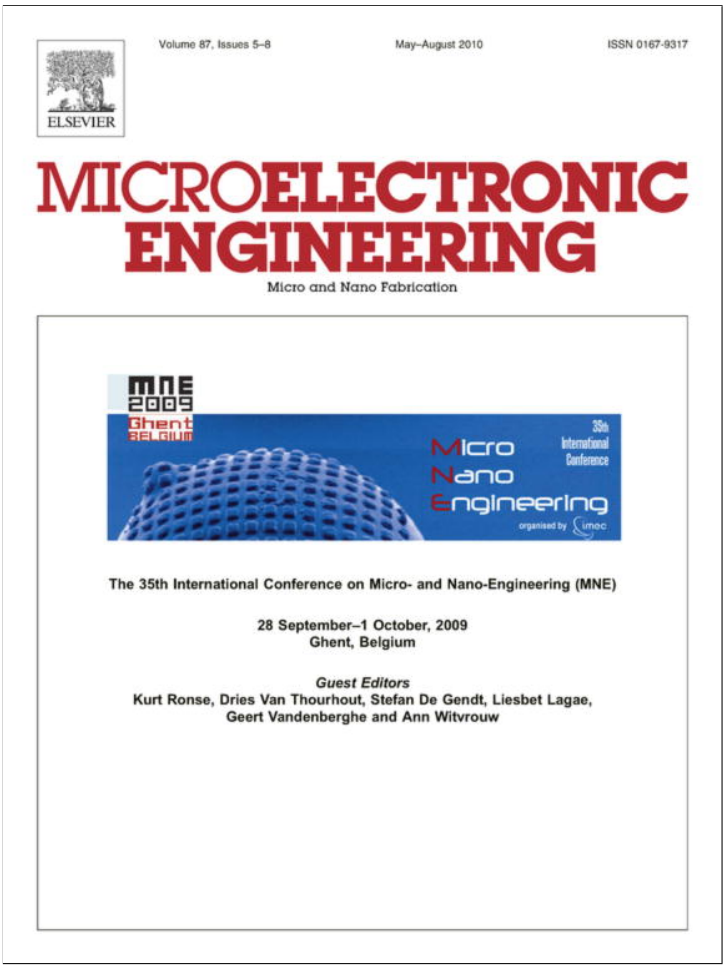

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Microfluidics structures for probing the dynamic behaviour of filamentous fungi

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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

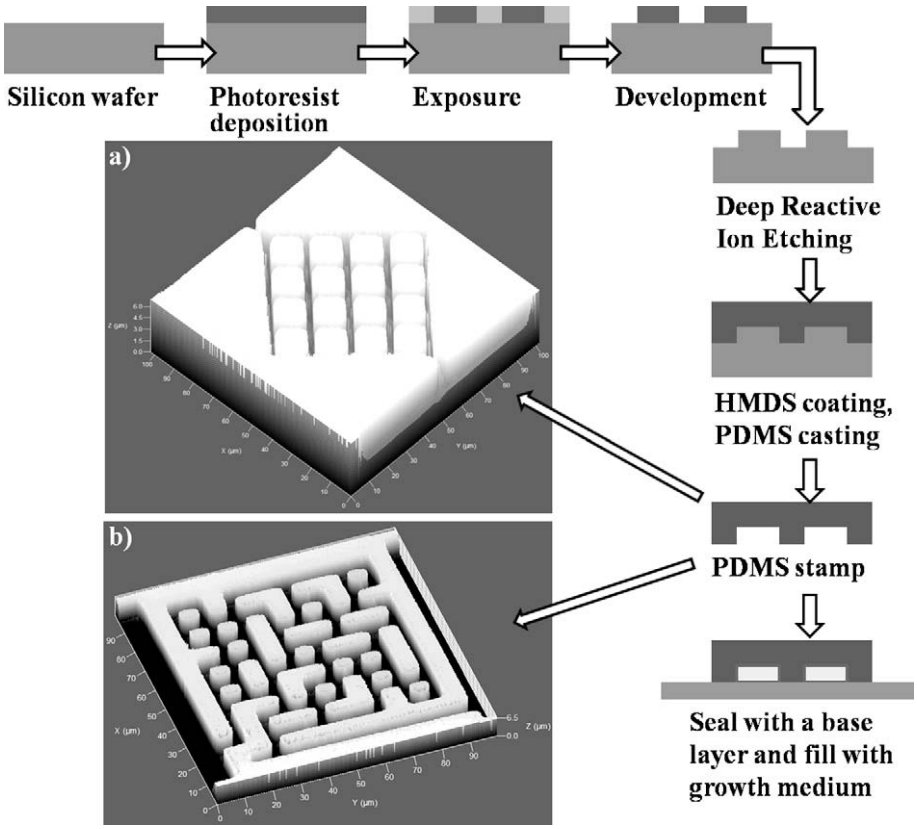

Fig. 1. Flow chart of the fabrication processes of microstructured Si wafers and subsequent PDMS molds. (A and b) show three-dimensional representations of the (a) diamond structure and (b) maze-like structure used.

