Fungal Intelligence; Or on the behaviour of microorganisms in confined micro-environments

Marie Held¹, Clive Edwards², Dan V Nicolau¹

¹Department of Electrical Engineering and Electronics, University of Liverpool, L69 3GJ, United Kingdom
²School of Biological Sciences, University of Liverpool, L69 7ZB, United Kingdom
Email: dnicolau@liverpool.ac.uk

Abstract. Filamentous fungi are very successful in colonising various microconfined environments, but their behaviour is usually tested on flat surfaces. This contribution presents the design, the fabrication and the use of microstructures, made of a biocompatible polymer (poly(dimethylsiloxane), PDMS) for studying the dynamic micro-scale behaviour of the filamentous fungus Neurospora crassa. The proposed methodology is simple to implement and uses low cost fabrication methods. The observations of the fungus growing through a variety of fabricated micro-environments revealed distinct structure-dependent and structure-induced responses. Generalising the proposed methodology we propose a tool for high-throughput studies of numerous fungal species.

1. Introduction
Filamentous fungi, which colonize natural micro-environments that are geometrically, physically and chemically heterogeneous, optimize their behaviour at the individual cell level and within limited time frames, responding to stimuli that are spatially distributed at the micro-scale. They use sensing mechanisms [1,2] to gain dynamic information regarding the physical (e.g. available space) and chemical (e.g. distribution of nutrients, toxins) properties of the environment. Despite this micro-scale dependent behaviour, the microorganisms are usually studied in and on homogeneous media that bear little resemblance with their natural environment, for instance on plain agar plates [3]. Semiconductor microfabrication, including soft lithography, offers large possibilities for the design and fabrication of physically and chemically microstructured environments, which either mimic the natural ones, represent a collection of distinct environments - to be used in combinatorial experiments - or constitute platforms for biomedical devices. These purposefully designed micro-environments could be used to test the micro-scale dependent behaviour of filamentous fungi.

Microconfined environments fabricated from polymers offer many opportunities for biological studies, because of the controllable surface properties of the microstructures. One of the most versatile and therefore most common materials for the fabrication of microfluidic channels is the silicon based organic polymer poly(dimethylsiloxane) (PDMS) [4]. Compared to the conventional microfabrication materials, such as silicon and glass that are expensive to process and only present a limited biocompatibility, microfluidic devices made from polymers are cheaper and the fabrication is simpler. PDMS is transparent down to 280 nm, which makes it suitable for detection methods at a wide range
of wavelengths. It is biologically inert and non-toxic, oxygen permeable, it seals readily with other silicon based materials and has a controllable surface chemistry [5].

Fungal colony extension rests upon several parameters and mechanisms unique to filamentous fungi, including highly-polarised cell growth, i.e., the growth of the individual hyphae in a relatively fixed direction [6] determined at the branching point, periodic hyphal branching [7], negative autotropism [8] and the ability to transport nutrients in and between hyphae [9]. Next to a general description of the growth dynamics on and in solid and liquid homogeneous media, these studies also revealed dependencies on a number of external influences, e.g., toxins [10], temperature [11], light [12] and nutrient level [13].

2. Methodology
The *Neurospora crassa* strain used belongs to the culture collection of the School of Biological Sciences, University of Liverpool, and was maintained on malt extract agar (MERCK) at 4°C. Prior to each experiment, the fungus was subcultured onto fresh malt extract agar plates and incubated at room temperature for 24-36 hours.

The microfluidic structures used for the confinement of fungi were fabricated from PDMS (Sylgard 184, Dow Corning) according to the process flow chart in Figure 1. A positive relief master (Prof. Lee, University of California, Irvine) was fabricated using standard photolithography techniques and subsequent deep reactive ion etching (DRIE) [14]. Briefly, this positive relief master was then coated with HMDS (HexaMethylDiSilazane) to facilitate easy PDMS removal. The negative relief PDMS stamp was then fabricated by casting the degassed PDMS prepolymer and curing agent mixture (10:1 by weight) according to well-established procedures [4] against the coated silicon master. The PDMS was overcured at 65°C for at least 8 hours to ensure the full cross-linking of the polymer. After removal from the silicon master, the PDMS surface is inherently hydrophobic, but deep UV exposure causes the formation of silanol groups rendering it hydrophilic. The microfluidic structures were enclosed three-dimensionally by sealing them to an unpatterned, UV treated base glass layer. The silanol groups on both surfaces form covalent bonds resulting in an irreversible seal. The structures were designed such as to present lateral openings allowing the introduction of growth medium and fungal inoculation into the enclosed areas.

