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Video article

Spiral-based microfluidic device for long-term time course imaging of *Neurospora crassa* with single nucleus resolution

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ABSTRACT

Real-time imaging of fluorescent reporters plays a critical role in elucidating fundamental molecular mechanisms including circadian rhythms in the model filamentous fungus, *Neurospora crassa*. However, monitoring *N. crassa* for an extended period of time with single nucleus resolution is a technically challenging task due to hyphal growth that rapidly moves beyond a region of interest during microscopy experiments. In this report, we have proposed a two-dimensional spiral-based microfluidic platform and applied for monitoring the single-nucleus dynamics in *N. crassa* for long-term time course experiments.

1. Introduction

Population-averaged cellular probing masks cell-to-cell variability and can mislead interpretation of differences between individual cells. Single-cell analysis is critical for overcoming these limitations (Elowitz et al., 2002). Numerous analytical techniques (Wang and Bodovitz, 2010; Kalisky et al., 2011) have been established and allow dynamic studies at the single cell level. Furthermore, flow cytometry enables high-throughput quantitative analysis of protein expression and phosphorylation state at single cell level (Tarnok et al., 2010; Tracy et al., 2010). However, these technologies have limited applications for filamentous fungi that grow as thread-like structures, which cannot be separated without breaking the cell wall (Klein and Paschke, 2004). Flow cytometry has previously been applied to germinating conidia of filamentous fungi, but only for short duration of approximately 13 h (Bradner and Nevalainen, 2003). Thus, it is necessary to develop an optimum platform for long-term tracking of filamentous fungi with single nucleus resolution.

Microfluidic lab-on-a-chip (LOC) technology has been researched extensively for biological applications in the past ranging from cell sorting to drug screening (Craighead, 2006; El-Ali et al., 2006). Microfluidic devices provide unique platforms to solve fundamental and applied problems at the single cell or nucleus level by facilitating investigation of cellular operations on the micrometer scale (El-Ali et al., 2006). To date, only a few microfluidic devices have been established for filamentous fungi, which explored growth dynamics (Demming et al., 2011) and germination of fungal spores in different environmental conditions (Held et al., 2011). Importantly, there are no microfluidic devices that enable long-term tracking of filamentous fungi with single nucleus resolution.

Circadian rhythms are physiological processes repeating with a period of about 24 h. The circadian rhythm molecular machinery provides temporal information to other cellular processes and regulates a variety of metabolic and physiological functions including sleep/wake cycles (Gachon et al., 2004; Sahar and Sassone-Corsi, 2009; Baker et al., 2012). *Neurospora crassa*, a multinucleate filamentous fungus, has been effectively used to elucidate the molecular mechanisms of circadian rhythms (Baker et al., 2012) and molecular links between cell cycle and circadian rhythms. Recently, Hong et al. (2014) successfully demonstrated the circadian clock-gated nuclear divisions in *N. crassa* using time-course live cell imaging to track nuclear morphology with a histone H1-green fluorescent protein (hH1-GFP) reporter (Freitag et al., 2004; Roca et al., 2010). hH1 is a chromatin component and hH1-GFP enables visualization of nuclear morphology (Roca et al., 2010). Previous imaging methods including "inverted agar block" protocol enables live cell imaging of filamentous fungi without any microfluidic device (Hickey et al., 2002). However, these methods only provide a 2–6 h window for live cell imaging due to fast hyphal growth or cell death. Therefore, we have developed a simple and efficient polydimethylsiloxane (PDMS)-based microfluidic device with 2D spiral microchannels. This design overcomes the inherent technical limitations for long-term time course

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experiments (>24 h) with filamentous fungi. As a proof of principle, we utilized our 2D spiral design to demonstrate circadian changes of growth rates and circadian clock-dependent mitotic cycles in *N. crassa* expressing the histone hH1-GFP reporter.

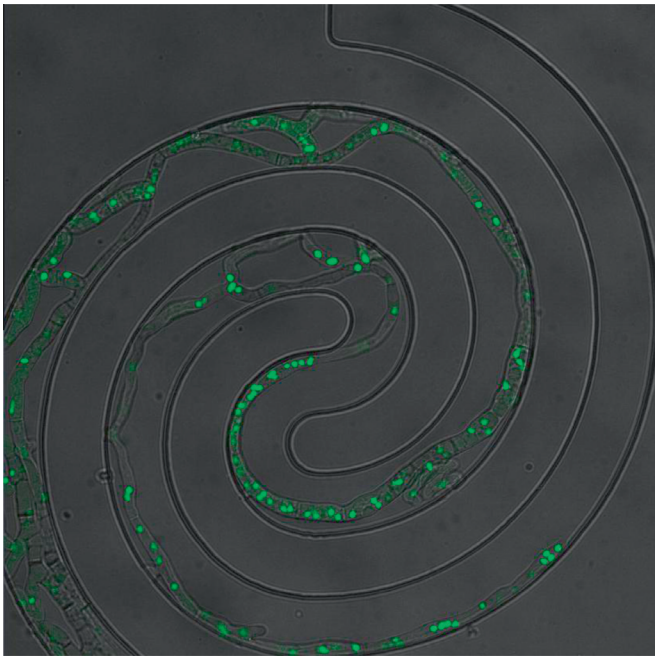
2. Results and discussion

The optimized depth and width of our 2D spiral channel in the microfluidic device is 10 μm and 15 μm, respectively. This dimension was optimized to the size of the *N. crassa* single hyphae (8–15 μm) (Freitag et al., 2004; Roca et al., 2010), as well as for the efficient loading of liquid culture media into the 2D spiral channels. A microchannel with <10 μm depth and <15 μm width was not feasible for efficient delivery of liquid media into the device as these channels were not subjected to hydrophilic treatments. Additionally, due to the dimensional limitations of the microchannels, *N. crassa* growth is constrained. Alternatively, devices exceeding the 10×15 μm dimensional window allowed for a more efficient delivery of the liquid media into the channel. However, the additional clearance also allows for a larger vertical degree of freedom and an unstable focal point. Therefore, the optimized device allowed for efficient loading of liquid media into the microchannel and real-time imaging of *N. crassa* with single-nucleus resolution.