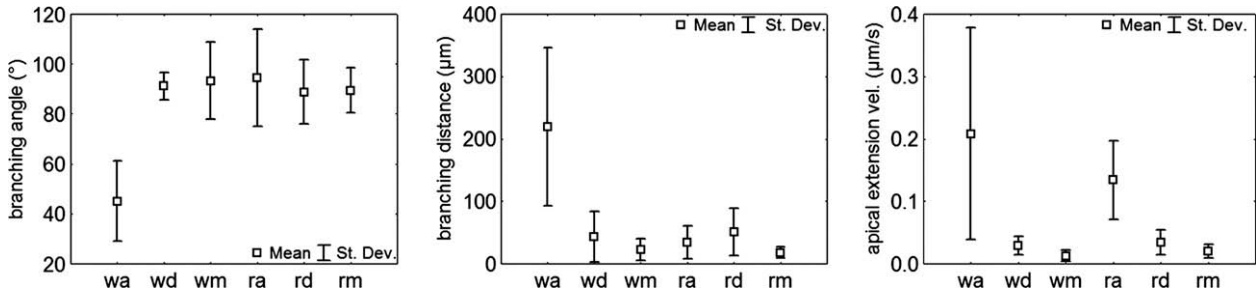

Fig. 2. Comparison of the average values (with error bars indicating the respective standard deviations) of the growth parameters of *Neurospora crassa* wild type and *ro-1* mutant strains on agar and in microfluidic test structures. Abbreviations: wa: wild type on agar; wd: wild type in diamond; wm: wild type in maze; ra: ro-1 on agar; rd: ro-1 in diamond; rm: ro-1 in maze.

the edge length of the test microfluidics structures (i.e., 219.1 ± 126.4 µm, compared with maximum 100 µm, respectively), it was expected that this growth parameter would be altered dramatically in confined geometries. The average apical extension velocity (0.21 ± 0.17 µm s^{-1}) resulted in a circular colony expansion of approximately 4 cm over 24 h. These parameters lie within the ranges given in the literature. While the colonies of the *ro-1* mutant expanded considerably less than the wild type one (approximately 1.5 cm over 24 h), the apical extension velocity of the *ro-1* mutant is slightly less (0.13 ± 0.06 µm s^{-1}) compared to the wild type strain. The explanation of this paradox lies in the alteration of the hyphal growth, and hence the morphology, of the *ro-1* mutant. While the individual hyphae of the wild type *N. crassa* grow into fixed directions, pre-determined at the respective branching points, those of the *ro-1* mutant do not maintain a fixed growth direction presenting curled paths. This observation

