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## Soil-on-a-Chip: microfluidic platforms for environmental organismal studies

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Soil is the habitat of countless organisms and encompasses an enormous variety of dynamic environmental conditions. While it is evident that a thorough understanding of how organisms interact with the soil environment may have substantial ecological and economical impact, current laboratory-based methods depend on reductionist approaches that are incapable of simulating natural diversity. The application of Lab-on-a-Chip or microfluidic technologies to organismal studies is an emerging field, where the unique benefits afforded by system miniaturisation offer new opportunities for the experimentalist. Indeed, precise spatiotemporal control over the microenvironments of soil organisms in combination with high-resolution imaging has the potential to provide an unprecedented view of biological events at the single-organism or single-cell level, which in turn opens up new avenues for environmental and organismal studies. Herein we review some of the most recent and interesting developments in microfluidic technologies for the study of soil organisms and their interactions with the environment. We discuss how so-called “Soil-on-a-Chip” technology has already contributed significantly to the study of bacteria, nematodes, fungi and plants, as well as inter-organismal interactions, by advancing experimental access and environmental control. Most crucially, we highlight where distinct advantages over traditional approaches exist and where novel biological insights will ensue.

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## Introduction

The field of microfluidics as we know it today originated from concepts and technological developments in the fields of microelectronics and chemical analysis due to the need to perform high efficiency chemical or biological experiments

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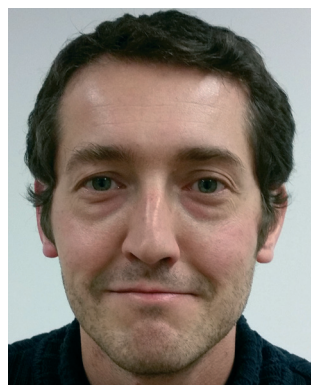
and perfusion platform for Arabidopsis roots.

on short timescales and with limited volumes of analyte.<sup>1,2†</sup> Crucially, the microfluidic revolution arose due to the distinct advantages offered by system miniaturisation, including the high analytical throughput, enhanced sensitivity, improved analytical performance, facile parallelisation through multiplexing, the ability to handle and process reduced reagent volumes and vastly reduced instrumental footprints. Since the pioneering developments of the early 1990s, microfluidic technology has found application in a variety of research fields, including chemical synthesis,<sup>3</sup> DNA analysis,<sup>4</sup> proteomics,<sup>5</sup> single cell analysis,<sup>6–8</sup> tissue engineering,<sup>9</sup> high-throughput screening,<sup>10</sup> environmental analysis<sup>11</sup> and medical diagnostics.<sup>12</sup>

Surprisingly, microfluidic technology has only been utilised for the study of whole (living) organisms in recent years, but already it is clear that such platforms provide new insights into biological processes and enable the efficient and rapid generation of novel biological information. Despite the immaturity of this field, studies of whole organisms using microfluidic platforms have been extremely diverse and are ever expanding, as highlighted by recent articles discussing the exploitation of microfluidics to explore bacterial microenvironments<sup>13,14</sup> and multicellular organisms (such as mammalian embryos, zebrafish, fruit flies and roundworms).<sup>15</sup> Indeed, microfluidic technology has also found application in new areas of organismal research such as microbial ecology<sup>16</sup> and plant sciences.<sup>17</sup> The adoption of microfluidic platforms in organismal studies has stemmed from a basic need to improve traditional methods or to conduct experiments that would otherwise be intractable. A key

feature of microfluidic systems relevant to organismal studies is the ability to confine an organism within a defined micro-environment in a precise and robust manner. At small length scales laminar flow becomes the dominant regime for fluid flow, and provides for exquisite control over both mass and thermal transport. This in turn allows the experimentalist to address specific parts of an organism with fine spatial and temporal resolution, and create defined concentration gradients in which to study phenomena such as bacterial chemotaxis<sup>18</sup> and embryonic patterning.<sup>19</sup> Moreover, the use of rapid prototyping methods (such as soft-lithography, hot embossing and 3D printing) means that microfluidic devices are bespoke and therefore specific to the organism of interest, allowing organism growth and distribution to be managed with ease.

In the current discussion we specifically consider organisms whose natural habitat is soil, namely bacteria, nematodes, fungi and plants, as well as inter-kingdom interactions such as those existing between bacteria and fungi or between microbes and plants. This heterogeneous environment is extremely complex, being composed of a porous network and having a non-uniform distribution of both water and nutrients. Environmental conditions in soil, such as temperature, pH or gas composition are also dynamic and often vary substantially between regions that are close to the surface and those deeper underground. Accordingly, all soil organisms have developed strategies to deal with abiotic stresses such as drought, anoxia, temperature changes, high salinity, nutrient deficiencies or over-fertilisation.<sup>20</sup> How these stresses are sensed and how organisms respond through physiological or



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† For the purposes of the current discussion, we define microfluidic systems as those that manipulate and control fluids geometrically constrained within environments having internal dimensions (or hydrodynamic diameters) most easily measured in microns.

developmental adaptations to cope with adverse conditions are central (and as yet unanswered) topics in ecological, biological and agronomical sciences.

The rivalry for resources has also led to the evolution of synergies and symbioses, in particular between plants and

microorganisms, which have a significant impact on plant nutrition. Mycorrhizae, a symbiosis between plant roots and fungi, help plants to effectively extend the soil volume that is being explored for nutrients by several orders of magnitude. Indeed, more than 80% of land plants, including most crop plants, use ecto- or endomycorrhizal symbiosis to ensure a sufficient supply of phosphorous.<sup>21</sup> Many plants associate with nitrogen-fixing rhizobia, and, in the case of legumes, can host these microbes inside symbiosomes; specialised intracellular compartments within root nodules.<sup>22</sup> The potential to improve plant nutrition and thereby crop yield by engineering such plant-microbe interactions has attracted much attention in recent years. Conversely, plants have to defend against pathogenic bacteria and fungi or parasitic animals, such as nematodes. How plants distinguish between beneficial and harmful organisms in the rhizosphere, how symbiotic interactions are established, and how plants defend against competitors and pests are subjects of extensive research with considerable economical significance. Moreover, free-living nematodes play a key role in soil ecology in their capacity as vectors, contributing to the persistence and spread of bacteria in soil.<sup>23</sup> When pathogenic, this raises public health concerns, specifically in anthropogenic or arable lands used for food production. Due to the heterogeneity of the underground biosphere, a comprehensive understanding of the nature of inter-organismal interactions and of the mechanisms of environmental sensing, acclimation and developmental responses to biotic and abiotic stresses remains a major challenge over the coming decades.



**Fig. 1** Cartoon representing “Soil-on-a-Chip” microfluidic technology. In this review, we consider microfluidic platforms that have been used to study the interplay between soil-dwelling organisms, including bacteria, nematodes, fungi and plants, and their environment. Importantly, inter-kingdom interactions are also discussed, such as those existing between bacteria and fungi or between microbes and plants. Illustration hand-drawn by C. E. S.

