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High-Throughput Monitoring of Pathogenic Fungal Growth Using Whole Slide Imaging for Rapid Antifungal Susceptibility Assessment

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Abstract

Invasive fungal infections are a major health threat with high morbidity and mortality, highlighting the urgent need for rapid diagnostic tools to detect antifungal resistance. Traditional culture-based antifungal susceptibility testing (AFST) methods often fall short due to their lengthy process. In our previous research, we developed a whole-slide imaging (WSI) technique for the high-throughput assessment of bacterial antibiotic resistance. Building on this foundation, this study expands the application of WSI by adapting it for rapid AFST through high-throughput monitoring of the growth of hundreds of individual fungi. Due to the distinct "budding" growth patterns of fungi, we developed a unique approach that utilizes specific cell number change to determine fungi replication, instead of cell area change used for bacteria in our previous study, to accurately determine the growth rates of individual fungal cells. This method not only accelerates the determination of antifungal resistance by directly observing individual fungal cell growth, but also yields accurate results. Employing *Candida albicans* as a representative model organism, reliable minimum inhibitory concentration (MIC) of fluconazole inhibiting 100% cells of *Candida albicans* (denoted as MIC_{100}) was obtained within 3h using the developed method, while the modified broth dilution method required 72h for the similar reliable result. In addition, our approach was effectively utilized to test blood culture samples directly, eliminating the need to separate the fungi from whole blood samples spiked with *Candida albicans*. These features indicate the developed method holds great potential serving as a general tool in rapid antifungal susceptibility testing and MIC determination.

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Disclosure statement
No potential conflict of interest was reported by the author(s).

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Keywords

Antifungal susceptibility testing (AFST); Candida albicans; minimum inhibitory concentration (MIC); rapid; whole slide imaging (WSI)

Introduction

Invasive fungal infections, especially those caused by *Candida* species, are associated with high morbidity and mortality to the immunocompromised population (Arendrup 2010; Bassetti et al. 2014; Kollef et al. 2012; Pfaller and Diekema 2007). In the past few decades, the empirical use of antifungal agents has promoted the emergence and spread of drug resistance in fungi which are normally susceptible to the treatment (Kanafani and Perfect 2008; Oxman et al. 2010). As recently reported, *Candida auris* have become multidrug resistant and thus life-threatening (Cortegiani et al. 2018; Forsberg et al. 2019; Lockhart 2019; Meis and Chowdhary 2018). The increasing prevalence of antifungal resistance in *Candida* species complicates treatment, underscoring the need for effective management and diagnostic strategies to mitigate the impact of these potentially devastating infections. Typically, healthcare providers employ antifungal susceptibility testing (AFST) to guide the prescription of antifungal drugs. The gold standard AFST methods, such as broth microdilution and disk diffusion, are based on the observation of visible fungal growth in the presence of antifungal drugs (Arendrup et al. 2012; Espinel-Ingroff 2007; Pfaller et al. 2011). To facilitate standardized AFST, commercial instruments with greater automation capability, such as VITEK-2 (bioMérieux, France), have been developed (Cuenca-Estrella et al. 2010). However, the culture-based methods typically take up to several days (Cantón, Espinel-Ingroff, and Pemán 2009), resulting in urgent need of rapid AFST to accelerate the initiation of appropriate antifungal treatment. Novel AFST approaches relying on the detection of specific genetic mutations or changes of proteome corresponding to antifungal resistance by molecular techniques, such as DNA sequencing, real-time polymerase chain reaction (PCR), and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS), could be promising alternatives (De Carolis et al. 2012; Dudiuk et al. 2014; Marinach et al. 2009; Pham et al. 2014; Sanguinetti and Posteraro 2017; Vatanshenassan et al. 2019; Vella et al. 2017; Vella et al. 2013; Zhao et al. 2016). Although molecular methods are known for their sensitivity and rapidness, phenotype based antifungal resistance to drug test is essential for reliable results (Sanguinetti and Posteraro 2017). Consequently, there is an urgent need for rapid antifungal susceptibility testing techniques that do not compromise on accuracy. (Song and Lei 2021)

In our previous research, we developed a WSI-based antibiotic susceptibility testing (AST) approach to determine the antibacterial susceptibility by high-throughput monitoring single-bacterium growth (Song et al. 2019). In this study, we expand this WSI platform by adapting it for the high-throughput monitoring of fungal growth at the single-cell level. Given the unique “budding” growth patterns of fungi, which significantly differ from those of bacteria in our previous study, we cannot correlate the bacterial surface area to the cell duplication. Instead, the new approach relies on tracking changes in the “budding” number of fungal cells to assess fungal replication. By focusing on cell number, we can more precisely

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determine the growth rates of individual fungal cells. To validate our counting approach for rapid antifungal susceptibility testing, we performed time-lapse imaging on hundreds of *C. albicans* cells, enabling simultaneous monitoring of their growth at the single-cell level. The minimum inhibitory concentration of fluconazole inhibiting 100% cells of *Candida albicans* (denoted as MIC_{100}) was determined within 3 h using the developed method, whereas the same reliable results obtained from the modified broth dilution method typically took 72 h. Moreover, our approach was effectively utilized to test blood culture samples directly, eliminating the need to separate the fungi from blood samples spiked with *Candida albicans*. These characteristics position the developed method as a promising tool with substantial potential for rapid antifungal susceptibility testing and minimum inhibitory concentration determination for fungi.

