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SIP-SRS Imaging of Cell Wall Synthesis Identifies a Synergy between Micafungin and Amphotericin B

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***andida* species causes life-threatening infections in immunocompromised individuals and presents a formidable challenge in clinical practice. This challenge is exacerbated by the growing prevalence of drug resistance, particularly against the last resort antifungals such as amphotericin B AmB). The fungal cell wall, known for its dynamic reorganization in response to growth demands and host threats, are recognized as a key drug target. In this study, stable isotope probe-assisted SRS microscopy SIP-SRS) is harnessed to directly visualize and interrogate the dynamics of fungal cell wall synthesis under various antifungal treatments. A striking observation is the thickening of the cell wall in newly synthesized daughter cells under AmB treatment. Based on this finding, a synergistic inhibition of fungal growth is demonstrated by AmB and micafungin, an antifungal agent targeting cell wall synthesis. These results not only advance the understanding of fungal physiology at the molecular level but also open promising avenues for combating drug-resistant fungal infections.**

1. Introduction

Fungal infections, particularly invasive candidiasis, affects millions of patients annually and has become an emerging crisis worldwide.[1] *Candida* species, one of the most significant opportunistic fungal pathogens, pose a global public health threat.[2] Several *Candida* species, including *Candida albicans* (*C. albicans*) and *Candida auris* *C. auris)*, cause opportunistic fungal infections and are responsible for 70 to 90 of all invasive fungal infections.[3] Early treatment with appropriate antifungal drugs is the key to reduce the mortality of severe candidiasis. Common antifungals that are used to treat invasive fungal infections either target fungal membrane sterols (polyenes), inhibit the synthesis of ergosterol (azoles), or inhibit the synthesis of β-glucan in the fungal cell wall (echinocandins).[4] Unfortunately, the widespread use of antifungals has resulted in increased antifungal resistance among different fungal species, which is an emerging threat to public health.[5] Fungal infections present a formidable challenge in clinical practice, exacerbated by the growing prevalence of drug resistance, particularly against antifungals such as amphotericin B (AmB). Despite intensive research efforts aimed at developing new antifungal agents and understanding resistance mechanisms, effective therapeutic solutions remain elusive.

AmB, despite its toxicity, has served as a last-resort antifungal with potent fungicidal activity against *Candida* infections for decades. However, the emerging resistance of *C. auris* isolates to AmB poses a significant threat of treatment failure for many patients. Numerous efforts have been made to enhance the efficacy and reduce the toxicity of AmB through the development of novel formulations. An alternative strategy involves combining AmB with potent enhancers, which may effectively overcome resistance without increasing the toxicity of AmB.[6]

Recent advances have also explored alternative approaches, such as the clinical translation of micafungin, with pivotal studies shedding light on its mechanism of action.[7] However,

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significant gaps remain in understanding the molecular mechanisms underpinning these therapies and the adaptive responses of fungi to combat treatment strategies.

Fungal cell walls are dynamic organelles that are essential for cell viability, morphogenesis, and pathogenesis.[8] In most fungal species the inner skeletal cell wall layer is composed of chitin, β-1,3-glucan and β-1,6-glucan. This branched β-1,3- and β-1,6-glucan is bound to proteins and/or other polysaccharides, whose composition may vary with the fungal species. In Candida species, the outer layer of the cell walls is heavily enriched with highly mannosylated proteins that are mostly attached via glycosylphosphatidylinositol remnants to β-1,6-glucan and to the β-1,3-glucan-chitin core. The cell wall composition is highly regulated in response to environmental conditions and external stresses. Cell wall structures can dynamically reorganize to accommodate growth demands and defend against host threats. Since the cell wall is absent in mammals, it has long been recognized as an attractive target for antifungal drug development.[9] This dynamic nature underscores the need for advanced imaging techniques to unravel the underlying processes and enhance our understanding of fungal cell wall adaptations.

Techniques such as stable isotope probing (SIP) allow for the precise tracking of isotopically labeled substrates within microbial cells, providing insights into metabolic pathways and substrate utilization.[7d,10] By incorporating stable isotopes like ^{13}C, D (^{2}H), and ^{15}N, SIP enables investigation of metabolic activities in a targeted and quantitative manner, offering a window into how microbiomes metabolize and respond to environmental changes and drug exposure.[10b,11] Raman-based SIP techniques have been hindered by their slow detection rates and low sensitivity to subtle metabolic changes, which can obscure real-time metabolic insights and limit the scope of dynamic observations. These drawbacks have constrained real-time observations of dynamic metabolic processes in complex biological systems.

To address the low speed issue in Raman-based SIP technique, SIP aided stimulated Raman scattering (SIP-SRS) imaging has emerged for meticulous metabolic investigations using isotopic tracers like D_2O and glucose-d7[12] and has demonstrated success in elucidating metabolism in bacteria, mammalian cells and animal models[12a,13] Despite these advances, SIP-SRS imaging of cell wall remodeling, a critical target of antifungal activity, remains underexplored.

