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The use of new probes and stains for improved assessment of cell viability and extracellular polymeric substances in *Candida albicans* biofilms

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Abstract

Phenotypic and genotypic cell differentiation is considered an important feature that confers enhanced antifungal resistance in candidal biofilms. Particular emphasis has been placed in this context on the viability of biofilm subpopulations, and their heterogeneity with regard to the production of extracellular polymeric substances (EPS). We therefore assessed the utility of two different labeled lectins *Erythrina cristagalli* (ECA) and *Canavalia ensiformis* (ConA), for EPS visualization. To evaluate the viability of candidal biofilms, we further studied combination stains, SYTO9 and propidium iodide (PI). The latter combination has been successfully used to assess bacterial, but not fungal, viability although PI alone has been previously used to stain nuclei in fungal cells. *Candida albicans* biofilms were developed in a rotating disc biofilm reactor and observed *in situ* using confocal scanning laser microscopy (CSLM). Our data indicate that SYTO9 and PI are reliable vital stains that may be used to investigate *C. albicans* biofilms. When used together with ConA, the lectin ECA optimized EPS visualization and revealed differential production of this material in mature candidal biofilms. The foregoing probes and stains and the methodology described should help better characterize *C. albicans* biofilms in terms of cell their viability, and EPS production.

Key words: biofilm, *Candida albicans*, confocal scanning laser microscope, differentiation, stain

Introduction

Candida is an important, opportunistic, fungal pathogen and *Candida albicans* in particular is the most frequently isolated species from humans. Candidal infections range from superficial diseases of the skin and mucosae to deep tissue infections, some leading to death. Indeed, *Candida* is known to be the fourth most common cause of bloodstream infections in hospitalized patients [1].

The capacity of *Candida* to cause disease is closely associated with their ability to grow as biofilm communities, in contrast to planktonic growth in suspension. Candidal biofilms are spatially organized communities of cells encased in a matrix of extracelluar polymeric substances (EPS) on a substrate and are particularly characterized by increased resistance to antifungals [2, 3]. Although the mechanisms of drug resistance of candidal biofilms are ill understood, biofilm heterogeneity is thought to be a contributing factor for this phenomenon. It is well known that biofilm communities develop differential gradients in nutrients and metabolic wastes, so that groups of cells in the same biofilm react variably in response to environmental cues, leading to a variety of phenotypes.

Previous workers have used a number of dyes or stains to visualize these varying phenotypes in

biofilms [4–6]. For instance, in studies investigating antifungal resistance or differentiated cell viability of candidal biofilms, FUN-1 is usually used [7]. With this dye, the nonviable yeast forms emit a diffuse, green–yellow fluorescence whilst the metabolically active cells convert this chemical to single or multiple orange–red cylindrical cellular, intravacuolar structures (CIVS). However, FUN-1 can yield multiple CIVS in a metabolically active hyphal phase *C. albicans* cell [7], leading to a possible overestimation of live cells especially when the cell density is high. Thus, FUN-1 is not ideal for evaluating cell viability within candidal biofilms and effort should be made to search for better viability indicators. In the current study we used a LIVE/DEAD *Bac*Light Bacterial Viability kit commonly used for bacterial viability assessment [8, 9] to evaluate cellular viability in *Candida* biofilms.

Additionally, previous work on biofilm heterogeneity has focused mainly on cell viability [4–6], and little attempt paid to assess the metabolites of biofilms such as extracellular polymeric substances (EPS). To visualize EPS in candidal biofilms, we attempted to use combination of two different labeled lectins, *Canavalia ensiformis* (ConA) and *Erythrina cristagalli* (ECA) which bind to specific polysaccharide residues in EPS.

A number of microscopic techniques have been used for examination of biofilm EPS, such as scanning electron microscopy (SEM) in combination with an ice-freezing technique [10] and fluorescence microscopy (FM) [7, 11]. The former is a time-consuming technique, and neither can demonstrate the three-dimensional distribution of EPS or the differential EPS components of the biofilms. We therefore employed confocal scanning laser microscopy (CSLM), which has been previously used to investigate candidal biofilm architecture [11, 12], to characterize their EPS and cells.