Materials and methods

Materials and instruments

Dextrose and peptone were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used to prepare Sabouraud dextrose broth (SDB). Agarose was bought from Promega, located in Madison, WI, USA. Silicon wafers were acquired from University Wafer in Boston, MA, USA. Chloramphenicol, fluconazole, polystyrene petri dishes with a diameter of 100 mm, and glass microscope slides were acquired from Thermo Fisher Scientific, based in Waltham, MA, USA. *Candida albicans* (*C. albicans*) (strain SC5314) is a laboratory strain originally isolated from the blood of a patient with disseminated candidiasis (PMID: 6394964). Human whole blood was purchased from Zen-Bio, Inc. (Research Triangle, NC, USA). Image acquisition was conducted using BZ-X800 All-in-One fluorescence microscope (Keyence Corporation, Japan).

Fungal cell culture

As the culture medium for *C. albicans*, SDB medium was prepared with 40 g/L dextrose, 10 g/L peptone, and 0.05 g/L chloramphenicol. The pH of the medium was adjusted to 5.6 and filtration-sterilized through 0.2 μm PVDF membrane (EMD Millipore, Burlington, MA, USA). After overnight culture of stocked *C. albicans* in a 37°C shaker, the cells were diluted to the required concentrations using phosphate buffered saline (PBS) for testing.

Sample preparation and whole slide imaging (WSI)

The general procedure for the fabrication of the culturing device and gel preparation for whole slide imaging have been reported elsewhere (Song et al. 2019). In brief, two microscope glass slides, spaced with 0.38 mm-thick silicon wafers, were set in a petri dish. SDB medium with 0.6% agarose was autoclaved, cooled, and mixed with fluconazole, and then poured into the dish between the slides. After gel solidification at room temperature, the top cover slide was removed to reveal a flat thin gel pad. 2.5 μL *Candidas* suspension was then applied to the gel, and once dry, the gel pad was cut out and transferred onto a new slide, ready for WSI. To conduct WSI, the prepared sampling slides were positioned on the imaging stage of a microscopy cell incubator, ensuring the fungal gel side faced the supporting slide. The microscope's auto-search function identified the sample area, followed by manual focusing of cells in 10 different fields of view (FOVs) for precise autofocus

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during scanning. The imaging software then directed the sample holder stage to capture bright-field image tiles across the entire area. Each tile, measured at 0.362 mm × 0.272 mm, was captured using a 40× objective lens. These tiles were then automatically stitched into a composite image by the BZ-X800 analyzer software. After the initial scan, the incubator was set to 37°C, and subsequent scans were performed at 0.5-h intervals up to 3 h.

Image analysis

The number of cells was read from phase contrast images. Numbers originated from the same cell were plotted over time to quantify the cell growth rate.

WSI-enabled AFST for C. albicans suspension and spiked blood samples

To assess the precision of the WSI-based AFST method for Minimum Inhibitory Concentration (MIC) determination, we employed *C. albicans* and fluconazole as the test fungus and antifungal agent, respectively. We treated *C. albicans* with a range of fluconazole concentrations (0, 4, 8, 16, 32, 64, and 128 μg/mL), incorporated into the gel pad of culture slides. Each fluconazole concentration was tested once with the aforementioned time-lapse WSI-based approach, enabling the growth monitoring of hundreds of individual fungal cells rapidly.

To demonstrate the feasibility of the as-developed approach in testing blood sample spiked with *C. albicans*, mixture of 1 μL overnight culture of *C. albicans* and 1 μL human whole blood was diluted into 1 mL PBS buffer. WSI-based AFST of this *ex vivo* blood culture was performed on the gel pad with 0, 32, and 64 μg/mL fluconazole, respectively.

AFST using broth dilution method

To validate the accuracy of the MIC_{100} determined using the developed method, AFST was also conducted using a modified broth dilution method (Wiegand, Hilpert, and Hancock 2008). In this procedure, fluconazole was mixed into SDB medium, generating a range of concentrations identical to those used in the WSI-based tests (0 to 128 μg/mL). An equivalent number of *C. albicans* cells, as in the WSI tests, were inoculated into 5 mL of both fluconazole-supplemented and control SDB media in test tubes. To align with the WSI-based tests and accurately determine MIC_{100} (100% inhibition), the incubation period was extended to 72 h at 37°C. The MIC_{100} was identified as the lowest fluconazole concentration showing no visible cell growth after this incubation. Cell growth was quantified by measuring OD_{600} with a UV-Vis spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific). This broth dilution test was replicated three times.

Results and discussion

WSI-enabled monitoring of fungal cell growth

In this study, we adapted the similar microbial culturing principle using "sandwich slides" demonstrated in our previous work (Song et al. 2018). *C. albicans* cells are immobilized on the interface between the gel and the supporting glass. Next, bright-field images of the entire sample area were acquired as the sample stage moved and seamlessly stitched together into one image. Figure 1 illustrates the WSI process and also presents a composite image of a

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sample area measuring 5.689 mm by 5.408 mm. This detailed image allows for the clear observation of individual *C. albicans* cells, all sharply in focus. Employing a 40× objective lens, the WSI imaging was efficiently completed in just 2 min. Given that *C. albicans* typically has a doubling time of 1 h in standard culture media, it is reasonable to assume that no significant cell growth occurred during this short 2-min imaging period under the scanning microscope.