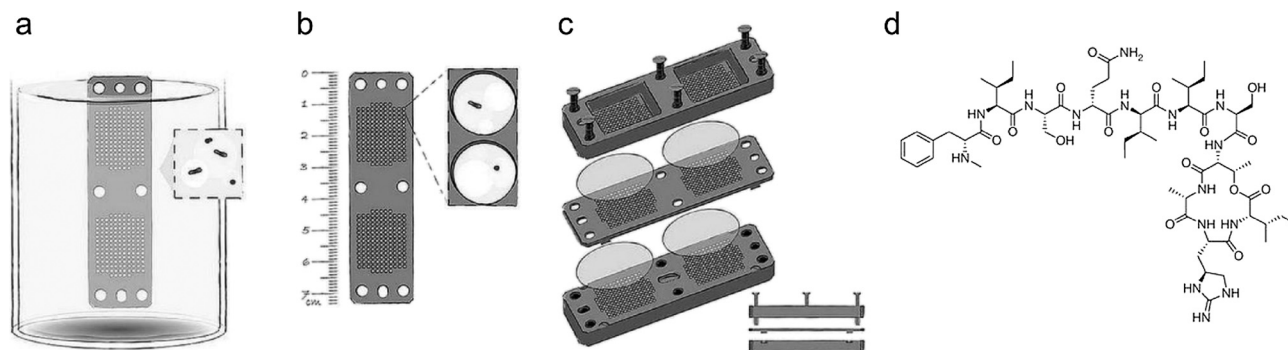
We have coined the term “Soil-on-a-Chip”, represented by the cartoon in Fig. 1, which we define as those microfluidic platforms used to study the interplay between soil-dwelling organisms and their environment. Clearly, there is a need for tools that can accurately simulate the complex soil environment and mimic variations in environmental conditions. For example, the ability to control the level of hydration within a network, apply combinations of stimuli or tune the spatio-temporal distribution of the interactions between co-localised species would be highly desirable. The opportunity to model soil environments, and control a variety of variables in a defined manner, is important with respect to enhancing our understanding of how soil-dwelling organisms interact with and adapt to their environment. Understanding how plants regulate root development, for example, has important implications with respect to improving crop yields.<sup>24</sup> Additionally, the ability to study how small organisms adapt to selective environmental-like cues can lead to a better understanding of their Darwinian evolution.<sup>25</sup> Herein, we describe the most recent developments in microfluidic technology within this young field and discuss how we believe microfluidics can be exploited within this field in the future.

## Bacteria

It is recognised that more than 99% of environmental microorganisms cannot be cultured under laboratory conditions, *i.e.* they fail to grow on artificial media in petri dishes.<sup>26</sup> This is most likely due to the fact that synthetic conditions do not provide many organisms with the correct combination of growth factors, chemicals and nutrients present in their natural environment. It is, however, clear that we do not yet fully understand the biology of these microorganisms. For example, antibiotic resistance continues to be an issue of immense concern with the number of new antibiotics reaching the market dropping year on year. Accordingly, the ability to tap into an unexplored source of potential antibiotic-producing bacteria and employ high-throughput droplet-based microfluidic screening technologies,<sup>27,28</sup> for example, is highly desirable, opening prospective new avenues for drug discovery.

Several attempts have been made to simulate the growth conditions of natural bacterial environments. Indeed Stewart *et al.*<sup>26</sup> provide an excellent overview of recent strategies employed to culture “uncultivable” bacteria, including co-culturing methods<sup>29,30</sup> and those that culture microorganisms in their original environment.<sup>31–33</sup> In 2010, Nichols *et al.*<sup>34</sup> reported the “isolation chip” (*iChip*), shown in Fig. 2, which allows for the isolation and cultivation of uncultivable microbes *in situ*. This device consists of a central plate containing 384 through-holes, each 1 mm in diameter and length. This central plate is then submersed in an aqueous, agar-containing solution containing an environmental cell sample of interest (Fig. 2a), which is diluted to a concentration such that, on average, one bacterial cell occupies one through-hole (Fig. 2b). A semi-permeable membrane is





**Fig. 2** The Isolation Chip (*iChip*). The central plate of the *iChip* is submerged in a dilute, agar-containing solution of bacteria (a), where single bacteria occupy each of the 384 through-holes (b). (c) Semi-permeable membranes are sandwiched on either side of the central plate with a top and bottom plate, thus forming 384 individual diffusion chambers. (d) Chemical structure of Teixobactin. Reprinted by permission from Macmillan Publishers Ltd: Nature (ref. 35), copyright 2015.

then placed on either side of the central plate, followed by a “top” and “bottom” plate (each containing 384 channels in equivalent positions to the through-holes) (Fig. 2c). The *iChip* can then be pressure sealed using a screw-tight mechanism, resulting in 384 individual diffusion chambers. The device is then submerged in the cells’ original environment and incubated over extended time periods. Using such an approach, the authors demonstrated that the percentage microbial recovery for the *iChip* far outperformed that of a Petri dish format, with almost 50% of cells assayed from a soil sample growing in the *iChip* (compared to only 1% on a petri dish).

In 2015, Ling *et al.*<sup>35</sup> used the *iChip* to mine for new antibiotics produced by soil bacteria. Soil harbours over 1 billion bacterial cells per gram<sup>36</sup> and represents an unexploited source for the discovery of new antibiotics, especially since only 1% of soil bacteria have been cultivated and explored and many antibiotics have been derived from soil microbes in the past.<sup>37</sup> To perform antibiotic mining, the *iChip* was loaded with bacterial cells, placed back into the soil from which the sample originated and left in place for a month. Extracts from 10 000 uncultured isolates were subsequently screened for antimicrobial activity on *Staphylococcus aureus* plates. In this way, the authors identified a new antibiotic, a cell wall inhibitor named Teixobactin (chemical structure displayed in Fig. 2d), from a new species of bacteria that displayed good activity. Such a discovery indicates that the microfluidic method has the potential to source novel bacterial strains that produce new antibiotics.

