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# Evaluation of a real-time impedance analysis platform on fungal infection

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ABSTRACT

End-point assays of *in vitro* cell proliferation and death have been employed to study the mechanisms of fungal pathogenesis and have shown the responses of host cells at individual time points. A new cell analysis technology has been developed that allows for the continuous measurement and quantification of cell activities, thus enabling the dynamic assessment of electrical impedance when various pathogens are cultured *in vitro*. In this study, this system was evaluated to determine the response of the cell line RAW264.7 to infection by several clinically relevant fungi *in vitro*, including *Aspergillus fumigatus*, *Candida albicans*, and melanized and albino mutant strains of *Fonsecaea monophora*. The results showed that infection resulted in rounding of the host cells with a loss of contact between individual cells and a decline in the electrical impedance of all test groups. However, changes in the electrical impedance were variable. *Aspergillus fumigatus* caused initial increases and later significant decreases in the electrical impedance, while for *C. albicans* and *F. monophora*, the effect was reduced. The melanized strain of *F. monophora* caused a faster change in the electrical impedance than the albino strain. Our data proved that this system can be used as an efficient tool for monitoring cellular responses to fungal infection.


## 1. Introduction

The interaction between host cells and pathogens includes internalization, phagocytosis, and the secretion of cytokines or oxygen radicals and results in different responses of infected cells in terms of their morphology and adherence, often eventually leading to apoptosis or necrosis (Galluzzi et al., 2009; Grassme et al., 2001; Kim et al., 2009; Muenzner et al., 2005). All processes may lead to disruptions in the overall structural and/or functional integrity of the involved tissues and organs. Modern techniques are available to allow the analysis of cell responses at the molecular level. Cell proliferation can be monitored by either direct cell counts or DNA synthesis (Abassi et al., 2009). Cytotoxicity can be observed *via* plasma membrane alterations of host cells (Ye et al., 2015), changes in intracellular metabolic activities by MTT/XTT assays (Bernas and Dobrucki, 2002), and by the release of enzymes such as lactate dehydrogenase (LDH) (Rayamajhi et al., 2013). Assays using genomic DNA fragmentation, apoptosis-induced proteases, regulators, inhibitors and cytochrome C have been developed to analyze apoptosis and/or necrosis (Galluzzi et al., 2009). However, these conventional end-point detection assays are insufficient because they only provide data at specific time points. As yet, no simple and accurate method is available for real-time dynamic recording and monitoring of these processes in a laboratory setting.

Real-time cell analysis (RTCA) was designed for the dynamic monitoring of cellular alternations (Atienza et al., 2005; Irelan et al., 2011). The technique has been demonstrated to be an effective tool for determining cytotoxicity, apoptosis, cell stress, cell invasion, and migration (Atienza et al., 2005; Solly et al., 2004; Xing et al., 2005; Yu et al., 2006). This platform first measures changes in electrical impedance and then transfers the data to a dimensionless cell index (CI) that allows for the quantitative analysis of the cellular status and for continuous monitoring of cellular responses in real-time (Atienza et al., 2005; Vistejnova et al., 2009). RTCA has been widely used in describing the infection processes of a variety of microbes (He et al., 2009; Ryder et al., 2010; Slanina et al., 2011) in cell toxicity induced by bacterial toxins (Ryder et al., 2010; Ye et al., 2015), in virus infections (Fang et al., 2011; Tian et al., 2012), and in screening drug resistance and neutralization assays for bacteria and viruses (Lehtoranta et al., 2009; Witkowski et al., 2010). The method has never been evaluated in fungi. In the present study, RTCA was used for the dynamic analysis of cellular responses over time induced in the frequently used immune cell line RAW264.7 by the pathogenic fungi *Aspergillus fumigatus*, *Candida albicans*, and

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*Fonsecaea monophora*. *A. fumigatus* and *C. albicans* are the most frequently isolated clinical fungi strains and are used to represent filamentous and yeast pathogenic fungi in experimental settings. *F. monophora* is one of the four pathogenic species of the genus *Fonsecaea*. Meristematic growth melanized and albino strains of *F. monophora* were obtained in our previously studies (Li et al., 2016; Xi et al., 2009). Compared with the melanized strain, the albino mutant was sensitive to environmental stress factors, including temperature, pH, UV irradiation and oxidative stress (Sun et al., 2011). The albino mutant induced the production of nitric oxide and Th1 cytokines in murine macrophages, resulting in persistent infection (Zhang et al., 2013). Therefore, the melanized and albino strains of *F. monophora* were used as models to evaluate the pathogenesis of these strains with the xCELLigence system.

## 2. Materials and methods

### 2.1. Fungal strains and cell culture

The strains of *A. fumigatus*, *C. albicans*, and *F. monophora* used in this study originated from clinical patients. They were identified by morphology and molecular methods and stored at −80 °C. *Aspergillus fumigatus* and *F. monophora* were cultured on potato dextrose agar (PDA; OXOID, Basingstoke, UK) at 25 °C for two weeks. *Candida albicans* was cultured on Sabouraud's Glucose Agar (SGA; OXOID, Basingstoke, UK) at 37 °C for 24 h. The mouse macrophage cell line RAW264.7 was cultured in DMEM with 10% fetal calf serum (Biochrom, Berlin, Germany) at 37 °C and 5% $CO_2$.