To observe single-cell growth on this platform, "sandwich" slides with *C. albicans* were maintained at 37°C and 90% humidity, ensuring that the gel pad did not lose water and remained a constant size during the experimental period. Time-lapse imaging of a designated sample area was carried out at 0.5-h intervals up to 3 h. From these time-lapse composite images, the progression of cell numbers originating from a single cell at various time points was analyzed. Due to the substantial size of each composite image, a smaller section containing 5 cells was selected to demonstrate cell growth monitoring, as shown in Figure 2. As a yeast *C. albicans* proliferates through budding. As illustrated in Figure 3, a small bud emerges on the mother cell and its size keeps growing until reaching a certain level. Then budding recurs on the daughter cell. This unique "budding" growth pattern of *C. albicans* leads to ongoing changes in caculating their surface area as captured in optical images. Relying on cell surface area as a measure of bacterial cell growth, as we did in our previous study (Song et al. 2019), could pose substantial challenges in accurately tracking fungal cell replication due to these continuous surface area changes. To accommodate this unique cell growth pattern, we came up with a solution where each "bud" is counted as an individual cell. Thus, we used cell numbers to determine the cell growth rates, which was more accurate to indicate fungal cell replication than using cell area change which was used for bacterial replication in our previous study. For each individual cell, the normalized growth rate at time t was calculated using Equation 1,

$$Normalized\ growth\ rate = \frac{N_t}{N_0} \tag{1}$$

where N_t and N_0 are the numbers of cells with the same location in the images captured at time t and time 0, respectively. Depicted in Figure 4 are the growth rate curves of 5 representative *C. albicans* cells. Apparently, at each time point, the growth rates varied, emphasizing the necessities of analyzing a large population of cells for accurate determination of antifungal susceptibility at single-cell level.

WSI-enabled AFST for rapid determination of the minimum inhibitory concentration (MIC)

To assess the effectiveness of our approach in determining antifungal susceptibility, we conducted experiments to observe the growth of *C. albicans* when exposed to various concentrations of fluconazole, thus determining the MIC_{100}. The tested fluconazole concentration levels in the gel pad were 0, 4, 8, 16, 32, 64, and 128 μg/mL, with cell counts for each concentration being 675, 638, 644, 564, 644, 578, and 611, respectively. This sample size was large enough to statistically represent the biological variability in growth rates. Analysis of time-lapse images taken at 30-minute intervals over 3 h revealed

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a wide range of growth rates at each concentration (Figure 5). To simplify the analysis, we adopted a similar method in our previous study (Song et al. 2019) to categorize these growth rates into distinct bins, as illustrated in Figure 6. By considering a threshold of 2 for indicating cells that replicated at least once, we observed that with increasing concentrations of fluconazole (e.g., 0 to 32 μg/mL), the proportion of growing cells decreased over time, as expected. However, cell growth was completely inhibited at fluconazole concentrations of 64 and 128 μg/mL. As shown in Figure 7, by plotting the percentage of replicated cells over time, we identified the MIC_{100} as 64 μg/mL. This finding aligns well with results from a modified broth dilution method over a 72-h period, as shown in Figure 8. It is worth noting that conventional broth microdilution is typically read after 24 h of growth and a 50% decrease in growth compared to the drug-free control to determine MIC_{50}. Therefore, MIC_{50} is typically much smaller than MIC_{90} (Eksi, Gayyurhan, and Balci 2013), which is expected to be further smaller than MIC_{100} under an extended incubation time. This result indicates that reliable MIC_{100} of *Candida albicans* against fluconazole was obtained within 3 h using the developed method, which is much faster than the modified broth dilution method requiring 72 h for the similar result and reported commercial Accelerate PhenoTest BC Kit which provides identification and susceptibility results with MICs in approximately 6 h.

WSI-enabled AFST for blood culture spiked with C. albicans

To increase the clinical relevance and potential utility of this method we further demonstrated the feasibility of antifungal susceptibility testing in blood samples spiked with *C. albicans*. For proof-of-concept, the tested concentrations of fluconazole were 0, 32, and 64 μg/mL, and the number of cells treated with each concentration was 220, 98, and 117, respectively. As a representative image, displayed in Figure 9 are cells treated with 32 μg/mL fluconazole. At time 0 shown in Figure 9, since *C. albicans* and red blood cells have similar size, it was difficult to distinguish *C. albicans* cells. After 15 min incubation, the red blood cells underwent lysis due to high dextrose concentration in the medium and *C. albicans* growth was monitored at 1, 2, and 3 h. Following the same procedure of single-cell growth rate analysis, as illustrated in Figure 10, the MIC_{100} was determined as 64 μg/mL, which is in good agreement with the result obtained from the background-free AFST.

Conclusions

Leveraging the whole slide imaging platform developed in our prior research, we have enhanced its application to enable high-throughput monitoring of fungal growth at the single-cell level. Recognizing the unique “budding” growth patterns of fungi, distinct from bacteria, we introduced a novel approach to calculate the cell growth rate based on changes in the number of individual fungal cells. This method marks a significant advancement in both the accuracy and speed of rapid antifungal susceptibility testing, allowing for precise measurement at the level of individual cell sensitivity. As a demonstration of employing this new method for rapid AFST, the MIC_{100} of *C. albicans* against fluconazole was determined within 3 h, whereas the modified conventional broth dilution method typically took 72 h for the similar reliable result. Moreover, our approach can test blood culture samples spiked with *C. albicans* without the need of separating *C. albicans* from the blood, further shortening the time for AFST. The significant improvement in rapidness would play

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a significant role in timely guiding antifungal prescription and preventing the spread of antifungal resistance.