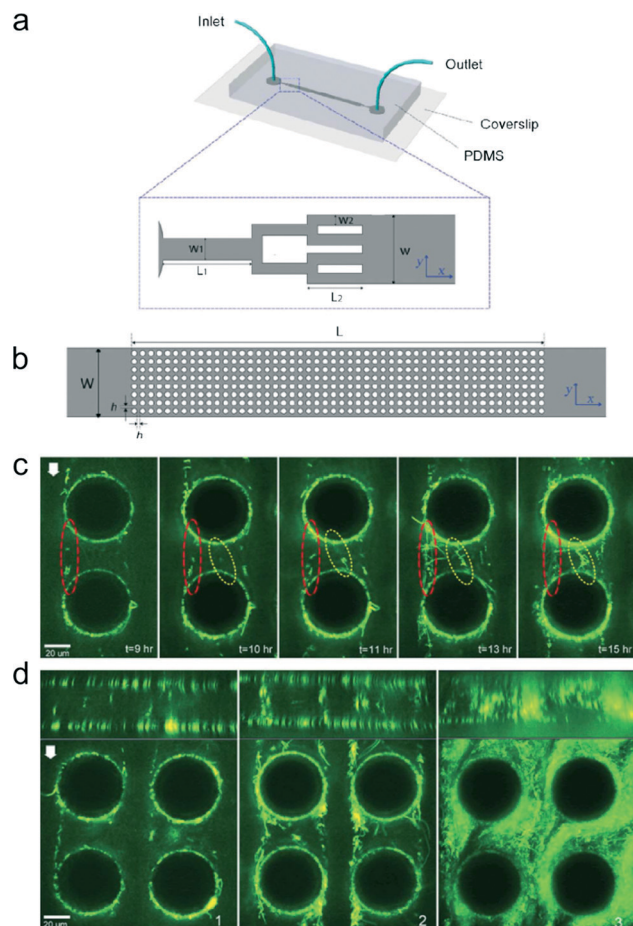
Bacteria do not solely exist as free-swimming cells. In nature, they often form multicellular communities known as biofilms.<sup>38</sup> These sessile entities are attached to surfaces and are enveloped in an extracellular polymeric matrix. Possible driving forces for the formation of biofilms by bacteria during infection, for example, have been postulated by Jefferson,<sup>39</sup> including the ability to offer a greater capacity for a community to defend itself, to exist in a favourable environment or to make use of cooperative benefits. Jefferson also suggests that biofilm formation could simply be the normal mode of growth in non-laboratory environments. Understanding the underlying quorum sensing mechanisms

driving biofilm formation has great importance to human health, both in tackling disease and contamination of medical devices,<sup>40,41</sup> as well as to processes such as bioremediation.<sup>42</sup> Biofilms also form in soil environments, being an important component of the rhizosphere for example.<sup>43</sup> However, our current understanding of biofilms in soil is limited, as they are difficult to study at the microscopic level.<sup>44</sup>

Microfluidic approaches have been used to study biofilm formation,<sup>45–47</sup> owing to the need to quantify biofilms under defined chemical and physical conditions, and have more recently been used to mimic soil environments. Markov *et al.*<sup>48</sup> used a simple microfluidic device, christened the *EcoChip*, to create microporous flow cells for environmental research. Perfusion of bacteria through a micropatterned, “microfluidic soil” region was achieved using a simple flow module, enabling long-term growth and transport of bacteria through the porous media, as well as biofilm formation, to be monitored. The authors highlight that the *EcoChip* can be used to house both bacteria and protozoa, where the effects of the micropatterned soil arrays on providing bacteria refuge for predation by protozoa can be investigated. A similar concept was used by Valiei and co-workers,<sup>49</sup> who developed a microfluidic device (Fig. 3a) containing a micropost array (Fig. 3b) embedded in a microchannel to investigate biofilm streamer development of *Pseudomonas fluorescens*, which inhabits and forms biofilms in soil environments. Streamers, defined as viscoelastic filamentous structures, are tethered to pre-formed, wall-attached biofilms.<sup>50</sup> Interestingly, the authors found that streamer formation was highly dependent on the fluidic conditions used (see Fig. 3c and d) and suggest that streamer development may be a necessity for the formation of higher ordered structures.

## Nematodes

*Caenorhabditis elegans* is a model organism commonly used in biological (particularly in the fields of evolutionary biology and aging research<sup>51</sup>) and toxicological screens.<sup>52</sup> The nematode is typically found in organically enriched anthropogenic soils and various microbe-rich habitats, such as decaying



**Fig. 3** (a) Schematic of the porous microfluidic device. The enlarged region displays the design used to equalise the pressure head (dimensions  $W = 625 \mu\text{m}$ ,  $W_1 = 200 \mu\text{m}$ ,  $W_2 = 100 \mu\text{m}$ ,  $L_1 = 1000 \mu\text{m}$ , and  $L_2 = 500 \mu\text{m}$ ). (b) The porous zone, having a length ( $L$ ), width ( $W$ ) and height ( $H$ ) of  $3750 \mu\text{m}$ ,  $625 \mu\text{m}$  and  $50 \mu\text{m}$  respectively. A  $50 \times 8$  array of microposts, measuring  $50 \mu\text{m}$  in diameter and height (inter-post spacing ( $h$ ) is  $25 \mu\text{m}$ ), forms the porous media within this zone. (c) Time series illustrating streamer formation at a constant rate of  $8 \mu\text{m h}^{-1}$ . Red and yellow ellipses represent parallel and traverse streamers respectively. (d) Dependence of flow rate on streamer development ( $8$ ,  $12$  and  $20 \mu\text{m h}^{-1}$  in picture 1, 2 and 3 respectively) at  $t = 9 \text{ h}$ . Reproduced in part from ref. 49 with permission from The Royal Society of Chemistry.

plant matter (compost and rotting fruit and plant stems).<sup>53</sup> *C. elegans* has been used as an experimental platform to study bacteria–nematode interactions, showing, for example, how these microbes affect the fitness of the worms they colonise<sup>54</sup> and demonstrating that *C. elegans* can enhance the spread of pathogenic bacteria in the environment.<sup>55,56</sup> In the wild, however, worms display a much wider range of phenotypes than observed in the laboratory. While the presence of microorganisms in the intestinal lumen of laboratory grown nematodes is rare, free-living nematodes often harbour a large microbial fauna. This ranges from eukaryotic cells (mostly yeasts) to live bacterial flora (which may proliferate and clot the intestinal tract or hinder pharyngeal pumping, illustrating how bacteria can behave as both a food source

and a pathogen) and even fungi and plant remains (which are both believed to be a source of sterols for the nematodes).<sup>57</sup>

Nevertheless, many aspects of the ecology of *C. elegans* remain unexplored. An example of this is the dauer stage, a developmental arrest characterised by reduced metabolism and increased stress resistance. The processes involved in deciding dauer entry and exit have been studied in the laboratory (both genetically and with environmental cues), but understanding regulation cues in the wild (*i.e.* in the presence of a much wider biodiversity), and how these responses evolve in different environmental contexts, are unresolved questions that require novel experimental approaches for their study.

To better understand the ecology of nematodes in their natural environment, it would clearly be advantageous to carefully reproduce in the laboratory the conditions in the wild. Ideally this would involve the use of experimental platforms that, under controlled conditions, can reproduce the complexity of the environmental cues experienced by free-living nematodes. Due to the small size of these organisms, miniaturised platforms have already been applied for neurophysiological, behavioural and toxicological studies. Indeed, several excellent reviews have elegantly described the diversity of microfluidic platforms developed for *C. elegans* screens.<sup>58–60</sup> Environmental studies utilising miniaturised platforms, though, are far scarcer and have been limited to the exploration of nematode behaviour in microstructured environments reminiscent of the porous and humid conditions of soil.