In this study, we harness glucose-d7 based SIP-SRS to directly visualize fungal cell wall synthesis and interrogate cell wall remodeling under antifungal treatment. By leveraging the high sensitivity and spatial resolution of SIP-SRS, we uncovered previously inaccessible details of metabolic interaction with antifungals. A striking observation was the thickening of the daughter cell wall after AmB treatment. This finding led to the development of a combinational AmB and micafungin treatment for synergistic inhibition of fungal growth. These results not only advance our understanding of fungal physiology at the molecular level but also opens promising avenues for combating drug-resistant fungal infections. By elucidating the intricate interplay between antifungal medications and fungal metabolic responses, our study contributes to ongoing efforts of developing effective treatments against resilient fungal pathogens.

2. Results

2.1. SIP-SRS Imaging Reveals Newly Synthesized Cell Wall via Glucose-d7 Metabolism

Glucose is a primary source of energy for *C. albicans* to synthesize its cell wall through glycolysis (**igure 1**A).[14] The fungal cell wall is composed mainly of β-glucan, chitin, and glycoproteins, with the proportions of these components dynamically changing in response to environmental conditions.[15] We first validate that glucose-d7, a glucose isotopologue with deuterium labelling on all its carbons, can be used as a metabolic probe for SRS imaging. This approach enables precise visualization of fungal cell wall synthesis, providing critical insights into the dynamic remodeling of the cell wall under varying conditions. The schematic of our SRS microscope is shown in Figure 1B. In brief, the spatially and temporally overlapped pump and Stokes pulses are tuned to match the vibrational frequency of Raman-active modes. The SRS signal appears as an intensity gain in the Stokes beam and an intensity loss in the pump beam, which is extracted through a lock-in amplifier. In our setup, the stimulated Raman loss is measured, in which most excitation power is in the 1040-nm Stokes beam having a high cellular damage threshold. The carbon-deuterium (C–D) vibrational band, located in the cell-silent region (1800–2600 cm^{-1}), is spectrally differentiated from endogenous Raman bands, enabling selective detection using SRS generated by chirped femtosecond laser pulses. Specifically, strong C–D signals in *C. albicans* SC5314 can be observed after incubation in glucose-d7-substituted medium within 1 h via hyperspectral SRS imaging in the C–D stretching vibrational region (2050–2300 cm^{-1}), confirming the incorporation of deuterium to form C–D bonds in individual cells (Figure 1C,D). Cell morphology was visualized using cross-phase modulation (XPM) images. As a control, minimal SRS C–D signals were observed in cells cultured in normal medium (Figure 1C,D). The C–D signal in both the control group and the non-cell regions of glucose-d7-treated samples was much lower than in the cellular regions of the glucose-d7-treated group, confirming that the observed signals are specific to newly synthesized biomolecules. To verify that the signals arise from metabolic activity, we compared conditions where cellular metabolism was active versus inhibited. Live *C. albicans* SC5314 cells cultured in glucose-d7-containing medium at 30 °C exhibited increasingly strong C–D signals over time (Figure S1, Supporting Information), consistent with active metabolism and incorporation of deuterium into newly synthesized biomolecules. In contrast, cells incubated at 4 °C, where metabolic activity is suppressed, showed negligible C–D signals, indicating that no significant abiotic H–D exchange occurs on preexisting C–H bonds. In a control group cultured without glucose-d7, no C–D signals were detected. Importantly, all cell samples were thoroughly washed with PBS multiple times prior to SRS imaging to remove any residual free glucose-d7. This step ensures that the detected C–D signals represent intracellular incorporation into macromolecules, not extracellular or unincorporated glucose. Collectively, these experiments confirm that the C–D signals are predominantly a result of deuterium incorporation into newly synthesized biomolecules through metabolic processes.