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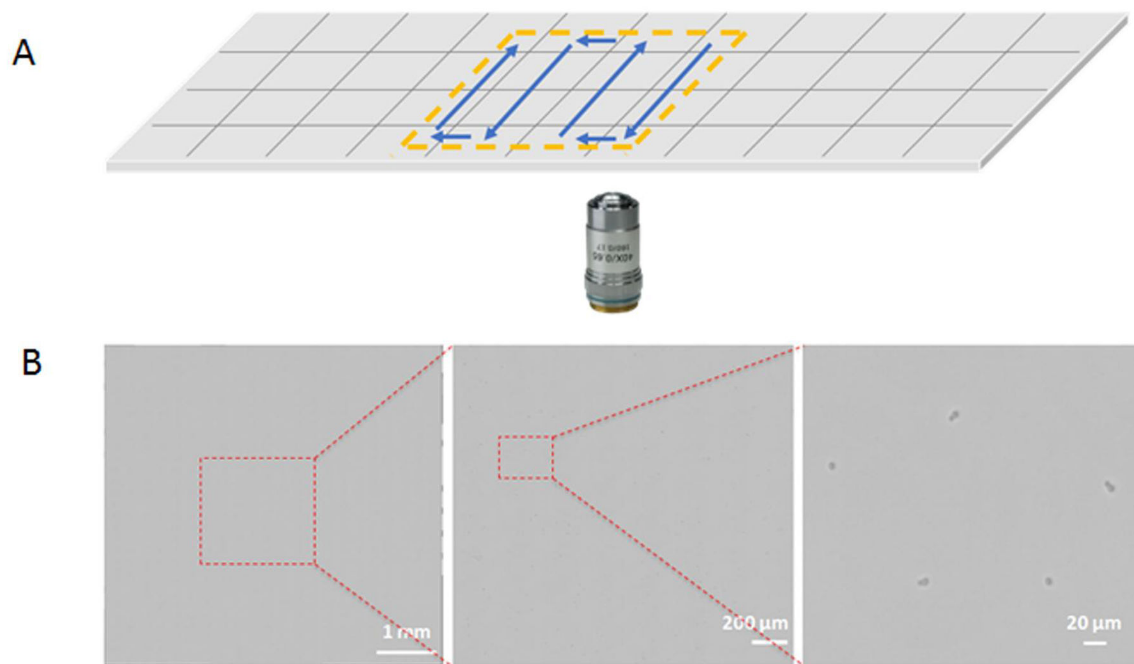

Figure 1.

(A) Schematic drawing for WSI and (B) A representative composite image captured by the reported platform. Zooming in the image allows the visualization of individual *C. albicans* cells.

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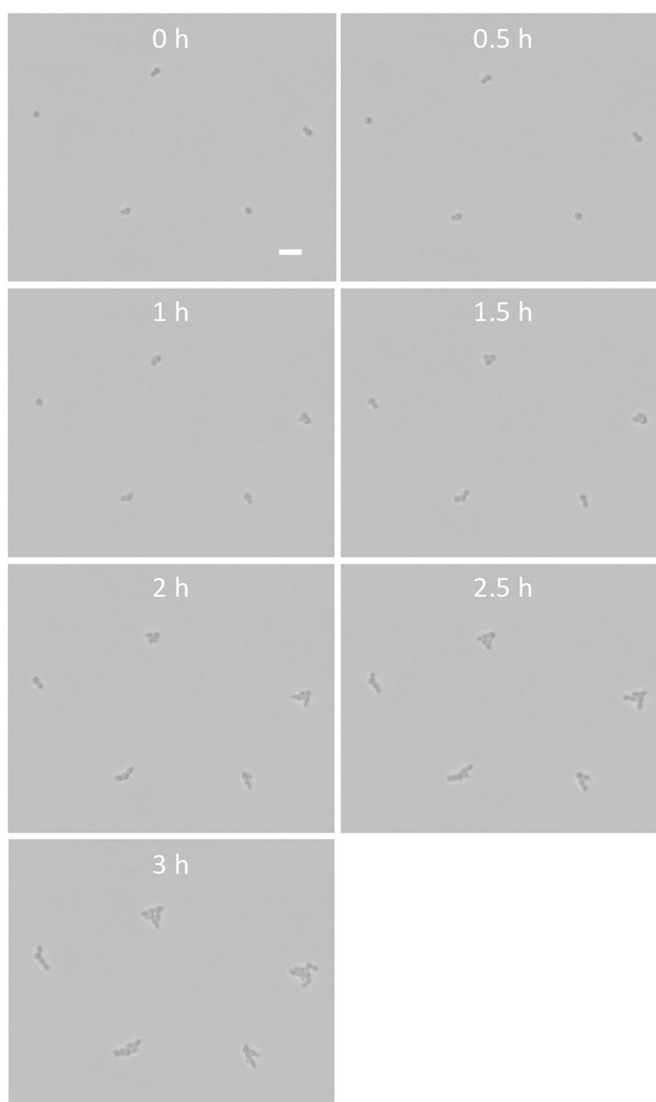

Figure 2.

Time-lapse images of *C. albicans* cells in one representative area selected from the composite image in Figure 1. Scale bar = 20 μm.

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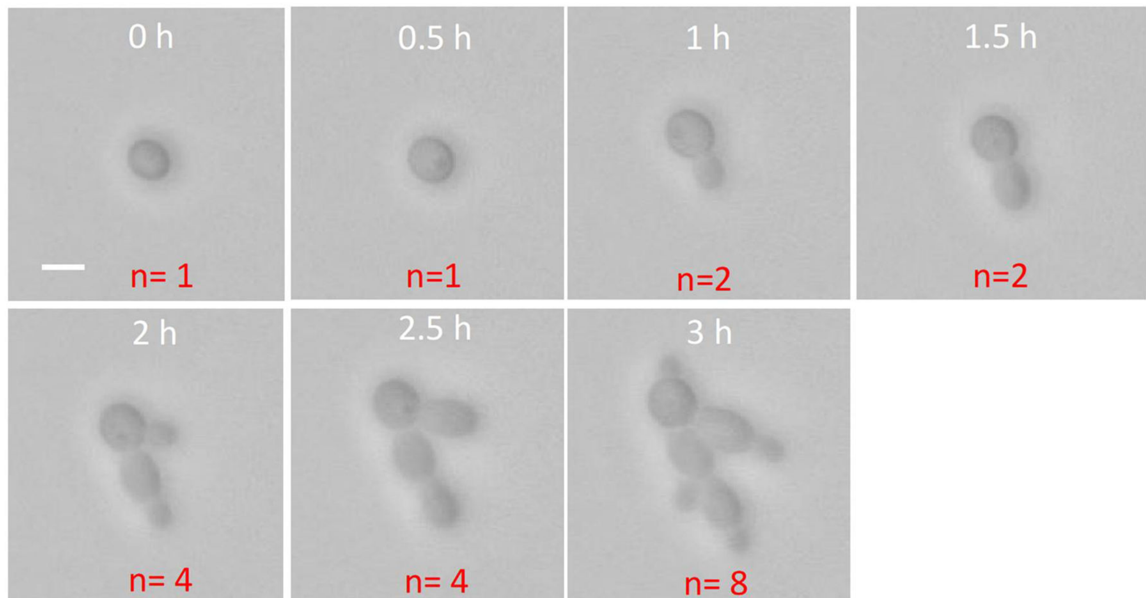