![Fabrication process](image)

**Figure 1.** Flow chart of the fabrication process; a)-c) 3D representation of three exemplary microconfined structures.

The microstructures were filled with nutrient-free growth medium (sterile distilled water) by depositing a droplet of the liquid next to the lateral opening. The channels fill due to capillary action and excess liquid surrounding the PDMS ensures the preservation of the filling. Fungal inoculation was achieved by placing an agar plug from the peripheral growth zone of a 24 to 36 hours old colony.
next to the lateral opening of the PDMS structure. This assembly was then enclosed in a Petri dish sealed with Parafilm creating an environment that retains moisture but allows exchange of oxygen and carbon dioxide.

Filament growth in the culture chamber was observed with a Brunel inverted microscope (SPI-98) equipped with a digital camera (Moticam 2300, 3MP). Images were typically collected as time series at regular intervals (usually one frame per 15 seconds) and subsequently imported into Image Pro Plus software (Version 6.1, Media Cybernetics) for further analysis. Image analysis comprised measuring the tip 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).

3. Results and Discussion

3.1. N. crassa on agar – reference data

We grew N. crassa on plain malt agar plates at room temperature and determined the average lateral branching angle as $45^\circ \pm 16^\circ$ (n=125, where n is the number of data points) whereas the distribution function corresponds to a normal distribution. This branching angle enables the expanding mycelium to explore and exploit an obstacle free, unconfined environment most efficiently. The distribution of the branching distances corresponds to a Weibull distribution with a mode of 158µm (n=107). This branching distance is large compared to the size of the micro-environments tested (Fig. 2). The tip extension velocity of N. crassa is one of the highest of the filamentous fungi. In our experiments the mode of the Weibull velocity distribution is 5.9µm/min (n=1884).

3.2. N. crassa in microconfined environments

We have observed the growth dynamics of N. crassa in seven distinct microconfined environments. The design of three of these test environments, presented in Figure 2a-c, shows varying degrees of confinement, complexity and periodicity. Every structure, with an edge length of 100µm, is connected to other structures of identical design by 100×100µm wide channels, thus enabling the study of the growth through up to five successive micro-environments. Our observations show that the microconfined environments induce distinct changes in the growth parameters depending on the confinement and the feature sizes. The smaller the features, i.e., the closer the feature size is to the hyphal diameter, the larger is the impact on the growth parameters. Briefly, N. crassa navigates the environments efficiently by decreasing the growth velocity and branching distance while increasing the branching angle. The growth characteristic of maintaining a fixed growth direction persists within the microconfined environments. In case a hypha hits an obstacle at an impact angle smaller than 55° to the wall, they adapt to the wall geometry in the direction closest to the original growth direction. As soon as the original growth direction becomes available again, it will readapt to it with a maximum deviation of $\pm 20^\circ$.

The confinement of the channels suppresses the formation of aerial hyphae, which usually indicate the transition from vegetative growth (increase of biomass, colony extension) to the reproductive cycle resulting in sporulation. To observe the vegetative fungal growth dynamics, this transition must be avoided. Most commonly, sporulation is circumvented by stress reduction, i.e., sufficient supply with nutrients and oxygen. The hyphae in our experiments are under starvation stress because the channels contain nutrient-free medium. However, we did not observe sporulation in any of the microconfined environments.

