SUPPLEMENTARY INFORMATION

Probing bacterial-fungal interactions at the single cell level

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Supplementary Materials

Medium	***Details***
Luria-Bertani (LB) medium	0.5 % w/v yeast extract, 1 % w/v tryptone and 0.5 % w/v sodium chloride, solidified with 1.5 % w/v agar supplemented with 100 μg/mL ampicillin
Yeast extract-Peptone-Dextrose (YPD) medium	1.0 % w/v yeast extract, 2.0 % w/v peptone, 2.0 % w/v glucose
Yeast-Malt extract-Glucose (YMG) medium	0.4 % w/v yeast extract, 1 % w/v malt extract, 0.4 % w/v glucose, 1.5 % w/v agar
C. cinerea minimal medium (CCMM)	5 g/l glucose, 2 g/l asparagine, 50 mg/l adenine sulfate, 1 g/l potassium phosphate monobasic, 2.25 g/l sodium phosphate dibasic, 0.29 g/l sodium sulfate, 0.5 g/l diammonium tartrate, 0.04 mg/l thiamine hydrochloride, 0.25 g/l magnesium sulphate heptahydrate and 5 mg/l p-aminobenzoic acid (pABA). CCMM agar plates contained 1.5 % w/v agar.

Supplementary Methods

Supplementary Method 1

Device operation, Design 1: Bacterial-Fungal Interaction (BFI) device. Bacteria, taken from an overnight culture, were diluted with LB using a 1:25 ratio and sub-cultured for 3 h at 37°C. Bacteria were washed once with a 0.9% w/v sodium chloride solution and resuspended in CCMM to an OD_{600} of 1. After incubating the device with the fungal inoculum for 18 h at 28°C, 10 μL of this bacterial suspension was introduced into the device inlet. Subsequently, the bacteria are able to explore the device architecture (due to their motile nature) and interactions between the bacteria and hyphae can be monitored.

Device operation, Design 2: fluid exchange device. Once the hyphae have passed the constriction junction and entered the main microchannel, the fluid in the main channel can be exchanged with a solution of interest. To introduce a new solution into the main channel (containing hyphae), any CCMM present in the inlet and outlet is removed and 30 μL of the new solution introduced into the inlet. If it is desirable to remove this new solution from the main channel, fluid from the outlet is removed and the inlet is exchanged three times with 30 μL aliquots of CCMM (the third addition of CCMM remains in the inlet).

Supplementary Method 2

Characterisation measurements were conducted using the fluid exchange device to: i) assess the time taken for fluid exchange and ii) to ascertain whether 100 % exchange of the fluid is achieved.

In order to access the kinetic profile of the exchange, a 30 μl aliquot of a fluorescein-containing solution (0.5 mM fluorescein sodium salt (Sigma-Aldrich, USA) solution in autoclaved double distilled water, containing 100 mM potassium phosphate buffer at pH 7.5, diluted 1:1 with *Coprinopsis cinerea* Minimal Medium (CCMM)) was introduced into the inlet of the fluid exchange device (after having removed the CCMM from both the inlet and outlet). Note that *C. cinerea* was grown into the device as detailed in the Materials and Methods. The device was mounted onto the microscope stage and a time-lapse (with time interval equal to 5 s) was started immediately after the addition of the fluorescein-containing solution to capture the exchange event. Supplementary Figure S1 a illustrates an increase in the fluorescence intensity over time for 5 experiments and Supplementary Fig. S1 b represents an average of this data. Each time point represents an average of the fluorescence intensity profiles measured at a distance of 300 and 500

μm from the beginning of the tapered observation channel using Fiji. Note that the regions of the fluorescence intensities profiles occupied by hyphae were discarded in these measurements. It can be observed that fluid exchange takes place within 3-4 minutes, after which an equilibrium value is attained.

In order to assess whether this equilibrium value is the true value, i.e. that 100 % exchange had occurred, two further experiments were conducted. First, an exchange of the fluid was made with four washing steps and second, a comparison was made with microfluidic devices that had been filled with the fluorescein-containing solution after assembly (no exchange). The inclusion of several washing steps ensures that there is no dilution of the new solution introduced into the inlet for exchange. Specifically, four 30 μl aliquots of the fluorescein-containing solution were introduced into the inlet and subsequently removed. The fifth aliquot remains in the inlet. Supplementary Figure S2 b represents the fluorescence intensity profiles of a time-lapse started 5 minutes after the exchange, with washing steps (data analysed in an identical manner to that described above). As a result, the initial increase in the fluorescence intensity, due to exchange of CCMM with the fluorescein-containing solution, is not observed.