Figure 3.
Budding growth pattern of *C. albicans* is demonstrated in representative time-lapse images. Scale bar = 5 μm.

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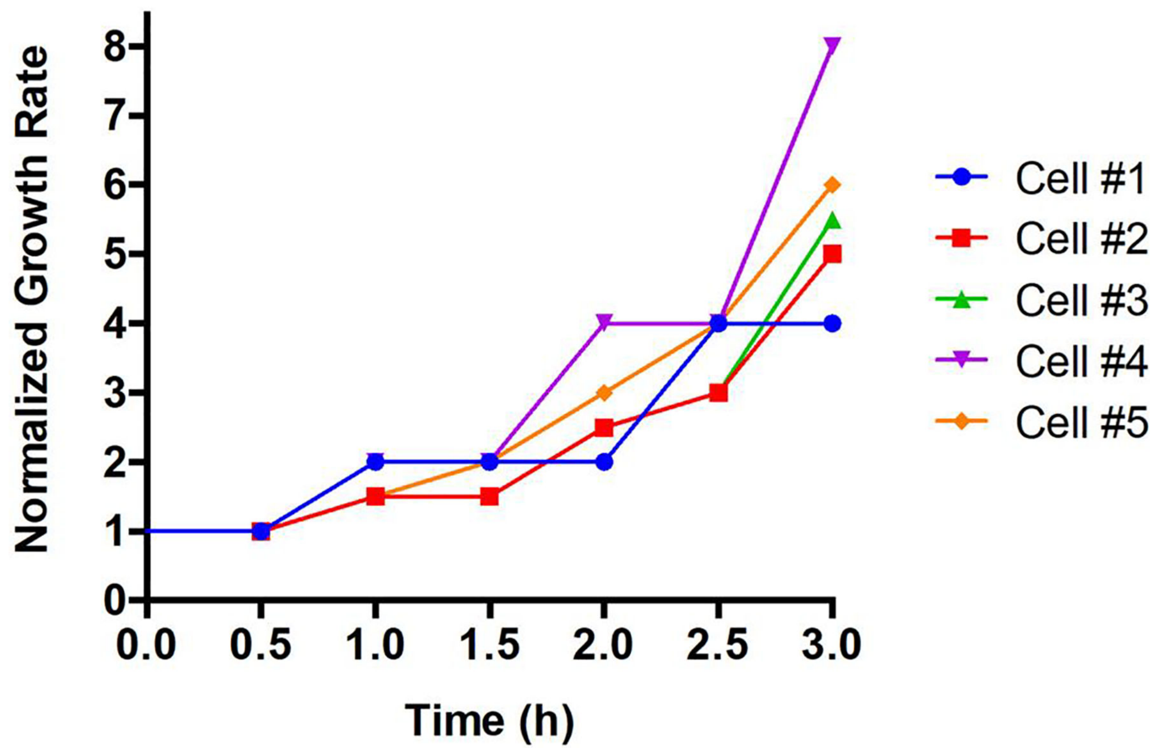

Figure 4.

Normalized growth rate of individual *C. albicans* cells versus incubation time for 5 representative fungal cells depicted in Figure 2.

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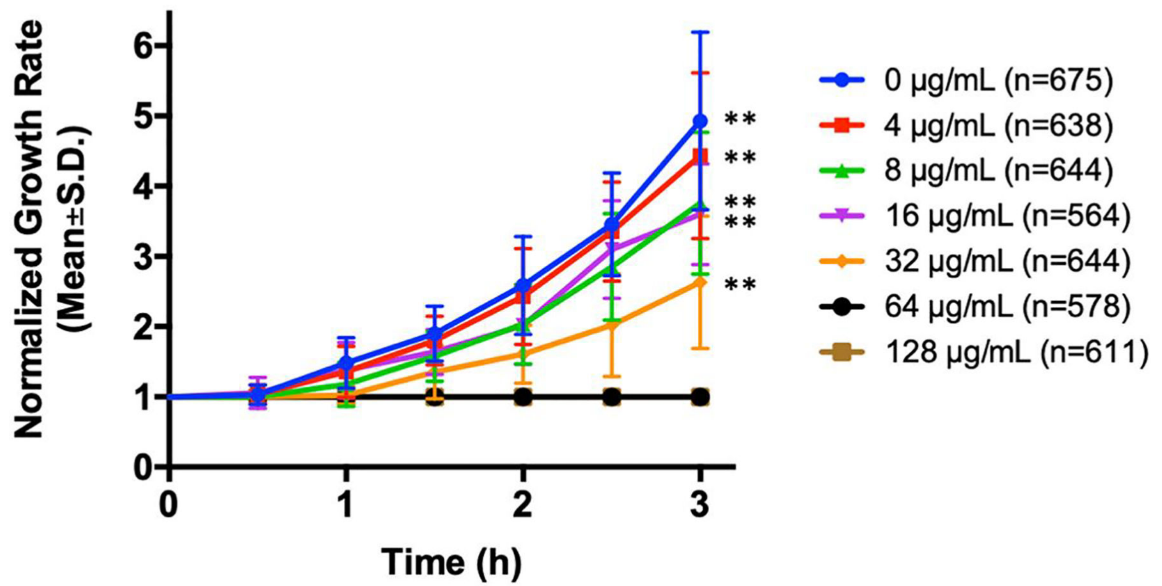

Figure 5.

