PKC) cell wall integrity pathway, including modification of the chitin layer [18,35,36,45,50,51]. The response increases echinocandin tolerance both *in vitro* and *in vivo* [18,36,45]. Quantitative analysis of *Candida* cell wall components has therefore become an important parameter in the assessment of stress responses potentially mediating resistance to antifungal therapy. Current methodological approaches rely on alkaline, acidic or enzymatic cell wall disruption to obtain individual components or isolated analysis of chitin and derivatives by spectroscopic methods [52-54,56]. Such approaches demand complex and time-consuming analytical methods such as high-performance anion-exchange chromatography and pulsed amperometric detection (PAD) [35,36,52,53,57-59].

Only recently, flow cytometry-based methodologies have emerged, enabling quantitative carbohydrate analysis of certain cell wall components [42,60-63]. However, current methods do not permit comprehensive analysis of all major cell wall components in a single assay. In addition, the analysis of live cells has not been possible. Here, we present a triple-staining flow cytometry-based method to determine the quantitative composition of the entire fungal cell wall. Six clinically relevant and phylogenetically diverse *Candida* spp., including *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. lusitaniae* and *C. guilliermondii* were employed to validate the method, demonstrating that exploitation of flow cytometry enables rapid and inexpensive quantitative analysis of all carbohydrate cell wall components.

## Materials and Methods

### Strains, Growth Conditions and Preparation for Staining

*Candida* strains used in this study are listed in Table 1. Strains were grown overnight in sterile-filtered yeast extract peptone dextrose (YPD) medium at 30 °C with agitation at 220 rpm. The cultures were subsequently diluted in fresh YPD medium to an OD<sub>600</sub> of ~0.3. After 4 h of incubation at 30 °C with agitation at 220 rpm, caspofungin (Merck & Co., Whitehouse Station, NJ) was added at the concentrations indicated in Table 1 and Figure S1 (1/12.5 of the corresponding Minimal Inhibitory Concentration (MIC)). The cultures, untreated or treated with caspofungin, were incubated for an additional 3 hours, and were then harvested by centrifugation at 0.4 g for 2 min. Cell pellets were washed once with 2 mL of 1x phosphate-buffered saline (PBS – NaCl, KCl, Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, dH<sub>2</sub>O, pH 7.4) and then resuspended with 500 µL of 1x PBS. Aliquots of 100 µL were transferred to fresh Eppendorf tubes, and the cells were harvested by centrifugation at 0.8 g for 2 min for subsequent single- or triple-staining of the cell wall components. Amber Eppendorf tubes were used for fluorescent staining to prevent dye degradation by exposure to light, while unstained controls were processed in standard Eppendorf tubes. The cell wall components were either stained individually or sequentially ( $\beta$ -glucans >mannans >chitin), if triple-staining was performed, as outlined below. All centrifugation steps for cell collection were performed at 0.8 g for 2 min.

Strain	Designation	MIC Caspofungin (µg/mL)	Caspofungin Stress (ng/mL)
<i>C. albicans</i>	SC5314 (77)	0.03125	2.5
<i>C. glabrata</i>	ATCC2001 (www.atcc.org)	0.125	10
<i>C. krusei</i>	ATCC6258 (www.atcc.org)	0.0625	5
<i>C. parapsilosis</i>	ATCC22019 (www.atcc.org)	0.25	20
<i>C. lusitaniae</i>	KK007 (clinical isolate)	0.0625	5
<i>C. guilliermondii</i>	KK167 (clinical isolate)	0.25	20