Observing N. crassa navigating the mazelike environment in Fig. 1c, we conclude that it has the ability to solve mazes through the application of efficient space searching algorithms. The fungal response to this structure is very complex due to the initiation of a large number of branches induced by two parallel occurring mechanisms 1) lateral branching and 2) apical splitting (Fig. 2d). The lateral branching distance as observed on plain agar decreases to approximately 1/10 15.5µm (n=77). The branching angle increases to $93.3^\circ \pm 15.4^\circ$ (n=84), which we suggest is induced by the majority of
rectangular corners of the environment features. Additionally to the aforementioned branching mechanism, the wall-collisions with an impact angle larger than 55° induce apical splitting at the collision point, giving rise to two daughter hyphae, which try to simultaneously grow around the obstacle. This behaviour suggests a high level of adaptation at both small spatial- and time-scales. To achieve this dynamic adaption and an effective distribution of ‘building material’ to the numerous exploring hyphae, the tip extension velocity reduces nearly 15-fold to 0.4µm/min (n=2215).

Figure 2. Snapshots of *N. crassa* recorded in different types of microfluidic structures. Images a-c show the designs of the three example structures. Images d-f show snapshots of *N. crassa* growing through these strcutures. In every case, the hypha originally entered the image from the left.

The representative environment in Figure 1e was designed to test the flexibility of fungal hyphae and whether they can perform u-turns. The snapshot taken shows that *N. crassa* is not only able to perform u-turns but that it can perform turns larger than 360°, if enforced by the geometry, indicating that the hyphal apex is very flexible, whereas more mature parts are stiff. The suggested flexibility is supported by the fact that hyphae are able to find imperfections in the seal of the PDMS cast and the base layer, penetrate them and split the layers apart thereby tunneling obstacles and walls. The design in Figure 1e presents only few channel crossings, which results in a very low number of branches (branches usually emanate into a crossing channel) and head-on wall collisions and therefore the data set is not sufficient to fit a distribution. Throughout the individual environments, *N. crassa* exhibits a distinct affinity to the cell walls which enables it to navigate this structure successfully, but it can also lead to the curl up observed in the adjacent connecting channel.

The third environment represented in Figure 1c consists of an array of 4×4 pillars of equal size and distance arranged in a square pattern. The entrance and exit are located at opposite corners and only 5µm wide in a 100µm wide wall. The first challenge this environment poses to the fungus is therefore to find the entrance and the second challenge is to master the highly-periodic pattern. Most hyphae show a meandering behaviour as represented in Figure 1f. This is induced by the characteristic of hyphae following fixed growth directions [6], thereby resulting in a trajectory through the environment close to the original growth direction. We suggest that the maintenance of the growth direction for *N. crassa* is very robust in both the spatial and the temporal domain.

3.3. Device for high throughput testing of the growth of filamentous fungi

The observations of the growth dynamics of *N. crassa* compared to a previous study focussing on a different species [15] in the same set of microconfined environments shows that the respective species present very different responses, suggesting that there could be more, unrevealed space searching algorithms than previously thought. This motivated us to develop the design of a stage mountable device generalising the previously used experimental setup. Figure 3 represents a scheme for the proposed device consisting of two parts, a structured PDMS cast and an unstructured base plate. The base plate comprises four stubs to ensure alignment and fixation of the PDMS cast. The PDMS cast is
fabricated in a similar fashion as described above. The positive relief master containing the microconfined environments is placed in a structure containing the relief for the stub indentations and the culture chambers and this whole set is then cast from PDMS. The base plate should be fabricated from a solid material like a non-corroding metal comprising a gap at the position of the micro-environments. This gap will accommodate a microscope cover slip ensuring the sealing of the channels and transparency for detection with a microscope (see Figure 3 Base plate side view). This setup does not require any additional sealing to ensure moisture due to the enclosed culture chambers and microconfined channels. In case this setup does not result in sufficient supply of oxygen, the culture chambers can be pierced with tubes connected to a pump circulating oxygen or possibly other gaseous or liquid chemical agents. This circulation can occur either within the individual chambers or possibly through the microconfined channels between the chambers. Using a microscope cover slip as the base layer will also facilitate fluorescence studies that require close vicinity of immersion objectives to the sample.