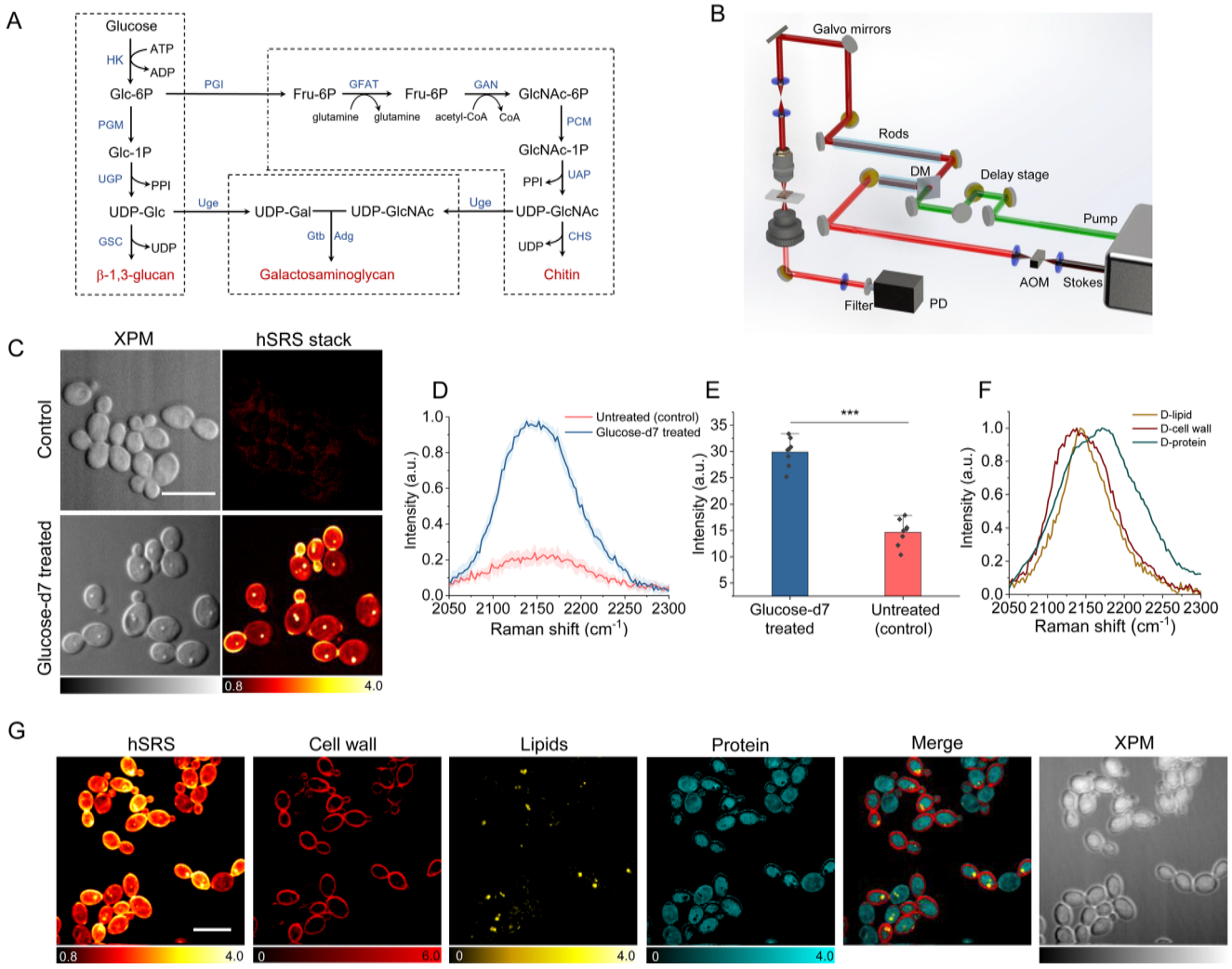

Figure 1. SIP-SRS imaging of newly synthesized cell wall via glucose-d7 metabolism. A) Scheme of cell wall polysaccharides synthesis via glycolysis in *C. albicans*.[14] B) Schematic illustration of the SRS setup. AOM: acousto-optic modulation. DM: dichroic mirror. PD: photodiode. C) SRS and corresponding XPM images of *C. albicans* after culture in normal and glucose-d7-containing medium for 1 h. Scale bar: 10 m. D) Average SRS spectra of individual *C. albicans* cells after culture in normal and glucose-d7-containing medium. N ≥ 15 per group. E) Average SRS C–D intensity plot for *C. albicans* in C) with N ≥ 10 per group. Significance was evaluated using an unpaired *t*-test (***, $p < 0.001$). F) Reference spectra for hSRS spectra unmixing analysis using LASSO. G) The decomposed hyperspectral SRS images into cell wall, deuterated lipids, and deuterated protein channels, and the corresponding XPM images of *C. albicans*. Scale bar: 10 m.

Newly synthesized daughter cells were identified based on their smaller size, budding morphology, and spatial attachment to larger mother cells.[16] In budding yeast, cells divide asymmetrically by orienting the spindle perpendicularly to the site of cell division (bud neck) during the early stages of the cell cycle.[17] Interestingly, the daughter cells showed stronger SRS signal contrast than the mother cells, indicating that the C–D signals originated from newly synthesized macromolecules (Figure S2, Supporting Information). We examined the budding area between the mother cells and the daughter cells which showed strong C–D intensities. We then acquired fluorescence imaging after labeling multiple layers in the cell wall of *C. albicans* (Figure S3, Supporting Information). The fluorescence image of chitin is remarkably strong between the mother cells and the daughter cells, while the mannan layer is a uniform ring shape in individual fungal cells. Further quantification analysis of single cell indicated distinctly higher C–D intensities at the glucose-d7 treated cell than the untreated cells (Figure 1E). These results were further confirmed by spontaneous Raman spectra (Figure S4, Supporting Information), showing a broad peak (2050–2300 cm^{-1}) at C–D vibration for *C. albicans* cultured in glucose-d7-substituted medium. The single cell SRS spectra from the cell wall region, cytoplasmic region, and the lipid droplets regions are largely different (Figure S5, Supporting Information).

To reveal the specific chemical mapping at the single cell level, we performed spectral unmixing using a pixel-wise least absolute shrinkage and selection operator (LASSO) regression algorithm. This method applies a sparsity constraint during spectral decomposition, enabling the selective inclusion of only the most relevant reference spectra at each pixel. Specifically,