Normalized growth rate versus incubation time in the presence of different antifungal fluconazole concentration (n represents the number of individual cells in each experiment). the growth rates in different treatment groups at 3 h were statistically analyzed using one-way ANOVA test (** $p < 0.01$).

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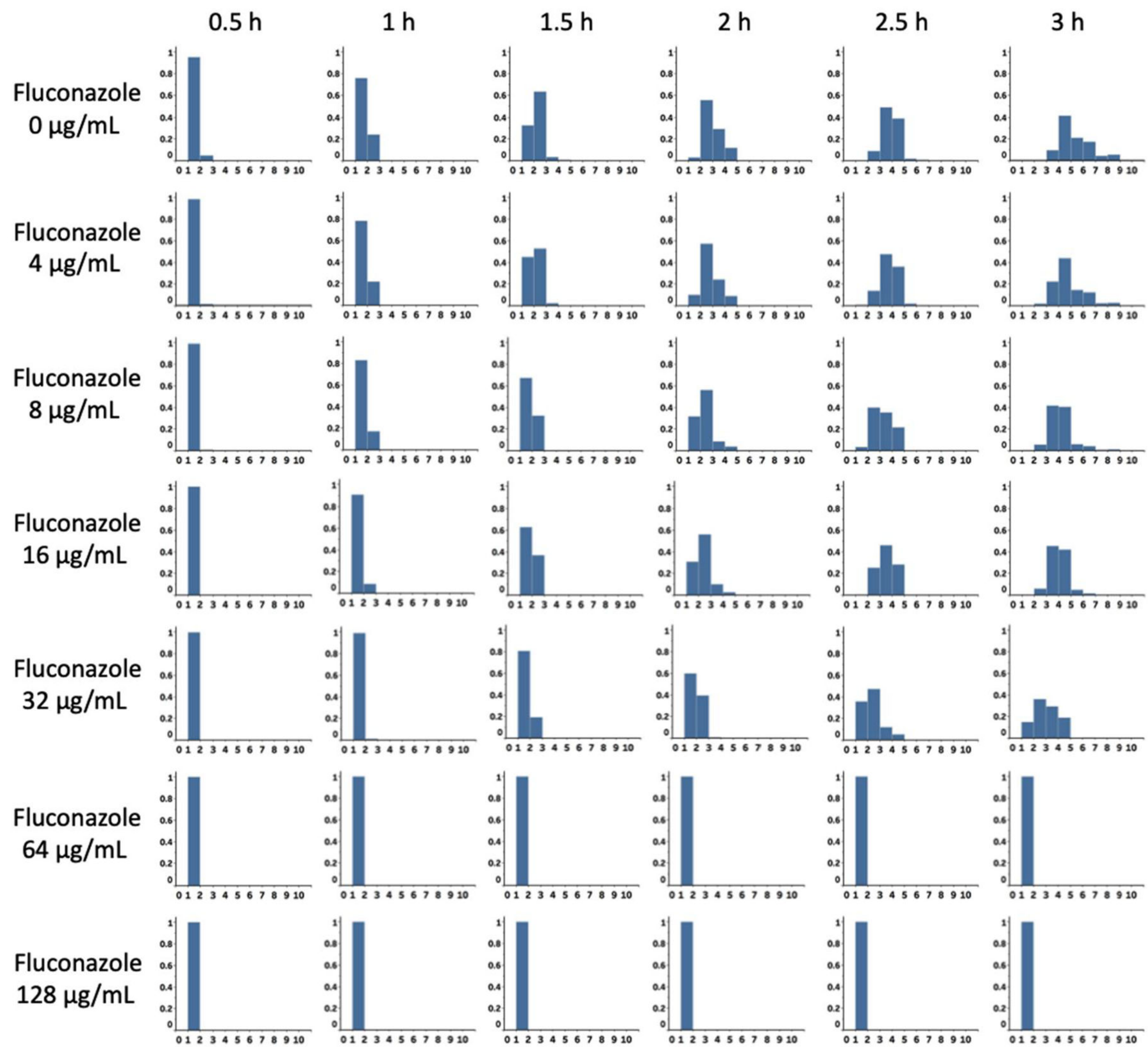

Figure 6.

Number-based distribution of the normalized single-cell growth rate for *C. albicans* cells treated with 0, 4, 8, 16, 32, 64, and 128 μg/mL fluconazole, respectively. In each histogram, X-axis indicates the normalized growth rates at different fluconazole concentrations and Y-axis indicates the number-based fraction of cells.