![Figure 3: proposed device for generalised testing of the behaviour of filamentous fungi in microconfined environments.](image)

The proposed device for the generalised experimental setup could be the basis of a number of new developments for biological applications or for whole cell-based biosensors that use filamentous fungi as the sensing agent. Possible applications include force sensors, e.g., hyphae pushing beads through channels or pushing levers, a tool for testing quorum sensing/chemotaxis and a tool for testing the fungal response to toxins, growth inhibitors or drugs. Punching holes through the PDMS and connecting one of the culture chambers to a pump facilitates the filling of this chamber with the respective chemical agent at any chosen time. Since PDMS is transparent to light down to 280nm, it can be used to test the fungal morphological responses to and sensitivity to light throughout the entire visible spectrum and parts of the UV spectrum. This approach can be a useful addition to the genetic studies that approach the problem by attempting to knock out genes for light sensing proteins. The behaviour towards certain wavelength could hint towards the expressed light sensing proteins.

Using fungi in whole cell sensors has several advantages towards those based on bacteria, including physical robustness, more extreme pH, temperature and osmotic/ionic strength tolerances, rapid growth and the fact that fungi are eukaryotes. So far, only yeast fungi immobilised on a substrate have been used as the biological agent. Using filamentous fungi circumvents the necessity of immobilisation on a substrate and enables the observation of changes in the growth dynamics, which requires less specialised equipment.
4. Conclusions
The proposed methodology proves valuable for testing the fungal growth dynamics in microconfined environments. The work shows that the microconfinement of the natural environment is a non-negligible parameter for fungal growth. From the absence of aerial hyphae within the test environments, we conclude that with the help of microconfinement it is possible to study vegetative fungal growth dynamics during nutrient and/or oxygen deprivation. Additionally, it revealed that the space searching algorithms differ vastly between fungal species.

Future work will continue to investigate the growth dynamics of a variety of filamentous fungi and the exploration of the feasibility of some of the applications proposed. The characterisation of the space searching algorithms of a large number of filamentous fungi might result in a general set of growth mechanisms for filamentous fungi. Additionally, we want to investigate the origin of the space searching algorithms and their biological basics, i.e., what are the mechanisms inside the cell that facilitate the adaptation. In order to achieve this we will use fluorescence techniques to observe the dynamics of cell organelles and the cytoskeleton.

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References
[1] Bowen A D, Davidson F A, Keatch R and Gadd G M, 2007 Fungal Genetics and Biology, 44, 484
[2] Kumamoto C A, 2008 Nature Reviews Microbiology 6, 667
[3] Kasuga T and Glass N L 2008 Eukaryotic Cell 7, 1549
[4] Sia S K and Whitesides G M 2003 Electrophoresis 24, 3563
[5] Makamba H, Kim J H, Lim K, Park N and Hahn J H, 2003 Electrophoresis 24, 3607
[6] Riquelme M, Reynaga-Pena C G, Gierz G and Bartnicki-Garcia S 1998 Fungal Genetics and Biology 24, 101
[7] Watters M K and Griffiths A J F 2001 Applied and Environmental Microbiology 67, 1788
[8] Bottone E J, Nagarsheth N and Chiu K 1998 Canadian Journal of Microbiology 143, 390
[9] Darrah P R, Tlalka M, Ashford A, Watkinson S C and Fricker M D 2006 Eukaryotic Cell 5, 1111
[10] Weitz H J, Campbell C D and Killham K 2002 Environmental Microbiology 4, 422
[11] Ryan F J, Beadle G W and Tatum E L 1943 American Journal of Botany 30, 784
[12] Linden H, Ballario P and Macino G 1997 Fungal Genetics and Biology 22, 141
[13] Brown A J P and Gow N A R 1999 Trends in Microbiology 7, 333
[14] Andersson H, van der Wijngaart W, Griss P, Niklaus F and Stemme G 2001 Sensors and Actuators B: Chemical 75, 136
[15] Hanson K L, Nicolau Jr. D V, Filipponi L, Wang L, Lee A P and Nicolau D V 2006 small 2, 1212