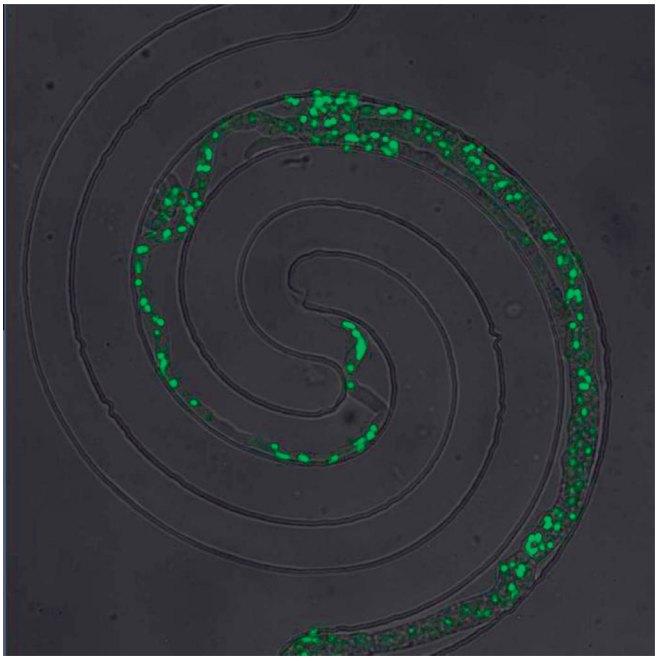
Fig. 1A shows a top view of the chrome mask with 15 μm wide 2D spiral microchannels followed by a 1000 μm wide channel for fabricating PDMS-based microfluidic devices. *N. crassa* conidia expressing either hH1-GFP or β-tubulin-GFP reporter were inoculated into the inlet port of the microfluidic device, and the 1000 μm channel allowed robust hyphal growth in constant light conditions (i.e. LL) for 12 h at 25 °C. The device was moved to a 36NL Percival incubator in constant darkness (DD) at 25 °C, where the *N. crassa* circadian clock is reset to the dusk, to observe molecular phenotypes that are under the control of endogenous circadian rhythms in the absence of external time cues. DD0 indicates the beginning of the subjective night, which is also labeled as CT12. CT designates circadian time in a free-running condition (i.e. no external time cues), where the subjective day begins at CT0 and subjective night at CT12. In other words, DD25 (CT15) and DD35 (CT2) represent subjective night and subjective day, respectively. Mats of mycelia grew through the 1000 μm channel and individual hyphae were captured by 15 μm channels (Fig. 1A). Single hyphae reached the 15 μm channel during the DD condition. Using the above device, we measured growth rates of the *hH1-gfp* strain in a 2D spiral microchannel at three different time points. Intriguingly, we discovered that *N. crassa* grows faster during the subjective night (DD25 and DD47) compared to the subjective day (DD35), which was not observed in a circadian arrhythmic mutant, frq^{ko} strain (Fig. 1B and D). These results clearly demonstrate that the circadian clock influences the rhythmic changes of growth rates in *N. crassa*.

In order to confirm circadian clock-gated nuclear divisions in the microfluidic devices with 2D spiral design, we measured percent of nuclei undergoing mitosis at different circadian time points (Fig. 1C). The hH1-GFP reporter was used to monitor dynamic changes in nuclear morphology, which reflected approximate cell cycle stages (Roca et al., 2010; Hong et al., 2014). The populations of nuclei were classified into two categories, interphase and mitotic phase as described in Fig. 1E. During the subjective day (DD35), most nuclei at the leading edge/growth front were in the interphase as shown by a round-type nuclear morphology (Fig. 1F, Video 1). In contrast, a significantly larger amount of nuclei were in mitotic phase during the subjective night (DD25 and DD47) (Fig. 1G, Video 2), which are not observed in *hH1-gfp*;frq^{ko} strain (Hong et al., 2014). These data indicate that faster growth rates at DD25 and DD47 correlated with higher percentage of nuclei in mitosis. With the above experiments, we successfully demonstrated that the proposed 2D spiral microfluidic device could be used for long-term time course (>24 h) imaging of *N. crassa*.

In summary, a simple PDMS-based microfluidic device containing 2D spiral microchannels has been designed, fabricated, and successfully applied for a long-term real-time imaging of single-nucleus behavior in *N. crassa*. We successfully performed



Video 1. Movie of *Neurospora crassa* hyphae with an hH1-GFP reporter from DD35. The video covers 80 min starting at DD35 and can be extended over 24 h. Most of the nuclei are in interphase.



Video 2. Movie of *Neurospora crassa* hyphae with an hH1-GFP reporter from DD25. The video covers 80 min starting at DD25 and can be extended over 24 h. Most of the nuclei at the growth front demonstrate elongated morphology, indicating synchronized mitosis.