Supplementary Figure S3 c details the results gained when the fluorescein-containing solution was introduced to the microfluidic device directly after assembly. When compared with Supplementary Fig. S3 b it can be observed that the fluorescence intensities are equivalent, therefore meaning that 100 % exchange is achieved when washing steps are included in the procedure.

Supplementary Method 3

Bacteria were prepared as described for the BFI device operation. 100 μL of this bacterial cell suspension was placed in a petri dish and the bacteria killed by UV light (254 nm) illumination using a Stratalinker cross linker 2400 (Stratagene, Canada) for 20 min. The lid from the petri dish was removed during the exposure to UV illumination. Bacteria were plated onto an LB agar plate and inoculated overnight at 37°C to confirm that the cells were not viable.

Supplementary Method 4

Bacteria, taken from an overday culture, were diluted into CCMM to an OD_{600} of 0.5 and sub-cultured for 20 h at 28°C with aeration. Bacteria were removed by centrifugation for 10 min at 3860 *g*. The supernatant was sterilized by filtration through a syringe filter (TPP, Switzerland) with a pore size of 0.22 μm. Conditioned medium was introduced into the fluid exchange device. Extraction of substances from the cell-free conditioned medium with *n*-butanol was

performed as described by Yazgan *et al*.[1] Briefly, the conditioned medium was extracted three times with one-fourth volume of *n*-butanol by vortexing for 1 min. The two phases were separated by centrifugation at 3860 *g* for 5 min. The collected *n*-butanol fraction was evaporated to dryness and resuspended in 1/40th of the starting volume in CCMM.

Supplementary Method 5

The plasmid pMA412 consists of two plasmids, pRS426-pAbgpdII-i-dTomato-tPcMNP and pMA336,[2] resulting in a plasmid where the *A. bisporus gpdII* promoter drives the expression of dTomato and that harbours the pab1 marker in the pRS426 backbone. The *A. bisporus gpdII* promoter cassette, together with the intron was amplified by polymerase chain reaction (PCR) with the primer pair *Sal*I-GPDII/*Sma*I-GPDII (see supplementary table 4) and the Pc-mnp terminator with the primer pair *Sma*I-MNP/*Bam*HI-MNP (see supplementary table 4) from the plasmid p004iGM3. These two PCR products were cut with *Sal*I/*Sma*I and *Sma*I/*Bam*HI, respectively and ligated into pRS426 cut with *Sal*I and *Bam*HI resulting in plasmid pMK317.

The dTomato gene was PCR amplified from the plasmid pRSET$_B$-dTomato[3] using the primer pair GPD-dTomato_Rev and GPD-DTomato_Fwd (see supplementary table 4) that contain homology regions for recombination into pMK317. Plasmid pMK317 was linearized with the restriction endonuclease *Sma*I. Homologous recombination resulted in the plasmid pRS426-pAbgpdII-i-dTomato-tPcMNP. The promoter-intron-dTomato-terminator cassette was cut out of this plasmid with *Xho*I/*Bam*HI. This fragment was ligated into pRS426-pab1 that was cut with the same enzymes. All constructs that were PCR-generated were confirmed by DNA sequencing (Microsynth, Switzerland).

Supplementary Method 6

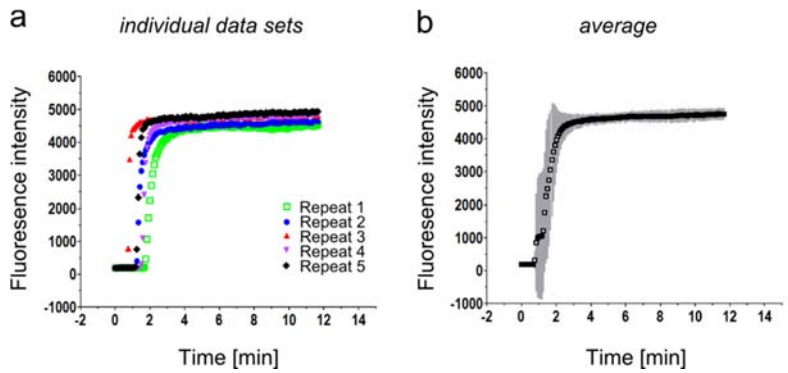
Master molds were manufactured as described herein. A 100 mm silicon wafer (Silicon Materials, Germany) was spin-coated with SU-8 photoresist (MicroChem, USA) with a target height of 10 μm and then baked at 95°C for hardening. The resist was then exposed in an MA6 ultrmovolet (UV) mask aligner (Suss Microtec, Germany) using a mylar film photolithography mask, with an exposure energy dose of 140 mJ/cm² measured at 365 nm wavelength, and again baked at 95°C. The combination of UV exposure and heat causes cross-linking of the photoresist in the exposed areas. Finally, the resist was developed using mr-Dev 600 developer solution (Microresist Technologies, Germany), where the unexposed resist is removed, lemovng the exposed microstructures on the wafer. The height of the SU-8 structures was 10.3 ± 0.1 μm for Design 1 (BFI device) and 10.2 ± 0.1 μm for Design 2 (fluid exchange device), as determined