For example, Lockery *et al.* developed a platform, coined “artificial dirt”,<sup>61</sup> to study the locomotive behaviour of nematodes within microstructured post arrays and channels. Their device allowed precise temporal and spatial delivery of a wide range of stimuli to freely crawling worms and experimental control of the worms’ locomotion waveform and trajectory. Using a similar platform, termed “micro-dirt”, Lee *et al.* studied the “nictating” behaviour of *C. elegans* (Fig. 4).<sup>62</sup> Nictation is a common (and conserved) behavioural response of dauer larvae (the most common stage of *C. elegans* in the wild) by which nematodes stand on the posterior part of their bodies and swing their heads in the air. This is believed to enable starved animals to establish contacts with carrier animals (such as snails, slugs, isopods and chilopods) so that they can access unexplored areas with richer food resources.<sup>63</sup> Using this platform, in combination with an optogenetic assay, the authors showed that signals from IL2 neurons are sufficient for nictation and that this behaviour was required for transmission of *C. elegans* to a new niche using flies as artificial carriers, suggesting the role of nictation as a dispersal and survival strategy.

Finally, Park and co-workers identified novel modes of locomotion for nematodes that were presented with arrays of microposts.<sup>64</sup> In this study, it was observed that *C. elegans* could move through an array of microposts ten times faster than through normal agar plates. When compared to wild type animals, mechanosensitive defective mutants failed to

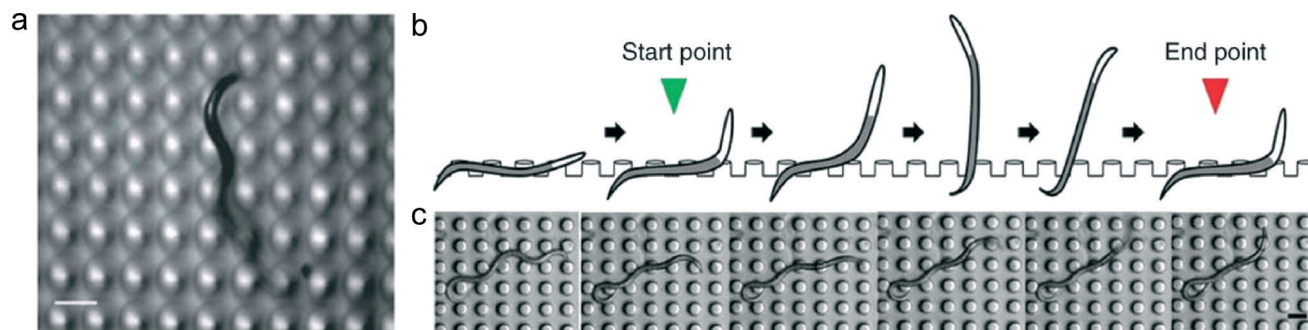


Fig. 4 The micro-dirt chip. (a) Image showing a nictating dauer (focussed on the nose tip). (b) Cartoon illustrating the various stages involved in nictation. The start and end points of nictation are indicated by the green and red arrows respectively. (c) Series of images representing a nictating dauer on the micro-dirt chip. The images correspond to the stages represented in (b). All scale bars represent 75  $\mu\text{m}$ . Reprinted by permission from Macmillan Publishers Ltd: Nature Neuroscience (ref. 62), copyright 2012.

show such enhanced locomotive response, which led the authors to conclude that worms rely on the transduction of touch for navigation and probably utilise this sensory pathway more routinely and subtly in their normal movements in the wild than previously expected. In a more complex study, Albrecht *et al.* characterised the locomotive behaviour of *C. elegans* under a wide range of temporal and spatial chemical stimuli.<sup>65</sup> Using a microfluidic platform, they confirmed known locomotory behaviours (such as klinokinesis and klinotaxis) and further identified three new locomotory behaviours: a coupled short reversal-curve, a 'surf' curve and a pure slowing response.

These few examples illustrate the potential of miniaturised platforms to reproduce some important environmental conditions of free-living nematodes. Nevertheless, recapitulating the complexity and diversity of the environmental cues and interactions typical of the nematodes' natural habitat (e.g. incorporating nematode–bacterial–fungi interactions) is a task still to be attempted, and a scenario that represents both a challenge and an opportunity for the study of the ecology of *C. elegans*.

## Fungi

One of the largest living organisms on earth is a fungus; namely the honey fungus *Armillaria solidipes*, found in the Blue Mountains of Oregon.<sup>66</sup> The above-ground portion of the fungus – its fruiting bodies – forms just one part of the entire organism. The remainder is a vast underground mycelial network, spanning a distance of over 2 miles (3.8 km) at its broadest point. This network is comprised of hyphae, filamentous tubes that grow in a polarised manner by tip extension, which branch to form secondary and higher order hyphae and fuse with other hyphae (anastomosis).<sup>67</sup> Fungi can sense a variety of physical and chemical stimuli, such as light, electric fields and nutrient availability.<sup>68</sup> Hence, this network can be highly dynamic, responding to changes in environment.