Materials and methods

Organisms and growth conditions

C. albicans 192887*g*, an oral rinse isolate from an archival collection, described previously [13], was used in all the experiments as it is known to produce a robust biofilm [14]. The isolate was subcultured from thawed suspensions of pure isolates stored at the Oral Bioscience Laboratory at the Prince Philip Dental Hospital of the University of Hong Kong, Hong Kong, SAR China. The isolate identity was reconfirmed using the standard germ tube test and the commercially available API20C-AUX method (Bio-Merieux, France).

The *C. albicans* isolate 192887*g* was first cultured at 37 °C for 18 h in Sabouraud's dextrose agar (SDA) before overnight broth culture in yeast nitrogen base medium (YNB) supplemented with 100 mM glucose. Afterwards, the yeasts were harvested in the late exponential growth phase, washed twice with 5 ml phosphate buffered saline (PBS; pH 7.2; Ca2+- and Mg2+- free), and the final suspension adjusted to an optical density of 0.38 at 520 nm (approximately 10^7 cells/ml).

Candidal biofilm formation in a rotating disk biofilm reactor (RDR)

C. albicans biofilms were grown in a RDR comprising a working chamber (250 ml) and a waste tank [15, 16]. In the working vessel was a magnetic stir disk carrying eighteen removable polycarbonate coupons measuring $5 \times 3 \times 2$ mm, on which biofilms were allowed to develop, 2.5 ml of the standardized yeast suspension (10^7 cells/ml) and 247.5 ml of YNB containing glucose (100 mM) or galactose (500 mM) was aseptically transferred to the working chamber so that the final suspension had 10^5 yeast cells/ml. The reactor was initially operated in a batch mode for 90 min (adhesion phase) and then switched into the chemostat mode at a dilution rate of 0.10/h [15]. Biofilm formation was allowed to proceed for 72 h at 37 °C. The assays were carried out on three different occasions in triplicate.

Confocal laser scanning microscopy (CLSM)

Following biofilm formation in RDR, the coupons were removed and gently rinsed in PBS for 15 s so as not to disrupt the biofilms. Half of the coupons were then transferred to the wells of a 96-well plate containing a mixture of two lectins, *Erythrina cristagalli* (ECA) labeled with fluorescein isothiocyanate (FITC) (Vector Laboratories, Burlingame, CA, USA) (250 μg/ml) and *Canavalia ensiformis* A (ConA) labeled with tetramethyl rhodamine isothiocyanate (TRITC) (Molecular Probes, Eugene, OR) (200 μg/ml), according to the manufacturers' instructions, and incubated for 90 min in the dark

at 30 °C. ECA is a 54,000 dalton glycoprotein, which has a specificity towards galactose and galactoside residues (green fluorescence). In contrast, ConA, a lectin commonly used to mark bacterial EPS, binds specifically to mannose and glucose residues of polysaccharides (red fluorescence). These two labeled lectins were selected because they possess different polysaccharide specificities, and yield distinct fluorescence colors.

The remaining coupons with the biofilms were stained using the Molecular Probes' LIVE/DEAD *Bac*Light Bacterial Viability kit which is composed of SYTO-9 and propidium iodide (PI) (Molecular Probes, Eugene, OR). SYTO-9 is a green-fluorescent nucleic acid stain, generally labeling both live and dead microorganisms. PI, in contrast, is a red-fluorescent nucleic acid stain and penetrates only the cells with damaged membranes, thus visualizing dead microbes.