using a Dektak XT stylus profilometer (Bruker, USA). The AutoCAD files for Design 1 and Design 2 are provided in Supplementary Data Files 1 and 2 respectively.

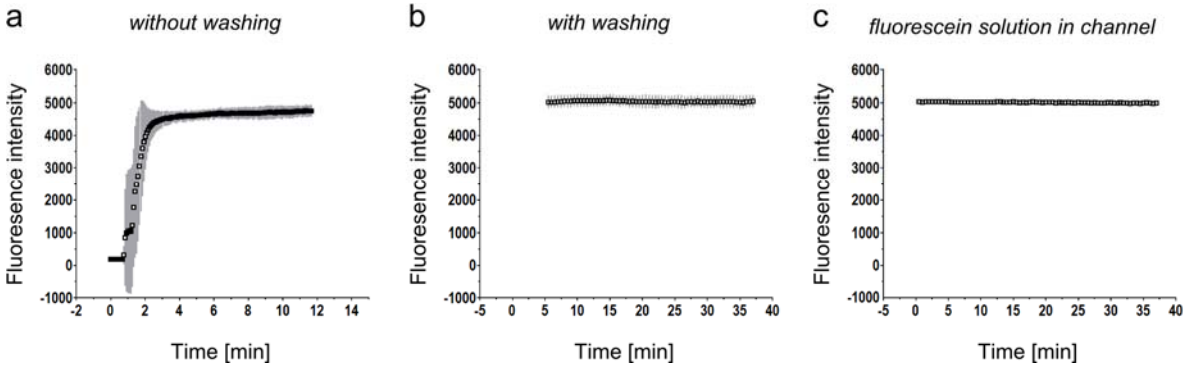
Supplementary Method 7

The poly(dimethylsiloxane) (PDMS) slabs and glass Petri dishes were washed in each of the following solutions (PDMS slabs were sonicated in each solution): 0.5 M sodium hydroxide solution (Sigma-Aldrich, Germany), 70 % v/v ethanol solution and autoclaved double distilled water (ddH_2O). Every washing step was followed by thorough rinsing with autoclaved ddH_2O. The PDMS slabs and Petri dishes were then dried with an air gun and placed in an oven at 70°C for 1 hour.

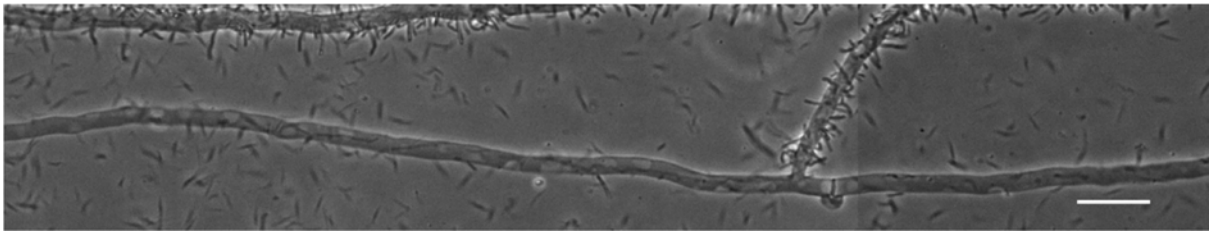
Supplementary Figures



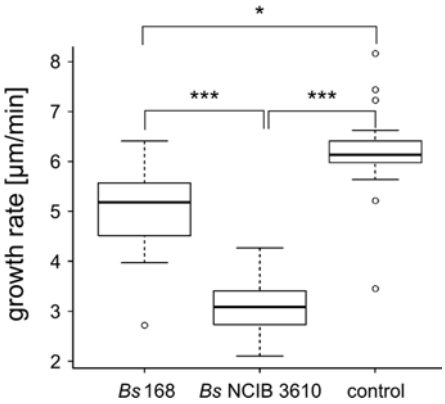
Supplementary Figure S1 | Characterisation of the fluid exchange device 1. (a) Graph illustrating the change in fluorescence intensity with time upon introducing a 30 µl aliquot of a fluorescein solution into the inlet. Five repeat experiments are displayed. *Coprinopsis cinerea* minimal medium (CCMM) was first removed from the inlet and outlet prior to addition of the fluorescein solution. **(b)** Graph illustrating the average change in fluorescence intensity for the data represented in **(a)**. The error bars represent standard deviations.