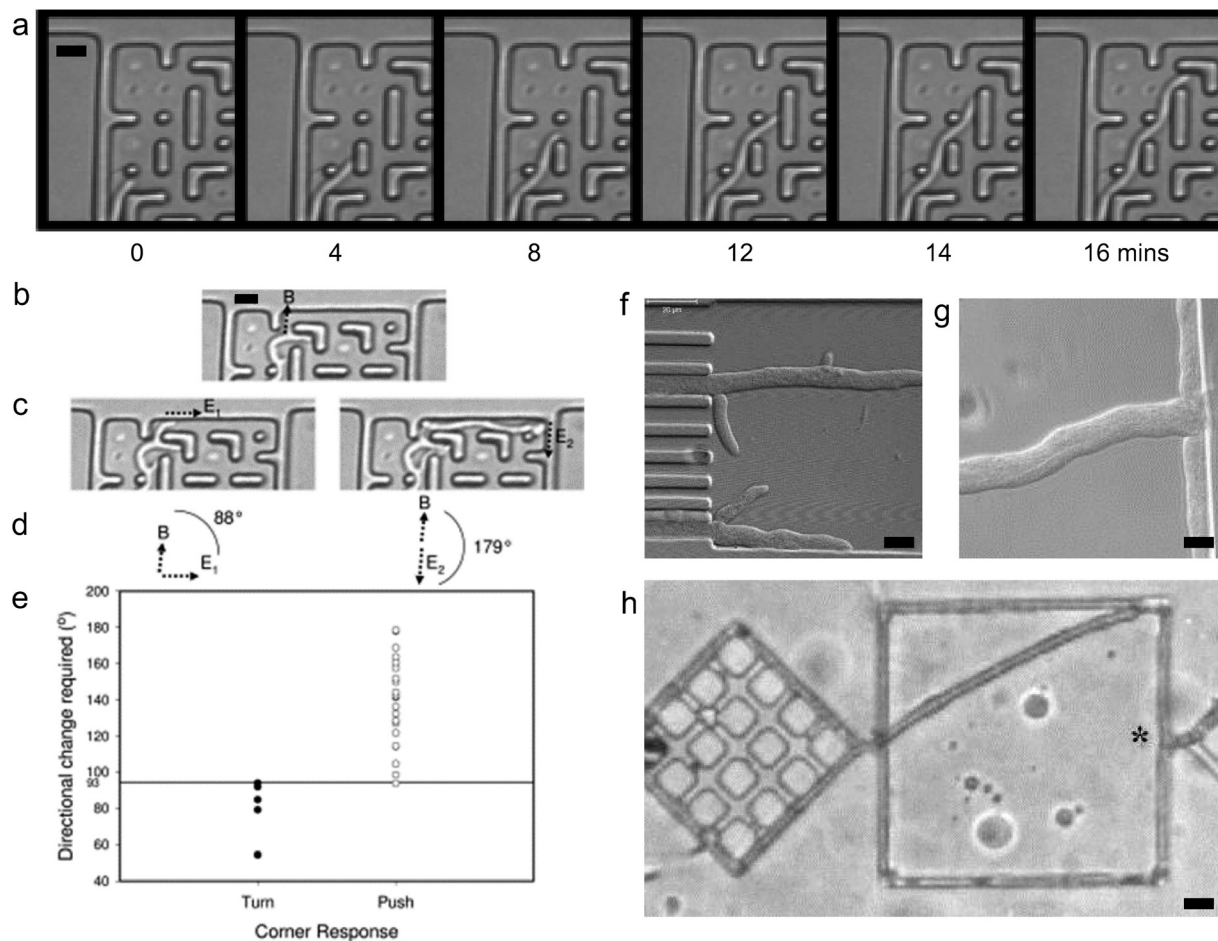
The use of microfluidics to study fungi is a nascent activity, with a strong emphasis on probing the growth dynamics

of filamentous fungi. The rationale for the body of work conducted by Nicolau and colleagues<sup>69–71</sup> stemmed from a need to represent more accurately the natural environment that a filamentous fungus encounters. They argue that most lab studies probing the growth dynamics of fungi are not characteristic of their natural environment. For example, traditional planar agar substrates, which contain a specific medium that is only applied homogeneously and lacks any representation of structure on the microscale, are typically used to study growth behaviour. As such, they used microfluidic technology to create structured environments for the investigation of fungal exploration and growth, where imaging can be conducted with ease.

Hanson *et al.*<sup>69</sup> measured differences in growth rates and branching frequencies of the basidiomycete *Pycnoporus cinnabarinus* in a microfluidic device containing patterned and non-patterned regions. Whilst growth rates remained constant at about 2  $\mu\text{m min}^{-1}$ , the average hyphal branching rate almost doubled within the micropatterned regions, suggesting that branching events can be controlled by physical cues. Further, they showed that growing hyphae have a directional memory, as they retain their original direction of growth after being diverted by a physical structure (Fig. 5a) and, upon collision with a corner, hyphae will only turn the corner if the resulting growth occurs at an angle of less than 93–94° compared to the original branching direction (Fig. 5b–e). Interestingly, Held *et al.*<sup>70</sup> have also reported that the ascomycete *Neurospora crassa* has a directional memory, as well as being able to increase its branching frequency after passing bottlenecks (Fig. 5f), achieve collision-induced apical splitting (where the collision angle is less than 55°) (Fig. 5g) and perform a phenomenon termed “nestling” (where hyphal extension occurs parallel to the confining wall, as illustrated in Fig. 5h).

In addition to exploring fungal cell growth within confined microenvironments, Held *et al.*<sup>71</sup> also used micron-sized, complex geometric networks to assess the space searching ability of fungi. Using stochastic modelling they demonstrated that long-term directional memory and the induction of branches upon physical obstruction – fungal





**Fig. 5** (a) Time series illustrating the directional memory of *Pycnoporus cinnabarinus* hyphae. (b–e) illustrate how the response of a hypha, upon coming into contact with a corner, depends upon the initial branching direction. (b) Growth direction of initial branch-emergence, indicated by B. (c) The hypha turns the first corner it meets, where  $E_1$  indicates the direction required to exit the corner (left image). The hypha does not turn the next corner, where  $E_2$  indicates the direction required to exit the corner (right image). (d) Diagrams illustrate the relative orientations of initial branching direction versus direction required to exit the corner. (e) Scatter plot illustrating the directional change required to see a turn or push response. Images illustrating (f) increased branching frequency after passing bottlenecks, (g) collision-induced apical splitting and (h) nestling of *Neurospora crassa* hyphae in microfluidic devices. In the latter image a hypha leaves the diamond structure and grows into the square box, continuing along its polarisation axis. After hitting the top right corner, the hypha makes a turn and grows alongside the wall. The main hypha grows past the next opening (a branch emerges from the hypha at this point, indicated by the asterisk) and continues to follow the wall geometry, making three more corner turns. All scale bars represent  $10\ \mu\text{m}$ . (a–e) Reproduced from ref. 69 with permission from John Wiley and Sons. (f–g) Reprinted from ref. 70, Copyright 2011, with permission from Elsevier.

traits they observed – are advantageous for the fungus. The authors suggest that fungi are not only able to solve mazes that encode nontrivial mathematic problems, but that a fungal species may actually be specifically engineered to solve a network more efficiently.

It must be remembered that not all fungi present themselves in a filamentous form. Yeasts are unicellular, eukaryotic microorganisms, forming part of the kingdom Fungi, and reproduce by budding or cell fission.<sup>72</sup> Soil yeasts have been reported to contribute largely to ecological processes and influence both plant<sup>73</sup> and microbial growth.<sup>74</sup> Microfluidic technology has been implemented to study yeasts, predominantly to enable high-resolution imaging at the single-cell level<sup>75,76</sup> and therefore allow cellular responses to be

measured upon perturbing the external environment in a defined manner.<sup>77–82</sup>