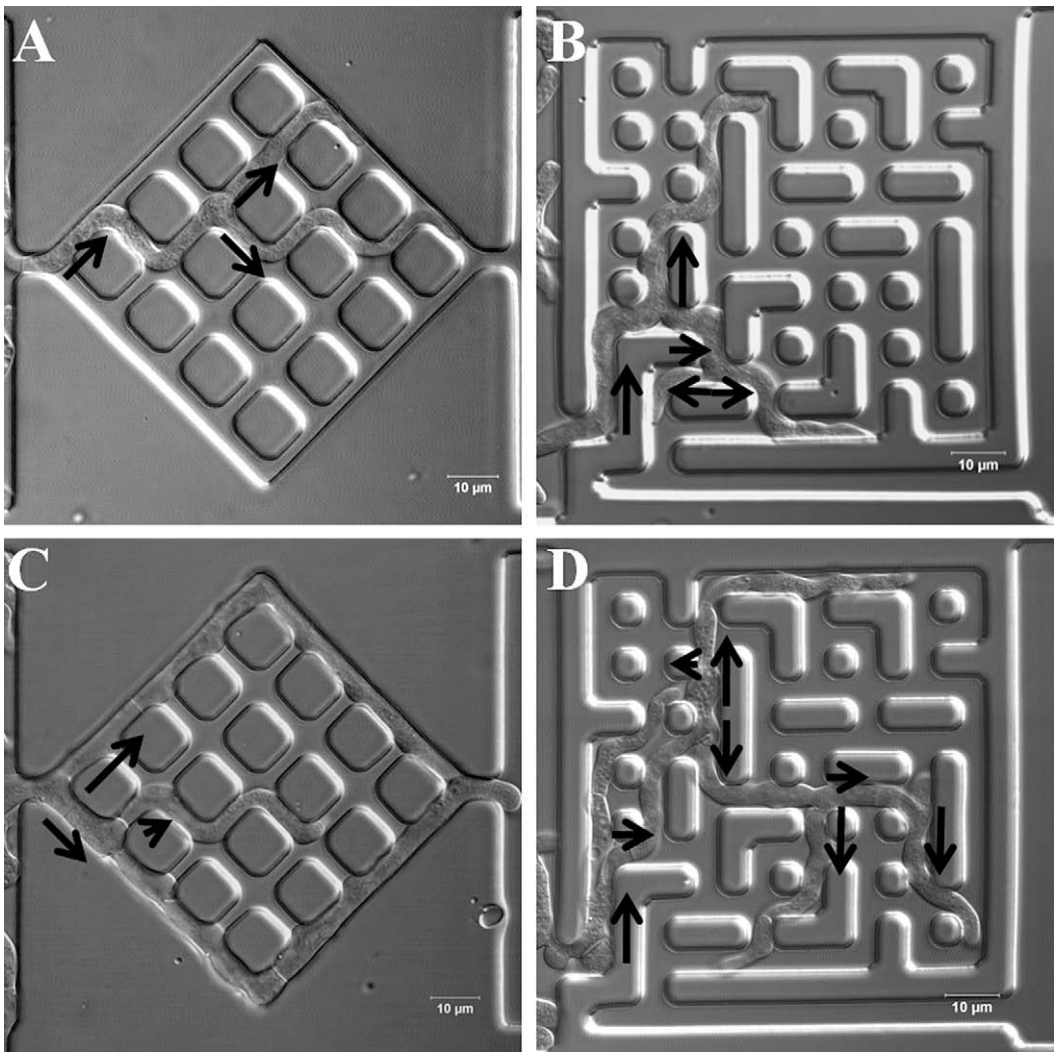

Fig. 3. Representative DIC images of the *N. crassa* wild type strain solving A the diamond and B the maze-like structure as well as the *N. crassa ro-1* mutant strain solving C the diamond and D the maze-like structure. Arrows indicate the growth directions of the hyphae at the entrance and at the branching points.

suggests that the Spitzenkörper contributes to directional growth but is not exclusively responsible for the polarized growth of fungi. The coupling of the large branching angle (94.4 ± 19.4°) and the small branching distance (34.3 ± 26.2 μm) results in a moderate rate of colony extension and a higher hyphal density of the colony of the mutant compared with wild type strain. Additionally, the mutation slightly alters the branching pattern from a principally lateral branching in the wild type to a partially dichotomous (apical) branching. The altered Spitzenkörper stability appears to mainly impact the branching distance, thus suggesting that the Spitzenkörper position and/or composition plays a crucial (but not exclusive) role in branch initiation.

3.2. N. crassa wild type and ro-1 in microfluidic structures

Both examined strains of *N. crassa* solve the microfluidic networks successfully and in similar periods (approximately 4 h for the maze-like geometry). The growth parameters, i.e., the branching angle, the branching distance and the apical extension velocity, presented in Fig. 2, reach similar values in a confined environment, as compared with the larger differences of the growth parameters on agar. This suggests that the space-searching algorithms of this species are robust and alteration by this particular mutation appears negligible. Fig. 3, representing still images of both strains within the microfluidic networks, shows the similarity of the solution paths.

In confined spaces, i.e., the diamond and maze-like structures, the branching angle for the *N. crassa* wild type increases by twofold in (91.2 ± 5.4° and 93.3 ± 15.4° respectively), while the periodicity of the diamond structure leads to a considerably smaller standard deviation, thus demonstrating a rapid and specific response to confined geometries. In contrast, the average branching angle of *N. crassa ro-1* remains at similar values (94.4 ± 19.4°, and 88.8 ± 12.9° and 89.4 ± 9.0° on agar, the diamond and the maze-like structure, respectively).

The branching distance of the wild type decreases considerably inside the microfluidic structures (43.1 ± 40.1 μm and 23.5 ± 15.0 μm, in the diamond and complex maze-like structures, respectively). This behaviour resulted in a vast increase of hyphal ramification inside the structures, as the fungus increased the possibility of finding the exit. The branches were primarily directed towards the channel openings and the branching towards solid walls or obstacles appeared suppressed. In contrast, the branching distance of the *N. crassa ro-1* mutant varies in the same range for growth on agar and in microfluidics networks (34.3 ± 26.2 μm, 50.8 ± 38.4 μm and 17.7 ± 9.0 μm, on agar, in diamond and maze-like structures, respectively).

