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Microfluidics structures for probing the dynamic behaviour of filamentous fungi

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ABSTRACT

Although filamentous fungi live in physically and chemically complex natural environments that require optimal survival strategies, both at colony and individual cell level, their growth dynamics are usually studied on homogenous media. This study proposes a new research methodology based on the purposeful design, fabrication and operation of microfluidics structures to examine the temporal and spatial responses of filamentous fungi. Two model fungal strains, the wild type of *Neurospora crassa* – a commonly used model organisms – and the *ro-1* mutant strain of this species impaired in hyphal growth and morphology, have been chosen to demonstrate the potential of this new methodology. Time-lapse observations of both species show that filamentous fungi respond rapidly to the physically microstructured environment without any detectable temporal or spatial adjustment period. Despite their genetic differences, and consequently different growth behaviour, both strains present efficient space-search strategies enabling them to solve the microsized networks successfully and in similar periods, thus demonstrating that the space-searching algorithms are robust and mutation-independent. Additionally, the use of the proposed methodology could put in evidence new biological mechanisms responsible for the apical extension of filamentous fungi, beyond the classical theory based on the central role of Spitzenkörper.

1. Introduction

Microorganisms, of which fungi are the most wide-spread eukaryotes, have a vast impact on the world's economies as important plant, animal and human pathogens and account for a large fraction of the decomposers present in the ecosystem [1]. Filamentous fungi colonize a variety of microconfined environments that are chemically, physically and geometrically heterogeneous. They have developed efficient strategies to optimize their growth at both the colony and individual cell level responding to stimuli that are spatially distributed at the macro- and micro-scale. This dynamic behaviour results in cell growth patterns that are interesting from more than a biological perspective. Though diverse in morphology, behaviour and habitat, the majority of species relies on polar tip growth for hyphal extension. At the individual cell level, the fungal growth dynamics is driven by sensing mechanisms at the growing tip [2] as a means to gain dynamic information about the physical (e.g., available space, distribution of light) and chemical (e.g., distribution of nutrients, toxins) characteristics of the environment. Despite this micro-scale dependent behaviour, fungal growth has been studied principally in and on homogeneous media [3] that bear little or no resemblance to their natural habitats.

Microfluidics structures have been used to probe individual cell shape and function [4] as well as their movement [5]. Here we propose a design, fabrication and application methodology for artificial microfluidic structures in order to probe the dynamic responses of the model organism *Neurospora crassa* and a mutant strain of this species with altered growth dynamics.

2. Materials and methods

The *N. crassa* wild type strain examined belongs to the culture collection of the School of Biological Sciences, University of Liverpool and the *N. crassa ro-1* mutant strain (FGSC #110) was obtained from the Fungal Genetics Stock Center. Both species were maintained on malt extract agar (MERCK) at 4 °C. The *ro-1* mutant is a dynein mutant that shows defects in the organization and stability of the Spitzenkörper [6], a cell site that supports hyphal growth [7–9]. This defect results in a variety of phenotypes including distortion of the hyphal morphology, disruption of nuclear movement and placement, reduced cell growth rates and altered microtubule organization and behaviour. Prior to each experiment, the fungal strains were sub-cultured onto fresh malt extract agar plates and incubated at room temperature for 24–48 h.

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The artificial microfluidic structures were fabricated from the PolyDiMethylSiloxane (PDMS, Sylgard 184, Dow Corning) according to the process flow chart in Fig. 1. A positive relief silicon master was fabricated using standard photolithography techniques and deep reactive ion etching (DRIE) [10]. This positive relief master was coated with HMDS and the negative relief PDMS stamp was then fabricated by casting the degassed PDMS prepolymer and curing the agent mixture (10:1 by weight), according to well-established procedures [11], against the coated silicon master. The PDMS was overcured at 65 °C for at least 8 h to ensure the full cross-linking of the polymer and for removal of any traces of toxic monomer. After removal from the silicon master, the PDMS surface, which is inherently hydrophobic, was rendered hydrophilic by deep UV exposure. The microfluidic structures were sealed irreversibly to a UV treated glass substrate. The microstructures were designed such as to present lateral openings allowing the introduction of growth medium and fungal inoculation into the test areas.

The microstructures were filled with nutrient-free growth medium (sterile distilled water) by immersing the structure in the medium and evacuation of the setup. Fungal inoculation was achieved by placing an agar plug from the peripheral growth zone of a 24–48 h old colony next to the lateral opening of the PDMS structure. This assembly was then enclosed in a Petri dish to retain moisture but allow the exchange of oxygen and carbon dioxide with the outside environment.