**Table 1:** *Candida* strains, Minimal Inhibitory Concentrations (MICs) and caspofungin-stress concentrations used

### Staining of $\beta$ -Glucans

*Candida* cells were resuspended in 1 mL of cold (4 °C) flow cytometry (FC) blocking solution consisting of 0.5% Bovine Serum Albumin (BSA), 5% HI-rabbit serum (Anti-rabbit IgG, New England Biolabs), 5 mM EDTA, and 2 mM NaN<sub>3</sub> in 1x PBS by incubation for 30 min in a sample mixer (Hula Mixer, Invitrogen) at room temperature (RT). Cells were then collected by centrifugation and washed three times by resuspending in 1 mL of cold FC washing solution consisting of 0.5% BSA, 5 mM EDTA, and 2 mM NaN<sub>3</sub> in 1x PBS. The cells were collected by centrifugation. The Fc: Dectin-1 protein [Fc (human): Dectin-1 (mouse) (recombinant.), Adipogen] diluted to a final concentration of 1 µg/ml in FC blocking solution was used for binding of  $\beta$ -glucans. An aliquot of 100 µL was used to resuspend the cells, and the solution was incubated for 60 min on ice. Cells were then collected by centrifugation and washed three times with 1 ml of cold FC washing solution. A 1:200 dilution of Alexa Fluor 488-conjugated anti-human IgG Fc antibody (Fc+488; Biolegend) was prepared in FC blocking solution. A total of 200 µL of this solution was then used to resuspend the cells, followed by incubation for 45 min on ice. Upon Dectin-1/Fc+488 binding, cells were collected by centrifugation, and washed three times with 1 ml of cold FC washing solution.

All centrifugation steps for cell collection described were performed at low speed (0.8 g) for 2 min. To avoid cell agglomerates, a solution of Tween 20 (0.05%) in 1x PBS or resuspension in FC buffer (1x PBS; 0.5 M EDTA; 0.5% BSA; 0.01% Tween 20)

was occasionally added. Alternatively, cell clumps were disintegrated by sonication (max power, 3 s) [64]. For experiments not requiring visualization and quantification of live cells, fixation with 1% formaldehyde was optionally performed.

### Staining of Mannans

ConcanavalinA Texas Red Conjugate (ConA-TRed) (LifeTechnologies) solution in 1x PBS was adjusted to a final concentration of 25 µg/mL. A total volume of 1 mL was used to resuspend the cells, and samples were incubated in the dark at 30 °C under shaking conditions at 500 rpm on a thermoshaker (Eppendorf) for 45 min. Cells were collected by centrifugation and the pellet was washed once with 1x PBS. Cells were finally resuspended in 500 µL 1x PBS and transferred into FC tubes.

### Staining of Chitin

A volume of 500 µL Calcofluor White (CFW, fluorescent brightener 28; Sigma) were added to the samples at a final concentration of 25 µg/mL, and samples were incubated for 10 min in the dark prior to flow cytometry analysis.

### Quantitative Measurement of Cell Wall Components by Flow Cytometry

The cell wall components of *Candida* species including chitin, β-glucans and mannans were quantified by a flow cytometry-based approach. Fluorescently-labelled cell wall components were measured in a BD Fortessa cytometer (BD biosciences). Triple-stained cells were measured using BV421 (violet laser, 405 nm wavelength, 50 mW power), FITC (blue laser, 488 nm wavelength, 50 mW power) and TRed (red laser, 640 nm wavelength, 40 mW power) channels to detect the fluorescence of chitin, β-glucans and mannans labelled with CFW, Dectin-1/Fc+488 and ConA-TRed, respectively. Controls included single-stained and unstained samples for each species tested: caspofungin-stressed, single-stained cells yielding strong fluorescence signals, and unstressed cells without staining. A total of 10,000 cells were analyzed according to the manufacturer's guidelines. The flow cytometry data were analyzed using the Flowjo software (Flowjo LLC, USA, version 7.6.5.) to assess the relative amounts of individual cell wall components.

### Fluorescence Microscopy

*Candida* cells were visualized under a confocal laser scanning microscope (CLSM, Zeiss LSM 700). The channels for bright field and fluorescence for DAPI, EGFP and TRed were applied to image the cell wall components chitin, β-glucans and mannans, respectively. A sample volume of 2.5 µL was pipetted onto a glass slide (VWR, Vienna, Austria) and mixed with 2.5 µL of CFW to yield a final concentration of 25 µg/mL. A 1.5 mm cover glass slip (VWR, Vienna, Austria) was placed on top. Imaging was performed under constant-time laser exposure for untreated and treated samples. Images were analyzed using the Fiji software (Open source Java image processing, NIH image).