## Plants

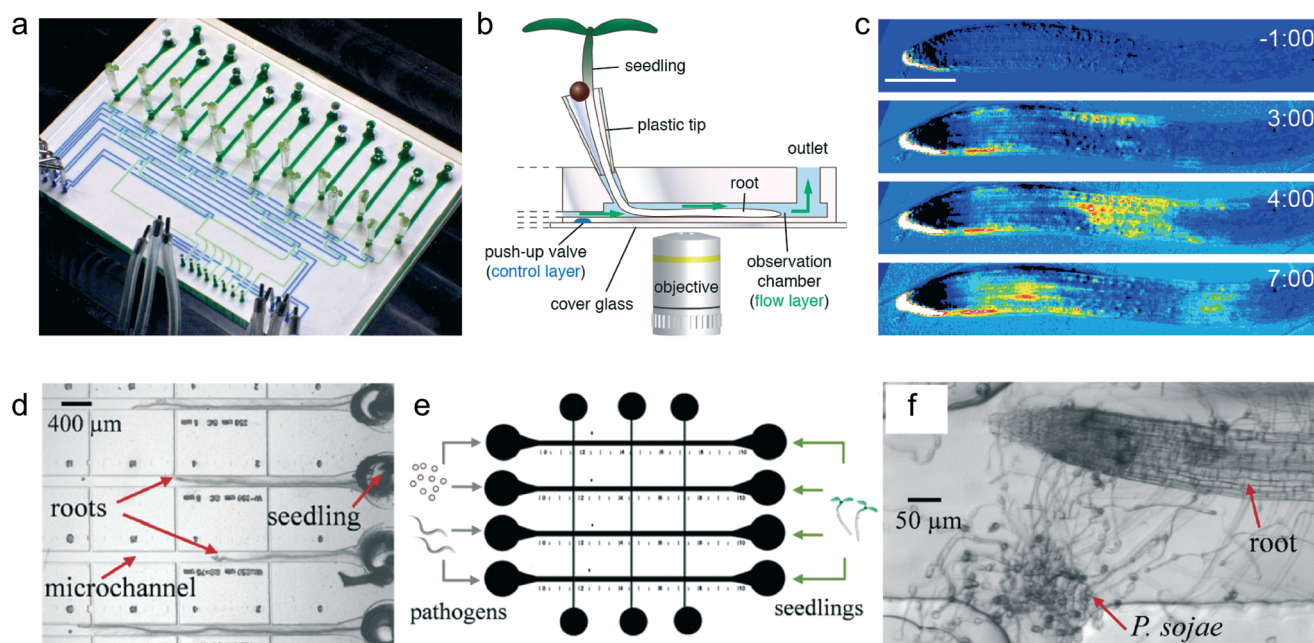
Plant roots explore the soil to provide anchorage and to take up water and mineral nutrients. They are also exposed to interactions with other soil organisms and various environmental stresses. Due to their fundamental importance in plant nutrition, the development of root systems in heterogeneous environments has always been of great interest for applied agronomical and basic plant sciences alike. Nevertheless, most breeding approaches in the past have focused on features of aerial plant parts. Despite the development of

shovelomics, large-scale phenotyping of root architecture of partly excavated plants in the field,<sup>83</sup> field-grown roots remain hardly accessible for direct analysis without causing major damage to the fine structure of the root system. It should be noted that minimally invasive imaging of root systems in soil has been achieved by magnetic resonance imaging (MRI) and X-ray computed tomography (CT) (see Metzner *et al.*<sup>84</sup>). Albeit expensive, these techniques present a substantial advance towards understanding how root systems develop under different conditions, such as soil composition, nutrient or water availability, or when exposed to stresses. A more accessible way to analyse root system architecture is through the use of rhizotrons, thin soil sheets that allow optical access from opposing sides. The recent development of the GLO-Roots system utilises bioluminescent roots, expressing fire-fly luciferase derivatives.<sup>85</sup> Such a system allows quantitative studies on root system architecture and even permits local manipulation or treatments through injection of solutions or microbe suspensions.

Although in-soil approaches are advantageous for studying root system architectures in a pseudo natural setting, many processes require imaging at far higher spatiotemporal resolution and a tighter control over the microenvironment. To understand the environmental impact on root development, the sensing and response to biotic and abiotic stresses, or the kinetics of nutrient uptake or molecular flux among tissues, one needs to observe these processes with cellular or

even subcellular resolution. Furthermore, fluorescence-based assays in cell biology and physiology require highly sensitive imaging setups and usually do not permit experiments in natural soil, which not only obstructs light collection but is also highly auto-fluorescent. One example, where high sensitivity and spatiotemporal resolution are required is the measurement of calcium signaling events in plants. The second messenger calcium is involved in numerous cellular signaling pathways and transient cytosolic elevations can be triggered by temperature changes, salt stress, bacterial elicitors and numerous other environmental cues.<sup>86</sup> The resulting calcium responses can be visualised using genetically encoded fluorescent sensors. Studies on roots have revealed their high sensitivity to environmental changes, in particular to mechanical stimulation.<sup>87</sup> This emphasises the need to minimise root handling, in particular during mounting for microscopy.

Microfluidic devices and plant growth in liquid synthetic media (hydroponics) have proven useful to ensure unperturbed root growth over several days under precisely controlled conditions. For example, the *RootChip* (Fig. 6a–c) consists of separate chambers for primary roots of seedlings of the model plant *Arabidopsis*.<sup>88,89</sup> These chambers are connected to an on-chip perfusion system with micro-mechanical valves that allow pulsed perfusion of each chamber individually. This system has been applied in several studies involving the use of fluorescent sensors for



**Fig. 6** Two examples of microfluidic devices specifically designed for on-chip cultivation and imaging of *Arabidopsis thaliana* roots. (a–b) The *RootChip*, a two-layer device consisting of a flow layer (green in a, b) for root perfusion and a control layer (blue in a, b) featuring micro-mechanical valves. (a) Reproduced from ref. 93 with permission, (b) adapted from ref. 88 with permission, www.plantcell.org, Copyright American Society of Plant Biologists. (c) Calcium response monitored over time in roots expressing genetically encoded calcium sensors upon stimulation with the bacterial peptide flg22. Reproduced from ref. 91 with permission. (d–f) A micro-device for co-cultivation of *Arabidopsis* roots and plant pathogens, such as the oomycete *Phytophthora sojae* (f). Reprinted with permission from ref. 110. Copyright 2011, AIP Publishing LLC.



measuring flux kinetics of small molecules such as signaling components (calcium<sup>90,91</sup>), nutrients (zinc<sup>92</sup>) or phytohormones (abscisic acid<sup>93</sup>).

Another microfluidic perfusion system, named the *RootArray*, was conceived as a large-scale gene expression analysis platform.<sup>94</sup> In this system, up to 64 roots can grow into a single large observation chamber where they can be imaged. Using various *Arabidopsis* lines with different promoter-GFP constructs and automated image acquisition, gene expression patterns were recorded upon changing conditions within the observation chamber.

Both the *RootChip* and *RootArray*, are optimised for imaging with high-numerical aperture objectives with short working distances, as commonly required for confocal microscopy. Inverted microscope mounts require horizontal growth of roots, but if used on a microscope stage that allows vertical mounting, such chip systems also allow growth along the gravity vector. More recently, a phenotyping platform was developed to allow trapping and germination of seeds, perfusion and vertical growth of roots.<sup>95</sup> The device was designed as a top-closed version, in which up to 26 seedlings could be observed for several days. To avoid the full submersion of the seedlings including the cotyledons the authors cut the top-part open, thus allowing cultivation for up to two weeks albeit with limited perfusion ability.