The apical extension velocity for both species exhibit similar values inside the microfluidics structures, which are also similar to those observed on agar. It appears that the mutation has the least impact on the apical extension velocity. This growth parameter is often proposed to be closely tied to the Spitzenkörper [7–9]. However, due to the alteration in the Spitzenkörper position and composition in the mutant, our observations obtained in confined spaces suggest that this might not be the sole factor responsible for fungal directed growth.

The response of both species did not follow a temporal or spatial adjustment period but seemed to occur instantaneous upon entering the microfluidic structures, which leads to the conclusion that the fungus relies on both wall contact-based and wall contact-independent space-searching mechanisms.

The results presented above suggest that there is a strong response of the growth dynamics of the filamentous fungus *N. crassa* to the geometrical properties of the microfluidic structures confining its growth, as suggested before for another species [12]. Moreover, this response to microfluidics 3D structures is far stronger correlated to geometrical parameters than that observed to open, 2D + microstructures [13].

Importantly, and in addition to these conclusions, the two *N. crassa* strains present different responses to confinement: the wild type decreased the branching distance and increased the branching angles without exception, but for the *ro-1* mutant this behavioural pattern required only small changes to reach similar solving efficiencies" of the networks. As both microfluidics test structures comprise only forced changes of directions at 90° angles, it appears that the wild type *N. crassa* "adapted" to the new confined environment, while the *ro-1* strain was "designed" (by accident) to solve this type of networks. This observation could have deeper significations, as it suggests that one can artificially mutate species designed to solve networks that encode a non-trivial mathematical problem, a suggestion made before [14].

4. Conclusions

We presented a methodology for the study of the dynamic behaviour of filamentous fungi based on the use of purposefully designed microfluidics test structures. The application of microfluidic structures with dimensions similar to the size of the fungal cell diameter instead of homogeneous media resulted in the geometry-induced and geometry-specific response of the fungal growth behaviour revealing distinct, so far unreported space-searching algorithms. An immediate result of this methodological study is the observation that that the growth response to confinement is instantaneous and that the Spitzenkörper is a crucial element for directional growth, but not necessarily for polarized growth. Most interestingly, the strain-specific response to confined networks suggests that directed mutation could lead to species designed to solve networks that encode non-trivial mathematical problems.

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References

[1] C.S. Evans, J.N. Hedger, In Fungi in Bioremediation, Cambridge University Press, Cambridge, United Kingdom, 2001. pp. 1–26.
[2] C.A. Kumamoto, Nat. Rev. Micro. 6 (9) (2008) 667–673.
[3] T. Kasuga, N.L. Glass, Eukaryot. Cell 7 (9) (2008) 1549–1564.
[4] R. Singhvi, A. Kumar, G.P. Lopez, G.N. Stephanopoulos, D.I.C. Wang, G.M. Whitesides, D.E. Ingber, Science 264 (1994) 696–698.
[5] S.E. Hulme, W.R. DiLuzio, S.S. Shevkoplyas, L. Turner, M. Mayer, H.C. Berg, G.M. Whitesides, Lab Chip 8 (11) (2008) 1888–1895.
[6] M. Riquelme, R.W. Roberson, D.P. McDaniel, S. Bartnicki-Garcia, Fungal. Genet. Biol. 37 (2) (2002) 171–179.
[7] S. Bartnicki-Garcia, F. Hergert, G. Gierz, Protoplasma 153 (1-2) (1989) 46–57.
[8] M. Riquelme, C.G. Reynaga-Pena, G. Gierz, S. Bartnicki-Garcia, Fungal. Genet. Biol. 24 (1–2) (1998) 101–109.
[9] G. Steinberg, Eukaryot. Cell 6 (3) (2007) 351–360.
[10] H. Andersson, W. van der Wijngaart, P. Griss, F. Niklaus, G. Stemme, Sensor. Actuat. B – Chem 75 (1–2) (2001) 131–141.
[11] S.K. Sia, G.M. Whitesides, Electrophoresis 24 (21) (2003) 3536–3576.
[12] K.L. Hanson, D.V. Nicolau Jr., L. Filipponi, L. Wang, A.P. Lee, D.V. Nicolau, Small 2 (10) (2006) 1212–1220.
[13] M. Held, A. Komaromy, F. Fulga, C. Edwards, R.I. Boysen, M.T.W. Hearn, D.V. Nicolau, Microelectr. Eng. 86 (4-6) (2009) 1455–1458.
[14] D.V. Nicolau, D.V. Nicolau Jr., G. Solana, K.L. Hanson, L. Filipponi, L. Wang, A.P. Lee, Microelectr. Eng. 83 (4–9) (2006) 1582–1588.