### Statistical Analysis

The significance of differences between individual cell wall components of stressed (caspofungin-treated) *versus* unstressed cells was determined by using the T-test with one-tailed distribution for paired samples (Excel software). *P* values <0.05 were considered significant. The calculations were based on three independent biological replicates.

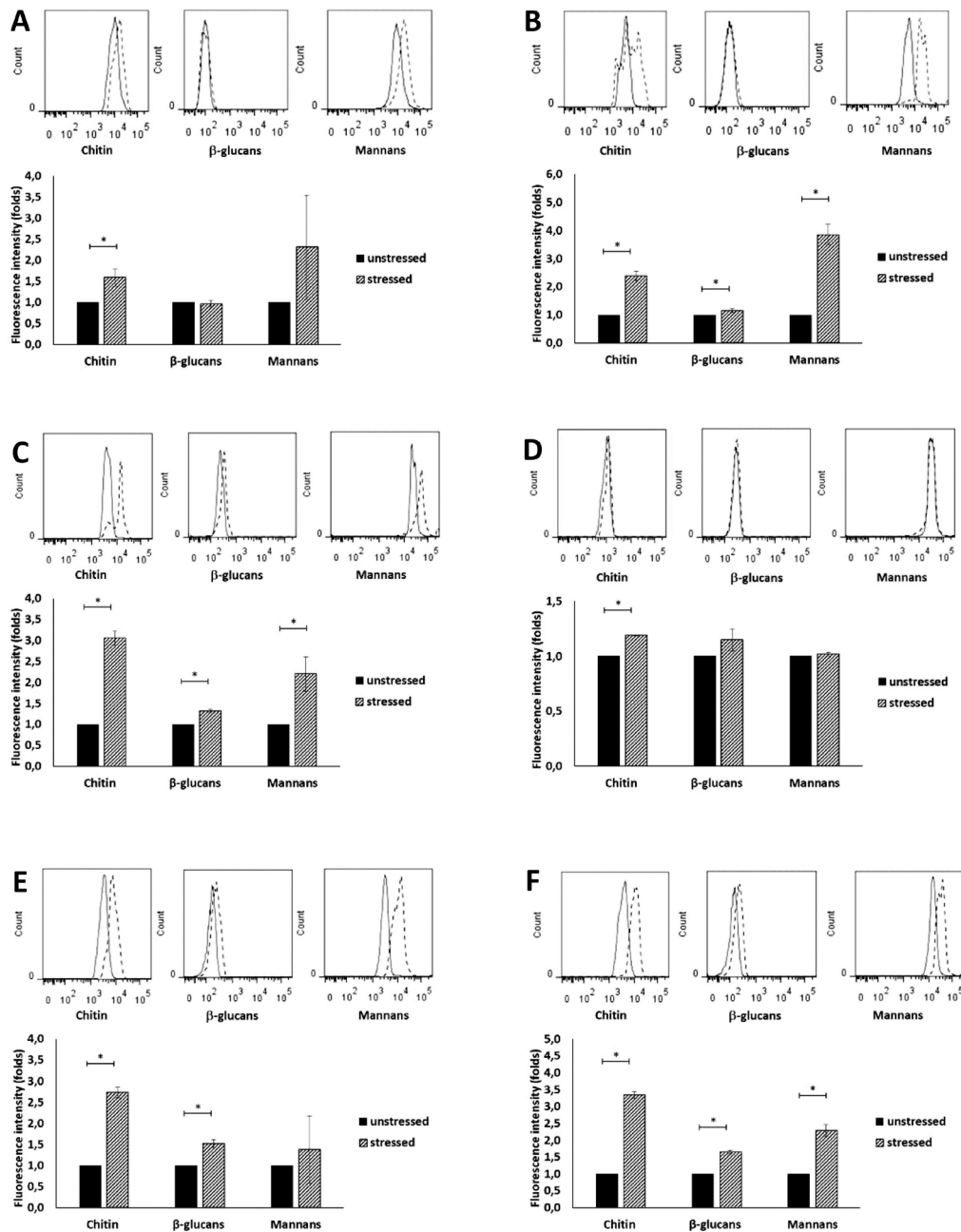
## Results

### Measurement of *Candida* Cell Wall Components by Flow Cytometry

We modified and improved a flow cytometric methodology for simultaneous quantification of all major cell wall components of *Candida* species. A triple-staining assay was used to fluorescently label chitin, β-glucans and mannans. Chitin was directly labelled with Calcofluor White (CFW) (blue color). β-glucans were detected by conjugation of anti-human Alexa Fluor 488 (green color) with the protein Fc-Dectin1, and mannans were labelled by conjugating ConcanavalinA to Texas Red (ConA-TRed). The spectral overlap of the fluorescent dyes used is limited, thus permitting simultaneous analysis. The efficacy of the method was demonstrated by testing six different clinical *Candida* species, including reference strains of *C. albicans*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*, as well as clinical isolates of *C. lusitanae* and *C. guilliermondii*, for which no commercial reference strains are available. Fungal cells were exposed to exogenous stress by treatment with caspofungin at concentrations corresponding to 1/12.5 of the respective minimum inhibitory concentration (MIC) values for each strain (Table 1, Figure S1). Fungal cells challenged with caspofungin and unstressed controls were stained for chitin, β-glucans and mannans and subjected to subsequent quantitative analysis