Perfusion chambers, as featured in the *RootChip*, apply any treatment to the entire root. An early device for *Arabidopsis* roots however demonstrated the potential of focused laminar flow to stimulate only a thin region, ranging from 10–800  $\mu\text{m}$ , on one side of the root.<sup>96</sup> Although this device was intended for root cultivation over 24 hours and required manual insertion of the root into the device, this proof-of-principle study may aid future designs for localised root stimulation. The ability for localised stimulation will undoubtedly allow researchers to distinguish between perception (local) and response (distal) or to mimic pathogen infection by localised application of bacterial or fungal elicitors.

While the described devices have been designed for control over the biological and chemical microenvironment of roots, physical interactions between roots and soil particles as well as obstacle-avoidance mechanisms of roots (thigmotropism) remain unaddressed. To this end, a microfluidic force sensor has recently been developed to quantify root growth forces based on the displacement of a PDMS-based cantilever by the growing root tip.<sup>97</sup>

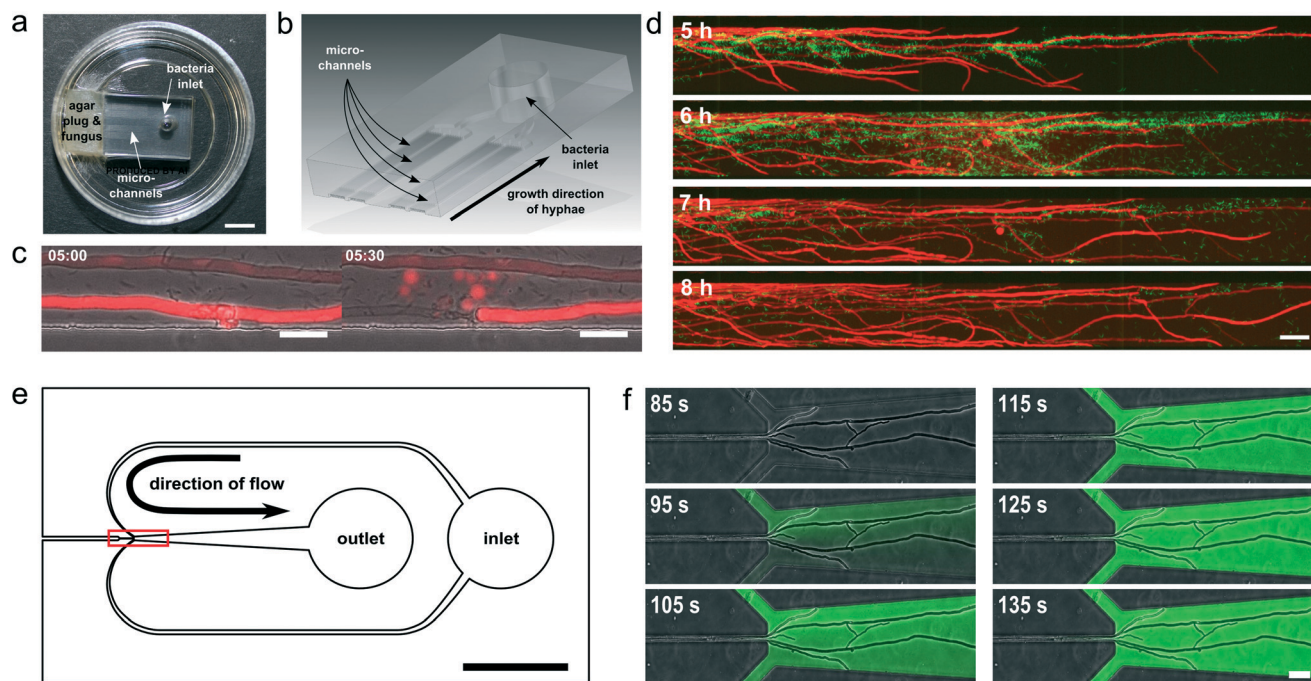
## Interaction analyses

Harbouring between  $1 \times 10^6$  and  $2 \times 10^9$  prokaryotic cells per gram of soil,<sup>98</sup> organisms in soil environments seldom prevail in isolation. Microbes often form synergistic communities, which are central, for example, to the survival and growth of plants.<sup>99</sup> Understanding the intricacies inherent to mixed microbial populations is clearly important, yet suitable methods for studying such interactions are rare. Recently, Park *et al.*<sup>100</sup> developed a microfluidic droplet platform for

detecting symbiotic relationships within mixed bacterial communities, comprised of up to three synthetic auxotrophs of *E. coli*. By tuning the relative ratios of each auxotroph in the seed cultures and controlling the average cell occupancy per droplet – an advantageous feature of droplet microfluidic technology – they were able to co-cultivate the bacteria in a highly parallelised and localised manner and detect symbiotic events among subsets of the community. Furthermore, they demonstrated that rare species, forming as little as 1% of the total microbial population, could be cultured successfully using this platform and that the detection of symbiotic events of these rare species with either abundant or other rare species within a microbial community is possible.

Understanding the interactions between different microbes present in a community is important for tackling polymicrobial infections, such as cystic fibrosis and urinary tract infections, for example. It is acknowledged that the approach taken to treat polymicrobial infections should be different to those employed for single microbial infections, due to the fact that the interactions present in polymicrobial communities can influence the response of the individual species to antimicrobials.<sup>101,102</sup> However, despite the seriousness of polymicrobial infection, treatment strategies and research efforts are still in their infancy.<sup>103</sup> Hence, a greater emphasis should be placed on extracting and implementing more representative information to afford more effective treatments. Very recently, Mohan *et al.*<sup>101</sup> described a multiplexed microfluidic approach to assess the effectiveness of antimicrobial treatments on bacterial co-cultures. The microfluidic platform contains a 48-well array, where each well is comprised of two half-wells. The setup therefore enables up to 12 unique co-culture conditions and 12 unique antimicrobial solutions to be assayed in quadruplicate and offers many advantages over conventional methods for assessing polymicrobial cultures, such as the ability to quantitatively measure bacterial growth of specific species within the co-culture in high-throughput and reduce minimum inhibitory concentration (MIC) assay times. Such a method is crucial to improving antimicrobial susceptibility testing for polymicrobial cultures and has implications in enhancing our understanding of factors underlying antimicrobial resistance.