Pilot studies were performed to validate the use of the latter kit for the discrimination of live and dead *C. albicans*, since these dyes have previously used for vital staining of bacteria only. For this purpose, 4 ml of the standardized suspension of *C. albicans* (10^7 cells/ml) was prepared as described above and one of two aliquots of the suspensions was heated at 135 °C for 20 min to obtain dead cell suspensions. Afterwards, the heat-treated dead cell suspensions and the untreated live controls were mixed, as per the following volume proportions, i.e. 0: 10, 1:9, 3:7, 5:6, 7:3, 9:1, and 10:0. Each suspension (500 μl) was mixed with 1 μl SYTO9 and 1 μl PI from the LIVE/DEAD *Bac*Light Bacterial Viability kit and incubated for 20 min in the dark at 30 °C. Afterwards, the stained samples were viewed using CSLM. For each mixture of live/dead suspensions, four independent fields were randomly selected and, the live and the dead cells in each field counted at 400× magnification based on their differential fluorescent colors. The CSLM-determined percentages of live cells were calculated, averaged, and compared with the known values.

As these dyes appeared to well differentiate live/dead *Candida* cells (see below), the LIVE/DEAD *Bac*Light Bacterial Viability kit was used to label the cells within candidal biofilms derived from the main study. Thus the biofilms were incubated with SYTO9 and PI for 20 min in the dark at 30 °C before the CSLM examinations, as follows. Images of single focal plane or vertical section (Figure 4) of the stained biofilms were captured using a CSLM system (LSM 5 Pascal, Zeiss, Jena, Germany) which was equipped with two lasers (488 and 543 nm), a beam splitter NFT545, two filter sets (BP515-530 and LP560), three lenses (10X/NA0.5, 40X/NA0.8 and 63X/NA1.4) and an upright microscope. To avoid interference between the emitted fluorescent lights, a two-track mode was applied when two lectins were used. For the first tack (ECA), the sample was excited by the laser at 488 nm and the emitting light was collected by the filter set BP515-530 to capture the signal from FITC; for the second track (ConA), the laser of 543 nm and the filter set LP560 were used for the signal from TRITC according to the manufactures' instructions (Molecular Probes, Eugene, OR).

Statistics

Paired *t* test, Pearson's correlation and linear regression analyses were performed using SPSS 11.0 for windows. *P* values of < 0.05 were considered statistically significant.

Results

The sensitivity of SYTO9 and PI combination for live/dead Candida *cell differentiation*

We adopted a LIVE/DEAD *Bac*Light Bacterial Viability kit to stain cells within candidal biofilms. With this assay, cell viability could be assessed since the healthy cells with intact membranes stain fluorescent green, whilst those with damaged membranes stain fluorescent red. In pilot experiments, we mixed heat-killed and healthy cells of *C. albicans* in a volumetric series of proportions, then stained these suspensions with SYTO9 and PI, and observed using CSLM. As can be seen in Figure 1a and 1b, the heat-killed *Candida* cells exhibited red fluorescence whilst the controls showed green fluorescence, in agreement with the staining patterns observed in previous studies with bacteria [8, 9]. We also quantified the viable and nonviable yeast cells according to their distinct fluorescence emissions by visual counting, and calculated the percentage of viable cells per sample. These values were then compared with the known values derived through the volume proportions of the viable and nonviable cells in the