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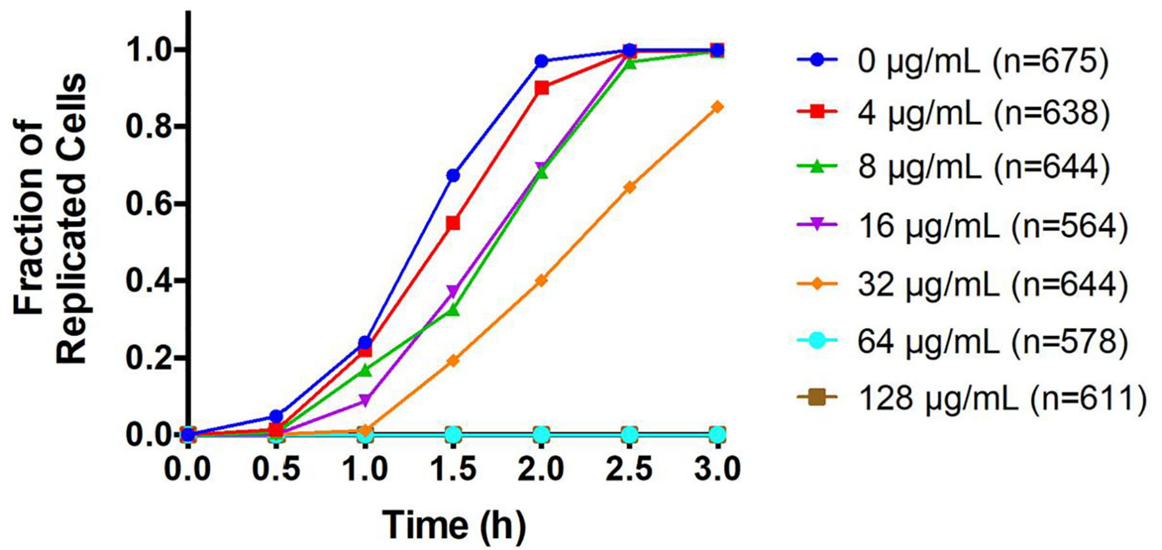

Figure 7.
MIC_{100} determination by WSI-based AFST approach. The curves were plotted with the fraction of replicated cells from each treated group versus time.

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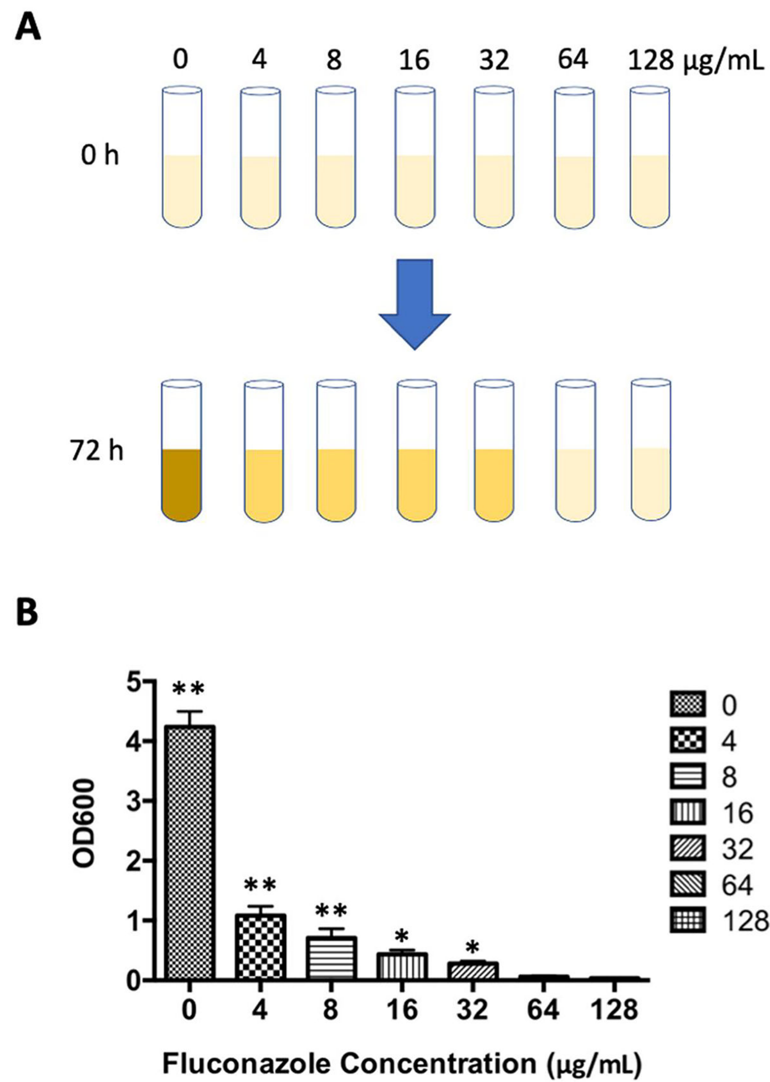

Figure 8.

(A) Graphic description of MIC_{100} determination by the modified conventional broth dilution method. (B) OD600 obtained experimentally after *C. albicans* cells were incubated for 72 h. Statistical analysis was conducted using one-way ANOVA test (*p<0.05, **p<0.01).