Interactions between fungi and microorganisms or bacteria and nematodes, are important in many ecological systems. A diversity of interactions exist, playing important roles in the control of plant disease and human infection for example.<sup>104,105</sup> Bioremediation, defined as the use of microorganisms to break down toxic compounds in soil,<sup>106</sup> is one example of how interactions of fungi with microorganisms are highly important with respect to the soil environment. For example, polycyclic aromatic hydrocarbons (PAHs) form a major group of toxic contaminants in the environment.<sup>107</sup> They are present in fossil fuels and are also produced as a result of incomplete combustion of carbon-containing fuels. As a result of their hydrophobicity, they readily accumulate in soils. Hence, strategies are required for their removal. One



**Fig. 7** (a) Photograph (scale bar, 3 mm) and (b) three-dimensional illustration of the bacterial–fungal interaction (BFI) device. Application of the BFI device to investigate a model interaction system, specifically the interaction between *C. cinerea* with *B. subtilis*, provided new biological insights including (c) bacteria-induced blebbing of hyphal cells in the presence of *B. subtilis* NCIB 3610 (scale bars, 25  $\mu\text{m}$ ) and (d) bacterial attachment to a subset of fungal hyphae (scale bar, 50  $\mu\text{m}$ ). (e) Overview of the fluid exchange device (scale bar, 3 mm) and (f) time series illustrating the exchange of the medium within the main channel upon introducing a fluorescein-containing solution to the device inlet (scale bar, 50  $\mu\text{m}$ ). Adapted from ref. 109 with permission from The Royal Society of Chemistry.

approach, for example, is to use bacterial–fungal co-cultures for the mineralisation of PAHs possessing more than five benzene rings, as opposed to a single microbial isolate, of which none were found to mineralise such PAHs successfully.<sup>108</sup>

Recently, Stanley and co-workers developed novel microfluidic platforms for studying dynamic interactions between living fungal hyphae and bacterial cells, specifically the bacterial–fungal interaction (BFI) device (Fig. 7a–d) and the fluidic exchange device (Fig. 7e and f).<sup>109</sup> These platforms enable the interplay between such soil-dwelling organisms to be studied with single cell resolution and in real-time, opening up the possibility for interaction analysis. The BFI device enables hyphae of a growing filamentous mycelium to be cultured and confined within fluid-filled microchannels into which bacteria can be introduced, whilst the fluidic exchange device enables the aqueous solution surrounding the fungal hyphae to be manipulated as a result of passively pumping fluid through the fluidic network (Fig. 7f). Using these devices, the interaction between the basidiomycete *Coprinopsis cinerea* and the soil-dwelling bacterium *Bacillus subtilis* was investigated, providing novel insights into this model interaction system. This included an arrest of hyphal growth and bacteria-induced blebbing of hyphal cells in the presence of the wild-strain *B. subtilis* NCIB 3610 (Fig. 7c) and also direct cellular contact mediated by polar attachment of bacteria to a subset of fungal hyphae (for both *B. subtilis* strains tested, specifically *B. subtilis* 184 and NCIB 3610)

suggesting a differentiation of hyphae with a fungal mycelium (Fig. 7d). Taken together, these devices present new opportunities for characterising the interaction of fungi with bacteria at a cellular level.

To investigate inter-organismal interactions, the design flexibility of microfluidic devices offers also the possibility to co-cultivate plant roots with different organisms and to observe the establishment of infection, herbivory, or symbiosis, for example. Such a co-cultivation of *Arabidopsis* roots with sugar-beet nematodes or the plant pathogenic oomycete *Phytophthora sojae* was pioneered by Parashar and Pandey, as illustrated in Fig. 6d–f.<sup>110</sup> On-chip growth for up to four days allowed nematodes feeding on roots to be observed, as well as the establishment of *P. sojae* infection sites and subsequent cell death in the affected root tissue.

## Future outlook

A variety of cases exemplifying the importance of microfluidic technology in studying the interplay between soil-dwelling organisms and their environment have been detailed. However, the possibility of studying interactions between many organisms in a controllable manner, such as single organisms, roots, hyphae and bacterial cells, will provide a fundamental contribution to the field. Such a prospect would enable a more complete understanding of the inter-organismal interactions involved in complex soil communities, in which microfluidic technology will play a crucial role.

Moreover, more sophisticated means to simulate the physical and chemical properties of this heterogeneous porous network would therefore be possible, where non-uniform distributions of water and nutrients and varying levels of hydration persist.

A single case study indicates the potential of microfluidics for studying root-fungus or root-herbivore interactions.<sup>110</sup> Devices for the investigation of symbiosis establishment between plants and microbes are, however, still missing. *Arabidopsis thaliana* has proven extremely useful as a model system for genetics and cell biology; thanks to their small size and transparency *Arabidopsis* roots are ideal specimens to be grown and examined in microfluidic devices. The use of *Arabidopsis* for the important field of symbiosis is, however, rather limited. While virtually all crop plants can establish stable symbioses with fungi through arbuscular mycorrhiza, many members of the plant family Brassicaceae, including *Arabidopsis*, have lost this ability during evolution. To study the molecular processes of symbiosis between plants and microbes and its impact on plant nutrition device architecture needs to be adapted to fit other model plants. The development of such platforms would open opportunities to probe additional questions concerning, for example, the role of arbuscular mycorrhiza networks and mechanism of signal transfer in the long-range communication between plants.<sup>111,112</sup>

The application of microfluidic technology to mycology, in particular to the field of BFIs, is an extremely new area of research. It has been demonstrated that the confinement and control of hyphal growth, offered by microfluidic platforms such as the BFI device,<sup>109</sup> reveals unique insights into a model interaction system at the single cell level, which would otherwise not be possible. It is envisaged that such a device could help unravel the complex antagonistic strategies utilised by microorganisms in these competitive environments, as well as being used in the location and quantification of antimicrobial production with both temporal and spatial resolution for example. Additionally, microfluidic platforms could be employed to mine for antifungal agents and secondary metabolites in high throughput. The opportunity to monitor *dynamic* interactions between bacteria and fungi at the single cell level and in real time means that we are now able to implement promoter-reporter fusion strains to study events such as quorum sensing of bacterial cells in BFIs. This platform would also enable studies regarding endobacteria in fungal hyphae to be explored.

Of particular interest is the characterisation of gene expression profiles of a fungal mycelium, which have been reported to change considerably upon challenge by bacteria<sup>113,114</sup> and nematodes.<sup>115</sup> However, new methods are required to study the dynamics and spatial distribution of gene induction within a fungal mycelium, which cannot currently be achieved. It is anticipated that the temporal and spatial regulation of potential genes involved in fungal defence may be monitored as a result of combining microfluidic technology with specific promoter-reporter fusion

strains, where potential defence genes of interest have been targeted using transcriptomics studies. Such studies would enhance our understanding of the defence response of filamentous fungi against bacteria and nematodes. Furthermore, microfluidic technologies such as IBM's microfluidic probe<sup>116</sup> offer the potential of collecting mRNA from single hyphae, thus elucidating which genes are being actively expressed at a particular time or position within a fungal mycelium. Coupling such information with bacterial attachment patterns, for example, would enable one to provide further evidence to support questions centred on differentiation in fungal mycelia.

The development of Soil-on-a-Chip technologies will be an important step in expanding our current understanding of the complex relationships that exist between soil-dwelling organisms. Not only will such developments provide further insights into our understanding of the fundamental biology, it will undoubtedly impact our society as a whole.

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