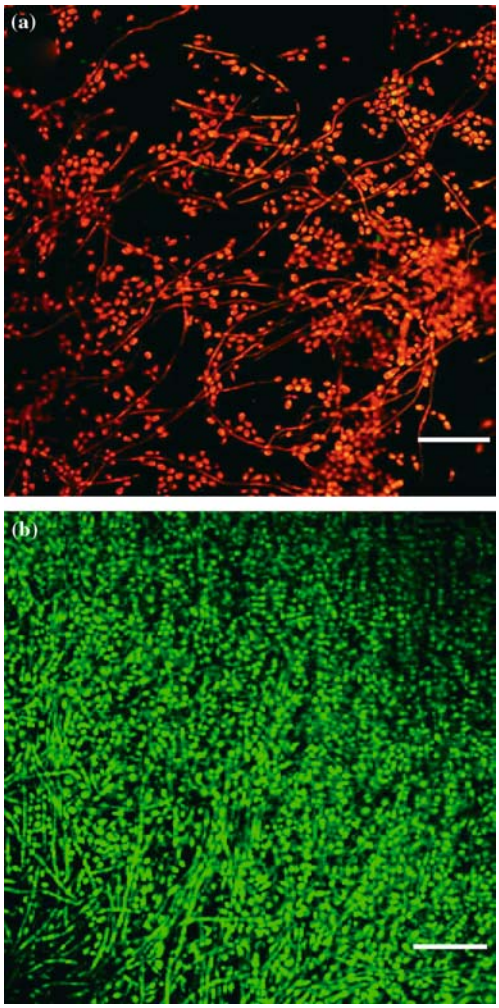

Figure 1. CSLM images of SYTO9 and PI stained *C. albicans* cells to show differential staining patterns of live and dead yeasts. (a) All the heat-killed cells stained red. (b) All the healthy cells were marked with green fluorescence. All scale bars represent 50 μm.

samples. As Figure 2 illustrates, there was a highly significant positive correlation between the results based on fluorescence emissions and the known values (Pearson coefficient = 0.994; $P < 0.0001$). Linear regression analysis revealed a regression coefficient of 0.944 and paired t test showed no significant difference between the two groups ($P = 0.424$).

CSLM illustrates biofilm heterogeneity in terms of EPS production and Candida *cell viability*

Candidal biofilms were grown in the RDR and examined using CSLM on day 1 and day 3. The

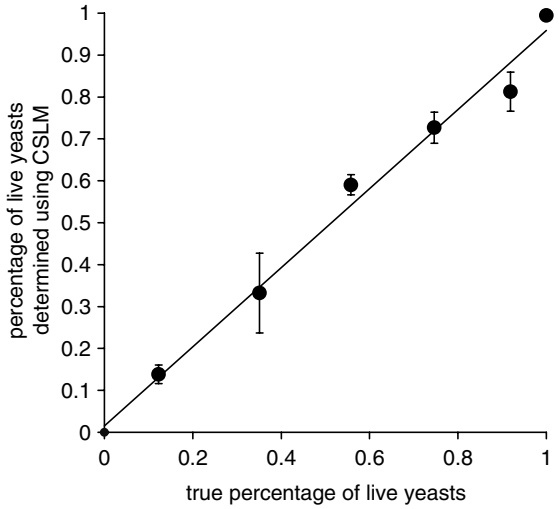

Figure 2. Correlation between CSLM-determined percentages of viable yeasts (using LIVE/DEAD *Bac*Light Bacterial Viability kit) and the known values derived through dilution of a calibrated suspension of *Candida* cells. Linear regression analysis revealed a regression coefficient of 0.944 ($P < 0.0001$) and paired t test showed no significant difference between the two groups ($P = 0.424$). Bars represent standard deviation (SD).

YNB medium was supplemented with either glucose (100 mM) or galactose (500 mM) so that biofilms grown under different nutrient supplies can be investigated. TRITC-labeled ConA and FITC-labeled ECA were used to detect biofilm EPS, while SYTO9 and PI were used for cell staining.

As seen in Figure 3, biofilm cells were encased in a grainy or diffuse mass of EPS. Interestingly, ECA and ConA exhibited distinct staining patterns: the former visualized EPS either on the cell surfaces or in between fungal cells; ConA, in contrast, highlighted fungal cell walls with a red ring appearance, and also stained the free-floating EPS, but to a lesser extent than ECA. The differential staining patterns between ECA and ConA may be due to the fact that the former has avidity for galactose and galactoside residues [17] whilst the latter binds specifically to mannose and glucose residues [18]. The distinct specificity of the two different probes makes it possible to qualitatively examine the heterogeneous cell wall structure/components and, EPS production in candidal biofilms. As shown in Figure 3, walls of biofilm cells took up the stain variably: some were positive with ConA alone (showing red fluorescence) and