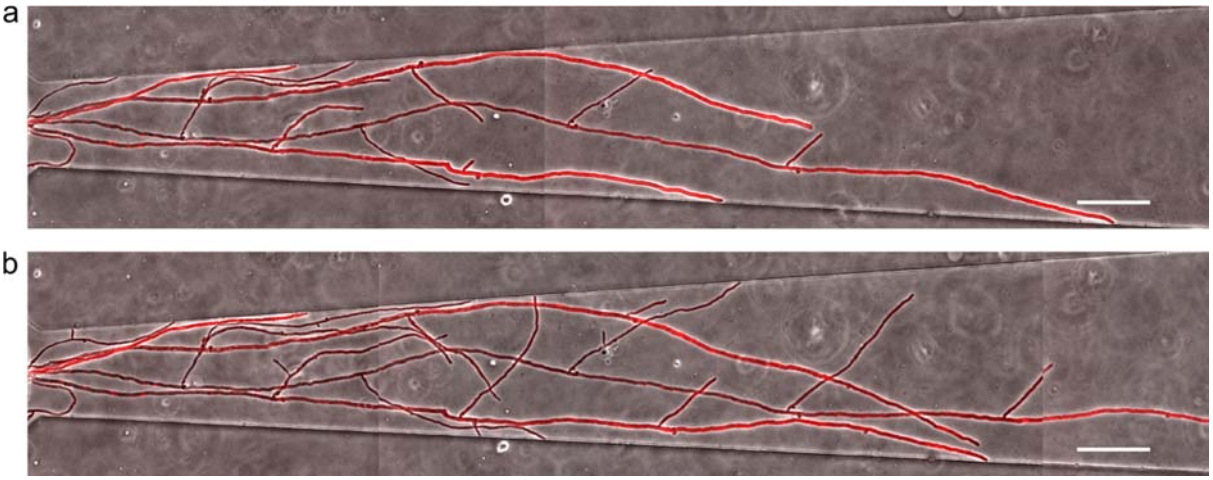
Supplementary Figure S2 | Characterisation of the fluid exchange device 2. (a) Graph illustrating the average change in fluorescence intensity with time upon introducing a 30 µl aliquot of a fluorescein solution into the inlet without washing. **(b)** Graph illustrating the change in the fluorescence intensity with time upon washing the inlet four times with 30 µl aliquots of the fluorescein solution. The fifth aliquot remained in the inlet and a time-lapse started 5 minutes after the first addition; hence, the initial increase in the fluorescence intensity was not captured. CCMM was first removed from the inlet and outlet prior to addition of the first aliquot. **(c)** Graph illustrating the change in the fluorescence intensity with time, where the microchannels of the fluid exchange device were filled with the fluorescein solution via capillary action immediately after the bonding procedure. This represents the situation where 100 % exchange of the fluid is achieved. The data displayed in **(a-c)** represent three independent repeat experiments and the error bars represent standard deviations.



Supplementary Figure S3 | Differentiated bacterial attachment on a hyphal branch. Image depicts polar attachment of *B. subtilis* NCIB 3610 to a hyphal branch of *C. cinerea* but not to the corresponding leading hyphae. Scale bar, 25 μm.



Supplementary Figure S4 | Aggregated average growth rates of the leading hyphae in presence and absence of *B. subtilis* strains. A linear mixed model was used to determine the aggregated average growth rates. Data are presented in Tukey Box plots representing the mean values. Code for p-values, * <0.05, ***<0.01 and *** <0.001.



Supplementary Figure S5 | Exchange of CCMM with conditioned medium from *B. subtilis* 168. (a) Image depicts hyphae before medium was exchanged. **(b)** The medium surrounding the hyphae was exchanged with conditioned medium from *B. subtilis* 168 with washing. Hyphae are shown 2 h after exchange and further growth of hyphae was observed. Scale bar, 100 μm. Brightfield and fluorescence images were merged.