The hyphal growth in the culture chamber was observed with a Brunel inverted microscope (SPI-98) equipped with a digital camera (Moticam 2300, 3MP) and with an inverted Zeiss Axio Observer Z1 equipped with a photomultiplier. The images were typically collected as time series at regular intervals (usually one frame per 15–30 s) and subsequently imported into Image Pro Plus software (Version 6.1, Media Cybernetics) for further image analysis. The statistical analysis of the images comprised the measuring of the apical extension velocity, branching angle (angle between the parent and the daughter hypha at the branching point) and the branching distance (distance between two daughter hyphae along the parent hypha). The statistical analysis included the mean and standard deviation values of the parameters.

3. Results and discussion

Observation and measurement of the growth parameters of both *N. crassa* strains were performed on plain agar and subsequently in two test structures. Both microfluidic networks had a total edge length of 100 μm and a channel depth of 10 μm. The channel and feature sizes within the networks had a dimension similar to the hyphal diameter (average: 7 μm). The diamond structure (Fig. 1a) consisted of a square pattern of 16 10 μm wide pillars positioned at regular intervals. The entrance and exit were positioned at two opposite corners, which resulted in two different kinds of solution paths including a periodic path through the pillars and a straight path around the pillar pattern. The second maze-like network (Fig. 1b) consisted of a variety of features with sizes ranging from 5–25 μm, comprising diverse complex and non-periodic shapes.

3.1. N. crassa wild type and ro-1 on agar

Fig. 2 depicts a graphical representation of the growth parameters of both examined strains measured on agar and in the two test structures. The wild type strain of *N. crassa* presented a growth dynamics on plain agar which is optimal for the exploration of unconstrained geometries, i.e., a branching angle optimal of 45.0 ± 16°. As the branching distance was, on average, double than

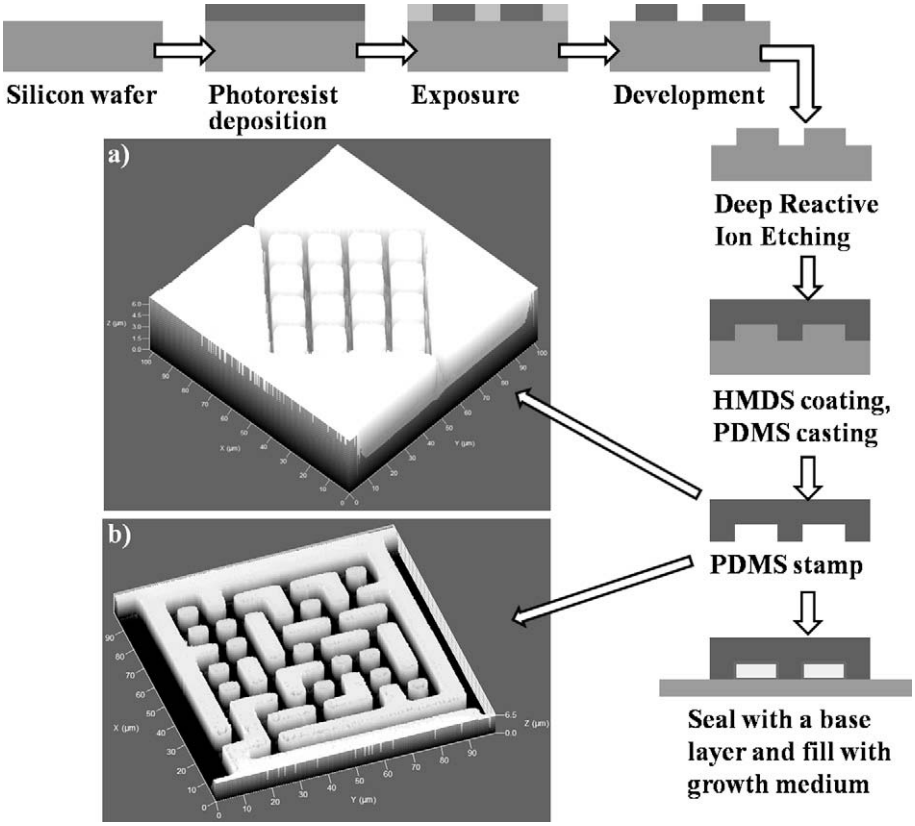

Fig. 1. Flow chart of the fabrication processes of microstructured Si wafers and subsequent PDMS molds. (A and b) show three-dimensional representations of the (a) diamond structure and (b) maze-like structure used.