of individual cell wall components by flow cytometry. The differential changes observed in the cell wall composition of chitin, β-glucan and mannan are displayed in Figure 1. For all measurements revealing significant differences, the calculated *p*-values were <0.05. Upon caspofungin treatment, *C. albicans* showed a significant increase of chitin levels (1.6-fold) when compared to unstressed cells, whereas no changes in the β-glucan and mannan components beyond the standard deviation were observed (Figure 1A).

*C. glabrata* revealed significant enhancement of all three cell wall components after exposure to caspofungin compared to untreated controls, (chitin 2.4-fold, β-glucan 1.2-fold, mannan 3.9-fold; Figure 1B), and similar observations were made for *C. krusei* (chitin 3.1-fold, β-glucan 1.3-fold; mannan 2.2-fold; Figure 1C). For *C. parapsilosis*, a significant increase in chitin content was observed

upon caspofungin treatment (1.2-fold), while no changes in  $\beta$ -glucan or mannan occurred (Figure 1D). In addition, clinical isolates of two *Candida* species including *C. lusitaniae* and *C. guilliermondii* were analyzed. While *C. lusitaniae* revealed significantly increased amounts of chitin (2.7-fold) and  $\beta$ -glucan (1.5-fold) upon caspofungin treatment (Figure 1E), *C. guilliermondii* showed significantly increased proportions of all three cell wall components (chitin 3.3-fold,  $\beta$ -glucan 1.7-fold, mannan 2.3-fold) (Figure 1F). To correct spectral overlap among the different fluorophores, compensation controls were set up independently for each *Candida* species, using single-stained and unstained samples. These served as a baseline for the assessment of changes in the cell wall composition mediated by exposure to caspofungin (Figure S2).