### 2.2. Impedance and cell index measurements

The RTCA assays were performed using an xCELLigence system according to the manufacturer's instructions (ACEA Biosciences, San Diego, U.S.A.) (Roche, 2008). Three main components are involved in this system, including the Real-Time Cell Analyzer (RTCA) computer, the RTCA Single Plate (SP) station and the disposable E-plate 16 (a 16-well plate with a glass bottom, coated by "circle-on-line" capillary gold electrodes). The E-plate and the signal receiving RTCA SP station remained in a standard cell culture incubator. The data are collected by a wireless link between the work station and computer (Fig. 1). Without cells, the impedance mainly shows the background of ion environments both at the electrode/solution interface and in the bulk solution (Fig. 1A). The initial monolayer cell attachment results in increased impedance (Fig. 1B). Later on, the proliferation or spreading of cells enhances the electrical impedance (Fig. 1C). Finally, infection by microbes (bacterial, virus or parasite) or toxicity (Fig. 1D) causes cellular detachment or death (Fig. 1E). The xCELLigence system measures the corresponding changes in impedance and calculates them as the dimensionless parameter CI. The simulation of the CI curve in different stages of monitoring is shown in Fig. 1 (right).

### 2.3. Cell growth and proliferation assay

Fifty microliters of DMEM with 10% fetal calf serum was added to the wells of the E-plate for background measurements. RAW264.7 cells were re-suspended in DMEM with 10% fetal calf serum and counted using a cell counter (Life Technologies, Carlsbad, CA, U.S.A.). The initial cell number was 160,000 cells per well, with a 2-fold dilution for the remaining wells (300 μl in total volume). To determine the influence of LPS on cell growth and proliferation, LPS was diluted in PBS and added to each well to obtain a 2-fold dilution series with final concentrations of 300, 200, and 100 μg/ml. Cell proliferation was automatically monitored every 5 min over 10 h, followed by every 15 min over 120 h. The electrical impedance was measured by the integrated software of the xCELLigence system as CI.

A parallel experiment with the same amount of RAW264.7 cells was carried out in a 96-well plate at the same time. The morphology changes of RAW264.7 cells were recorded at 1, 24, 48, 72, 96 and 120 h by using light microscopy (Leica, Wetzlar, Germany).

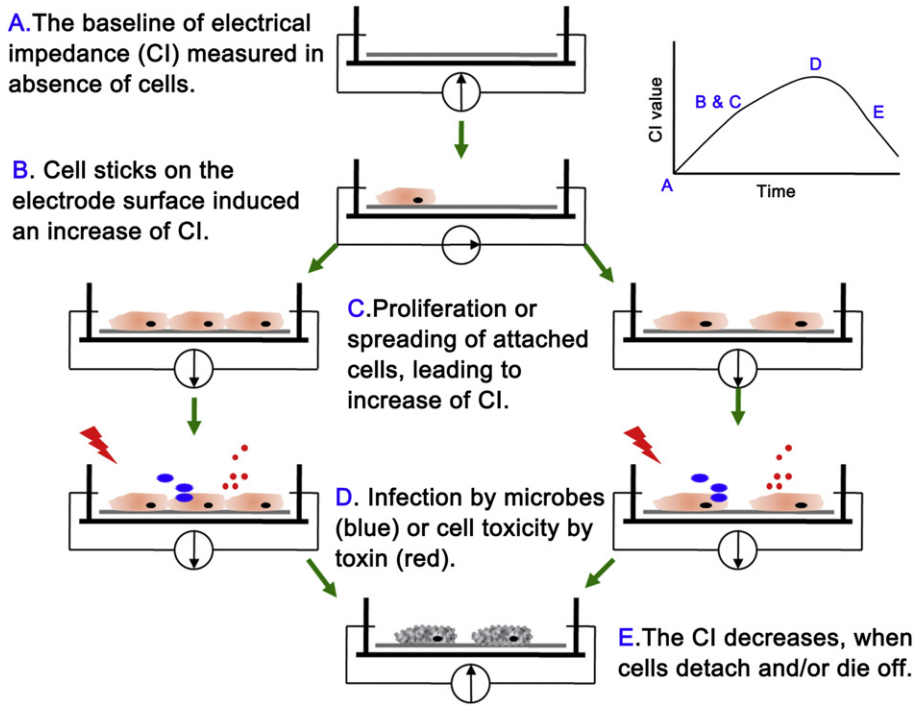


**Fig. 1.** Scheme of impedance measurement with the xCELLigence system. (A) The baseline impedance was measured in the absence of mouse macrophage RAW 264.7 cells. (B) A cell sticks on the electrode surface and acts as an isolator, inducing an increase in the electrical impedance. (C) If more cells attach to the electrodes or cells are spreading, there will be an increase in the electrical impedance, leading to higher CI values. (D) Cells were treated with microbes or cell toxicity. (E) The CI decreases when cells detach and/or die off.