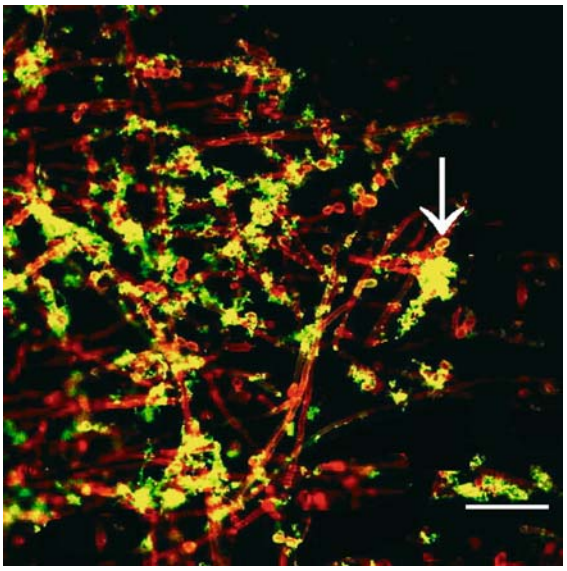

Figure 3. CSLM images of biofilm (72 h) EPS stained with the combination of ECA and ConA. Compared with ECA, ConA highlighted cell walls and stained EPS to a lesser extent. While the walls of most cells were positive for ConA alone (red fluorescence), some were both ECA and ConA (yellow fluorescence, arrow) positive. Thus, candidal biofilms appeared heterogeneous in terms of EPS production. All scale bars represent 50 μm.

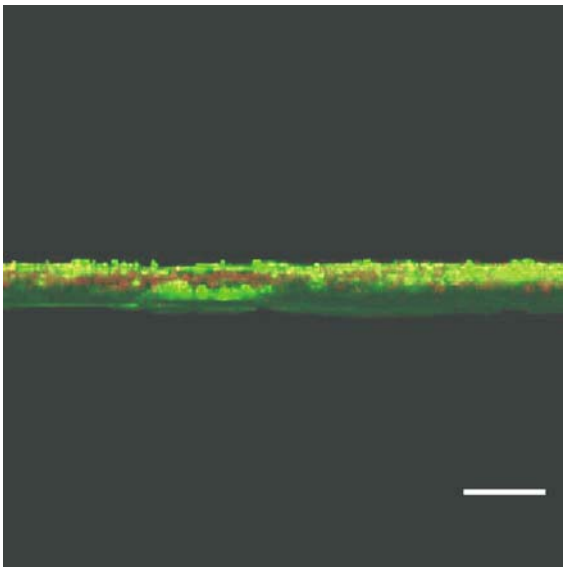

Figure 4. A lateral CSLM view of a *C. albicans* biofilm stained with the combination of SYTO9 and PI. The top and the bottom layers had relatively larger numbers of live cells (green) than the sandwiched middle layer (red). All scale bars represent 50 μm.

some with both (showing yellow fluorescence). A similar observation was noted for EPS interspersed between biofilm cells: some components with a green (ECA positive) and others acquiring a yellow stain (positive with both ConA and ECA). When the candidal biofilms grown in the media supplemented with glucose and galactose were compared, no apparent difference in EPS morphology or distribution was noted as in our previous studies (Jin et al., unpublished data).

In general, CSLM examinations of candidal biofilms cells, using the combination of SYTO9 and PI, demonstrated a mixture of hyphae, psedohyphae and buds. However, the viability of these biofilm components was not homogeneous: the majority of the biofilm cells was viable but a minority of red fluorescent nonviable cells was also noted. On visualization of the vertical axis of the biofilm, a tri-laminar distribution of the viable and nonviable cell mass was evident: the uppermost and the lowermost layers with predominant green, viable cells and, a middle, sandwiched, red-stained layer with nonviable cells (Figure 4). A temporal change in the proportion of dead cells was also noted: glucose-grown biofilms on day 3 exhibited higher dead/live ratio of cells than their day 1-counterparts (Figure 5c and 5d), although this trend was not observed with galactose-grown biofilms (Figure 5a and 5b). Further, the cells in the glucose-grown biofilms were distinctly denser than those grown in galactose, independent of the biofilm age. In addition, the aged, glucose-grown biofilms (on day 3) were somewhat less filamentous than their counterparts grown in galactose (Figure 5).