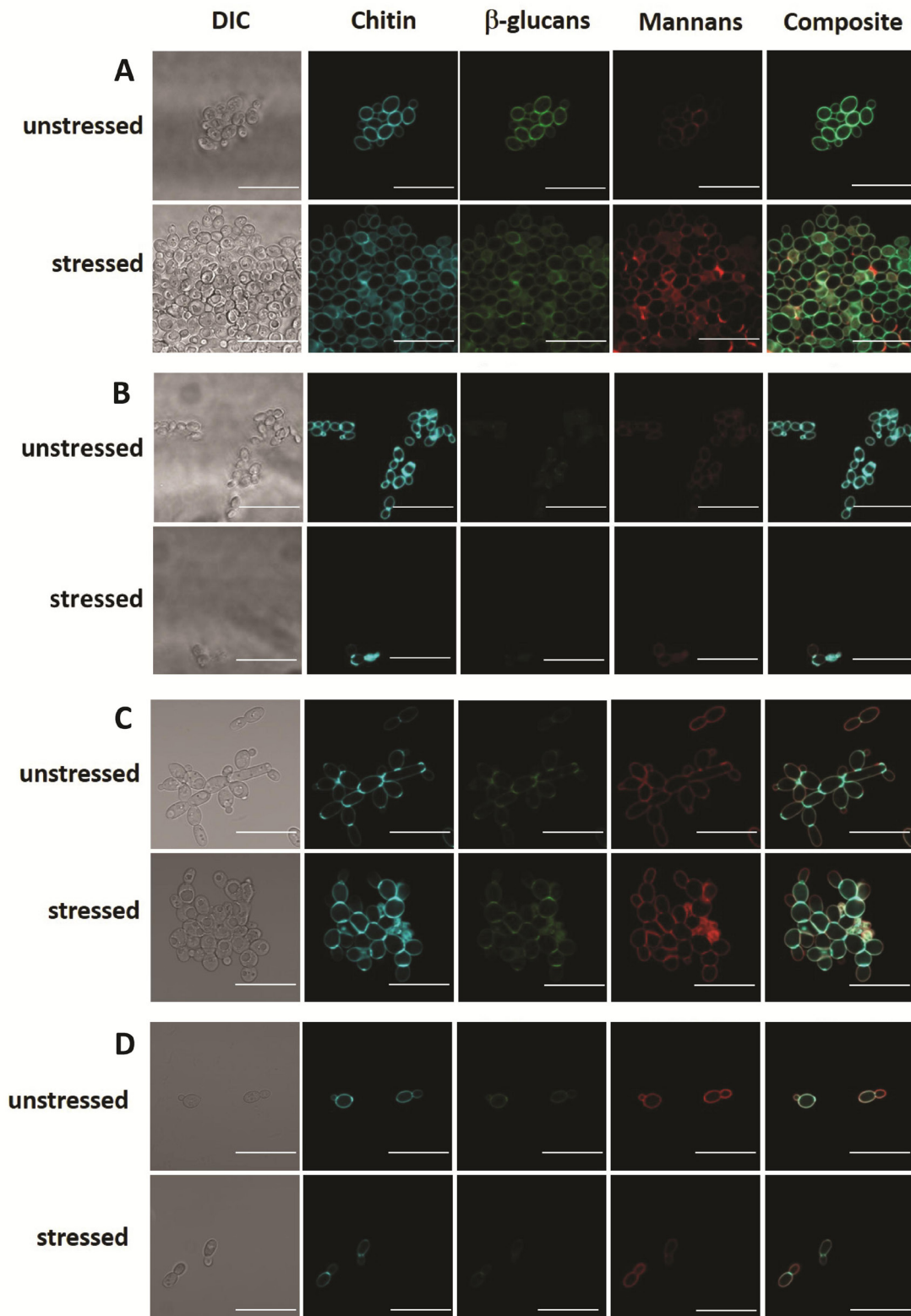
**Figure 1:** Quantitative analysis of cell wall components in *Candida* species by flow cytometry. The cell wall components chitin,  $\beta$ -glucans and mannans were quantified with a BD Fortessa cytometer using BV421, FITC and TRed lasers, respectively. A. *C. albicans*; B. *C. glabrata*; C. *C. krusei*; D. *C. parapsilosis*; E. *C. lusitaniae*; F. *C. guilliermondii*. The plots shown reflect unstressed (black peaks) and caspofungin-stressed cells (dashed peaks). Peak shifts on the flow cytometry plots from left to right indicate elevated fluorescence intensities reflecting an increase in the respective cell wall component. Quantification is indicated in folds of fluorescence intensity, with unstressed control samples set to 1. The data shown reflect results of three independent replicates and error bars represent the standard deviation (SD). Horizontal bars with an asterisk mark significant differences between stressed cells and controls ( $p$  value  $<0.05$ )