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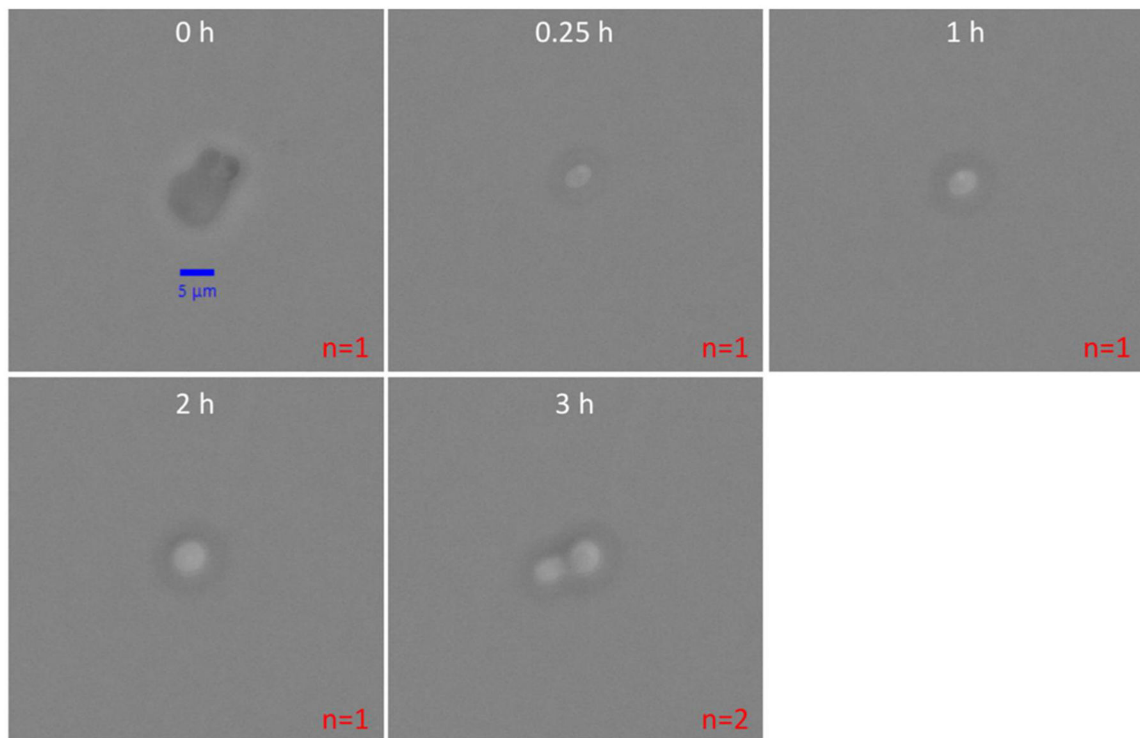

Figure 9.

Time-lapse images of *C. albicans* suspended in blood (further diluted in PBS buffer and cast on gel pad supplemented with 32 μg/mL fluconazole) in one representative area selected from a composite image. Scale bar = 5 μm.

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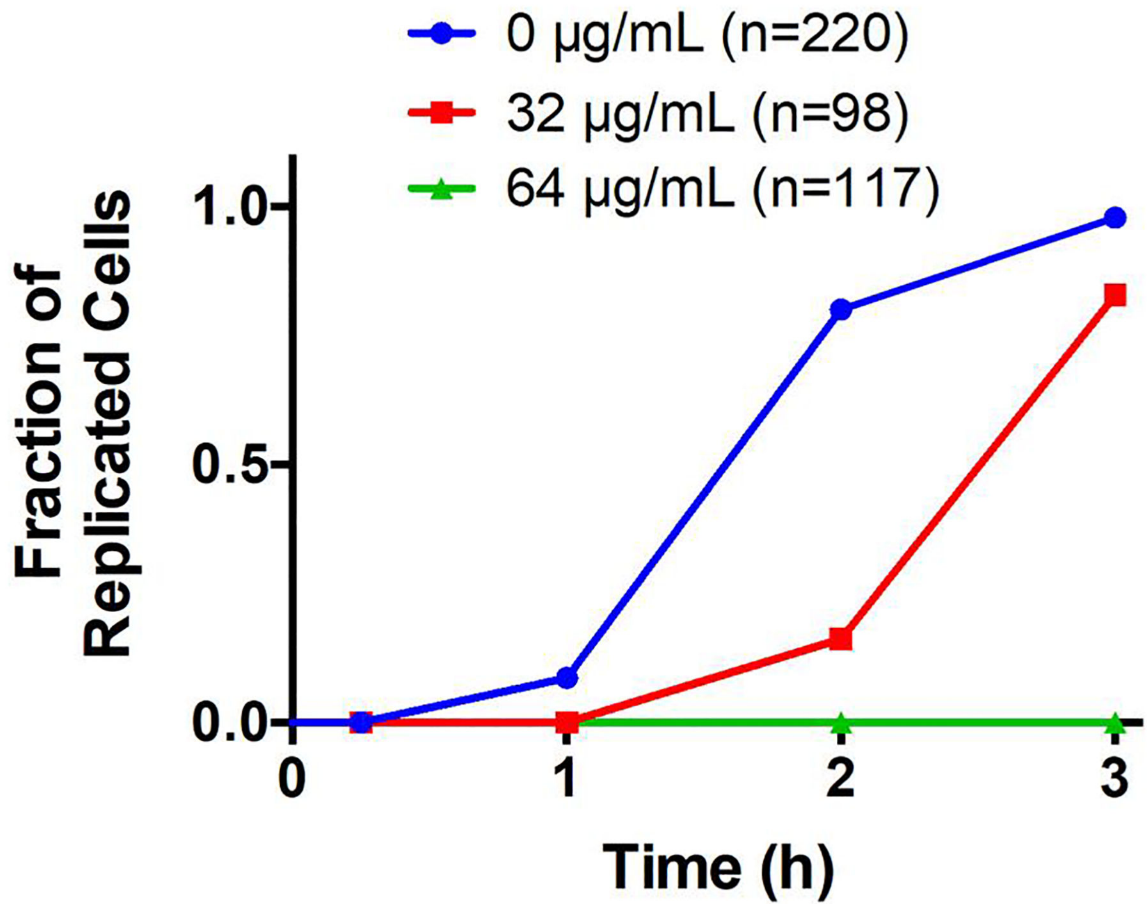

Figure 10.
MIC_{100} determination for *C. albicans* in blood culture by WSI-based AFST approach. The curves were plotted with the fraction of replicated cells from each treated group versus time.