Discussion

SYTO9 is a small molecule with the capacity to penetrate cell walls with relative ease, and stain the cells green, regardless of their viability. In comparison, PI cannot traverse intact cell membranes so that only cells with compromised membranes can be labeled with this red fluorescent dye. Although the *Bac*Light kit has been used to evaluate a wide spectrum of bacteria [8, 9], to our knowledge it has not been used in fungal studies. Yet, our pilot study demonstrates that the combined use of SYTO9 and PI appeared to be reliable for visualization and differentiation of live/dead *Candida* cells (Figure 1a and 1b).

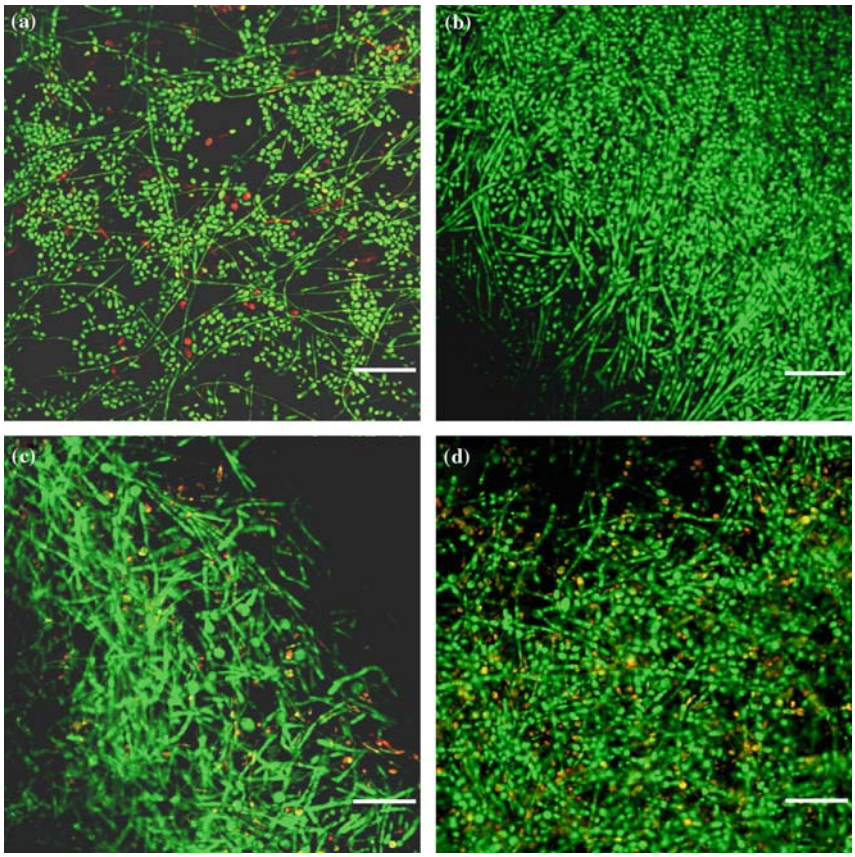

Figure 5. Microscopic characterization of *C. albicans* biofilms grown in media supplemented with glucose (100 mM) or galactose (500 mM). The stains used in CSLM examinations were SYTO9 and PI. CSLM images of (a) galactose-grown biofilms on day 1, (b) galactose-grown biofilms on day 3, (c) glucose-grown biofilms on day 1, and (d) glucose-grown biofilms on day 3. All scale bars represent 50 μm.