## Fluorescence Microscopy of *Candida* Species upon Caspofungin Challenge

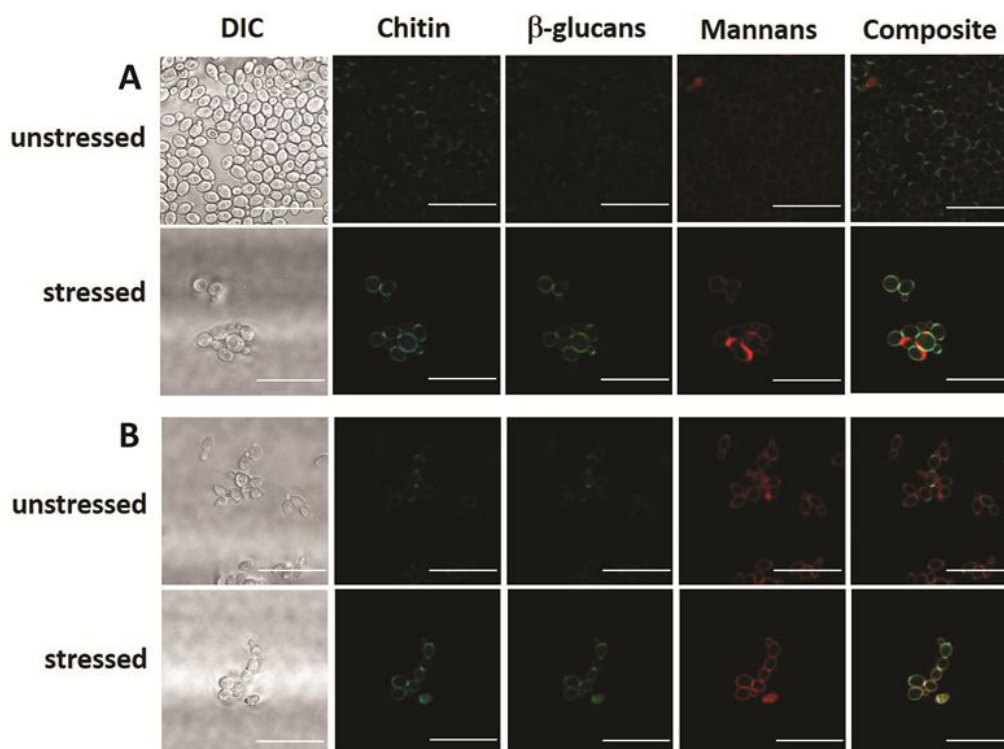
Triple-staining of *Candida* cell wall components was assessed by an independent technical approach using fluorescence microscopy. In addition to flow cytometric measurement, aliquots of each sample were subjected to imaging by confocal microscopy.



Simultaneous triple-staining of chitin,  $\beta$ -glucans and mannans could be visualized in all *Candida* species tested (Figure 2 and 3). Evaluation by microscopy did not permit precise quantitative assessment of cell wall components, but facilitated the imaging of changes in cell wall composition triggered by exposure to caspofungin (Figure 2 and 3).



**Figure 2:** Confocal microscopy of cell wall components in *Candida* reference strains. Triple-staining of chitin,  $\beta$ -glucans and mannans fluorescently labelled with CFW, Alexa Fluor 488 and TRed, respectively. **A.** *C. albicans*; **B.** *C. glabrata*; **C.** *C. krusei*; **D.** *C. parapsilosis*. Differential Interface Contrast (DIC) and fluorescent pictures are shown. The designation “unstressed” corresponds to non-treated cells whereas “stressed” corresponds to caspofungin-stressed cells. Higher fluorescent color intensity reflects an increase in the respective cell wall component. Laser exposure time was kept constant for each individual species. Images were processed using Fiji software. Scale bars represent 20  $\mu$ m