It is noteworthy that other workers have previously used FUN-1, a yeast-specific fluorescence dye (Molecular Probes, Eugene, OR), to evaluate yeast viability. However, the combined use of SYTO9 and PI has the following advantageous over FUN-1. First, a metabolically active hyphal phase *C. albicans* cell can contain multiple CIVS [7], leading to an over-estimation of live cells. In contrast, when labeled with the combination of SYTO9 and PI, the whole cell is uniformly stained either green (live) or red (dead), regardless of the cell morphology. Second, the fungal cells have to be provided with adequate nutrients during FUN-1 staining so that the viable cells can produce CIVS. This may not be the case of biofilm cells, particularly at the biofilm base. The combined use of SYTO9 and PI, in contrast, is based on the membrane integrity instead of the metabolic activity of cells, is not affected by the cell metabolism and, therefore, likely to be more suitable for viability testing of biofilms.

In this study ECA and ConA were used for the purpose of EPS examinations. ConA has been commonly used to visualize EPS in candidal biofilms [7, 11, 19] whilst ECA, to our knowledge, has not been used for this purpose. It has been revealed that glucose, galactose and mannose account for 31.4% of EPS dry weight [20]. Thus, ECA and ConA, which target the foregoing polysaccharide residues, are ideal candidates for visualization of EPS in candidal biofilms. More importantly, our data demonstrate that ECA and ConA bind to different moieties of EPS (Figure 3). Thus, these two lectins, in combination, can visualize more EPS components than either used alone. The different specificity to EPS components is important since a limitation of the lectin approach is that not all of the EPS compounds

present in the candidal biofilms can be stained. Therefore, EPS visualization can be improved by using lectins with different targets.

The use of the foregoing stains permitted exploration of candidal biofilm heterogeneity. As seen in Figure 5, even juvenile biofilm cells were not homogenously viable: a small proportion of cells being dead. Not surprisingly, this phenomenon was especially apparent with aging biofilms (on day 3) grown in glucose, possibly due to oxygen/nutrient starvation as a result of the increased cell mass. The vertical view of the biofilm (Figure 4) further showed that the dead cells were most frequently located in the mid-layer of the biofilm, sandwiched between the viable cells at the top and bottom. The top layer has ample oxygen and nutrients, thus its low proportion of dead cells is understandable. However, it is unclear why the basal layer contained less dead cells than the middle layer, given that the cells at the biofilm base are theoretically most inaccessible to nutrients and oxygen. One possible explanation for this phenomenon is the presence of intrinsic channels or canalliculi within *C. albicans* biofilms that permit transport of oxygen and nutrients to the bottom layers of the biofilm. Such structural features have been demonstrated in bacterial [21–23] and *C. dubliniensis* biofilms [24]. However, the presence of such structural elements does not explain the lower dead/live ratio in the biofilm base compared with the middle layer, since the latter can also benefit from the presence of the water channels. Further research is needed to investigate the factors associated with the intriguing phenomenon of cell death within biofilms.

The biofilm cells in our study were heterogeneous not only in their viability but also with regard to the cell wall components which are composed of predominantly polysaccharides (85%), proteins (5–15%), and lipids (2%) [25]. As Figure 3 clearly shows, some cell walls were ConA positive whilst others were positive for both ConA and ECA, providing further evidence for cell differentiation within candidal biofilms. Furthermore, our CSLM data reveal uneven EPS distribution, demonstrating heterogeneous EPS syntheses within biofilm cellular compartments.

Taken together, our study presents new data on the potential utility of multiple lectins and stains in the investigation of biofilm structure, EPS synthesis and cell viability in fungi. The combined use of ECA and ConA permits better characterization and visualization of biofilm EPS. SYTO9 and PI, which are usually used for bacteria, were proved reliable for discrimination of live/dead *Candida* cells. Their use will help better understand candidal biofilm heterogeneity in terms of EPS synthesis and cell viability.

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