**Figure 3:** Confocal microscopy of cell wall components in clinical isolates of *Candida* species. Triple-staining of chitin,  $\beta$ -glucans and mannans fluorescently labelled with CFW, Alexa Fluor 488 and TRed, respectively. **A.** *C. lusitaniae*; **B.** *C. guilliermondii*. Differential Interface Contrast (DIC) and fluorescent pictures are shown. “Unstressed” corresponds to non-treated cells whereas “stressed” corresponds to caspofungin-stressed cells. Higher fluorescent color intensity reflects an increase in the respective cell wall component. Laser exposure time was kept constant for each individual species. Images were processed using Fiji software. Scale bars represent 20  $\mu$ m

## Discussion

The fungal cell wall is a point of contact with the host immune system, and plays a key role in responses to various stresses, ranging from host factors to antifungal treatment. Modulation of the cell wall composition is an important mechanism affecting the fitness of *Candida* spp. and facilitates the survival of the pathogens or immune evasion. Many studies have therefore focussed on the investigation of cell wall changes upon exposure to stress both *in vitro* and *in vivo* [1-3,9,29,39,40]. Challenge of *C. albicans* by different echinocandins including caspofungin was shown to result in upregulation of chitin synthesis leading to increased content of this constituent in the fungal cell wall [18,32,36]. Indeed, some strains exhibit “paradoxical growth” with enhanced growth in the presence of concentrations of caspofungin above the MIC [65-69]. These effects are regulated by activation of multiple signal transmission cascades triggered by the MAPK-Hog1/PKC and  $Ca^{2+}$ /calcineurin pathways [35]. Increased chitin amounts lead to enhanced cell wall resistance, elevated tolerance to antifungal treatment, and escape from the immune system [18,36,45]. Treatment with caspofungin and growth on alternative carbon sources can also lead to higher  $\beta$ -glucan content in the cell wall, and unmask this layer, exposing it to interaction with dectin-1 [41,42,70-72]. Dectin-1 is a C-type lectin serving as the prime  $\beta$ -glucan receptor, which mediates phagocytosis of *Candida* cells, and stimulates inflammation, T-cell activation and proliferation [73-75]. Unmasking of  $\beta$ -glucans can vary during the infectious process [41]. Mannans contribute to masking of the  $\beta$ -glucan layer, thereby preventing immune recognition, and elevated contents of this cell wall constituent can therefore promote evasion from immune attack [63,75]. Hence, changes in the fungal cell wall composition can mediate differential effects on the overall functional properties of the pathogens including their ability to escape from host immune response [76]. While the elevation of chitin and/or mannan content would result in increased resistance and improved fungal survival, the net effect of the observed changes involving  $\beta$ -glucans is less clear. Since the extent of cell wall modifications and the altered proportion of individual components may provide important information on the expected effect of adaptive changes, techniques permitting quantitative analysis of the cell wall components can provide important insights into fungal responses to various stresses including antifungal treatment.

Most existing methodologies which include mechanic and chemical disruption of the cells are based on alkaline and acidic hydrolysis of the fungal cell wall, and require rather laborious and meticulous controls to ensure complete extraction [35,36,52,53,57]. Flow cytometry has more recently been employed for analysis of individual cell wall components [42,60-63]. Here, we present a flow cytometry-based methodology facilitating simultaneous quantification of chitin,  $\beta$ -glucans and mannans of different *Candida* species based on triple-fluorescent staining. The use of fluorophores with low spectral overlap permits simultaneous detection of all major cell wall components. An important advantage of this method includes the ability to analyze live fungal cells and their cell wall modifications occurring upon exposure to specific stresses such as antifungal treatment. The entire procedure including cell

preparation, staining and quantitative readout by flow cytometry with concomitant visualization by fluorescence microscopy for simultaneous analysis of two *Candida* species can be completed within one working day. The applicability of the approach presented has been demonstrated for some of the most frequently occurring and clinically highly relevant *Candida* species, including both reference strains and clinical isolates. The method can conceivably be applied to quantitative analysis of cell wall components in a broad spectrum of *Candida* species as well as other *Candida*-like fungi, and can contribute to improved understanding of stress responses in these pathogens, with important implications for research and ultimately for clinical management of fungal infections.

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