Supplementary Tables

Supplementary Table S1 | Effect of various treatments on *C. cinerea* hyphae using the fluid exchange device.

Treatment	*Blebbing*	*Growth stop*
Conditioned medium *B. subtilis* NCIB 3610	Yes	Yes
Conditioned medium *B. subtilis* NCIB 3610 100°C 15 min	Yes	Yes
Conditioned medium *B. subtilis* NCIB 3610 *n*-butanol extract	Yes	Yes
Minimal medium CCMM containing 1 % (v / v) *n*-butanol	No	No
Conditioned medium *B. subtilis* 168	No	No
Minimal medium CCMM	No	No

Supplementary Table S2 | Strains used in this study.

Strain	*Genotype*	*Source*
Coprinopsis cinerea AmutBmut	*A43mut B43mut pab1.2*	Swamy *et al.*[4]
Coprinopsis cinerea AmutBmut pMA412	Integrated plasmid pMA412	This study
Bacillus subtilis 168	*trpC2+*	Kunst *et al.*[5]
Bacillus subtilis NCIB 3610	Undomesticated wild-strain	Branda *et al.*[6]
Bacillus subtilis 168 pMF37	*amyE*::Phyper-spac gfp, spc^r	This study
Bacillus subtilis NCIB 3610 pMF37	*amyE*::Phyper-spac gfp, spc^r	This study

Supplementary Table S3 | Plasmids used in this study.

Plasmid	*Description*	*Source or reference*
pRS426	2µ-URA3	Christianson *et al.*[7]
$pRSET_B$-dTomato		Shaner *et al.*[3]
p004iGM3		Burns *et al.*[8]
pMK317	pRS426-pAbgpdII-i-tPcMNP	(C. Villalba, unpublished)
pMA336	pRS426-pab1	Wälti *et al.*[2]
pMA412	pRS426PAB-pAbgpdII-i-*dTomato*-tPcMNP	This study
pMF37	Phyper-spac *gfp* in pDG1730	M. Fujita, University of Houston

Supplementary Table S4 | Primers used in this study.

Primer Name	***Sequence (5' – 3')***	***Purpose***
*Sal*I-GPDII	ggggg<u>tcgacg</u>aagaagaattcagaggtccgc	Amplification of gpdII promoter including intron from p004iGM3
*Sma*I-GPDII	gggg<u>cccggg</u>tgctttgacctggaaagcgaag	Amplification of gpdII promoter including intron from p004iGM3 and introduction of a *Sma*I restriction site
*Sma*I-MNP	gggg<u>cccggg</u>taaattcatattccacgcggtttc	Amplification Pc-mnp terminator
*Bam*HI-MNP	ggggg<u>gatcc</u>cagtccacaatgacagctc	Amplification Pc-mnp terminator
GPD-DTomato_Fwd GPD-dTomato_Rev	<u>gtgctgacttcgctttccaggtcaaagcag</u>tgagcaagggcgaggaggt <u>ggtaagaaaccgcgtggaatatgaatttac</u>ttgtacagctcgtccatgccgtac	Amplification of dTomato gene, pMK317 homology regions are underlined

Supplementary Movies

Supplementary Movie 1 | Example of *C. cinerea* hyphae growing in the BFI device

Here, *C. cinerea* hyphae can be observed growing into the BFI device. Branching events, as well as clamp cell formation, can be observed. Time interval between successive frames is 30 s. The .mov file was recorded at 20 fps. Scale bar, 50 μm.

Supplementary Movie 2 | Clamp cell and septa formation in *C. cinerea* hyphae growing in the BFI device

This movie illustrates clamp cell and septa formation in *C. cinerea* hyphae at an effective magnification of 60x (40x plus an additional 1.5x) in the BFI device. Time interval between successive frames is 10 s. The .mov file was recorded at 20 fps. Scale bar, 10 μm.

Supplementary Movie 3 | Exploration of the BFI device (containing *C. cinerea* hyphae) by *B. subtilis*

Movie illustrating *B. subtilis* 168 exploring the BFI device. The time interval between successive frames is 200 ms. The .mov file was recorded at 20 fps. Scale bar, 5 μm.

Supplementary Movie 4 | *C. cinerea* hyphae tracking the channel edge

C. cinerea hyphae grow into the BFI device and the leading hyphae tracks along the edge of the microchannel. Time interval between successive frames is 30 min. The .mov file was recorded at 5 fps. Scale bar, 100 μm.

Supplementary Movie 5 | Clustering of bacteria in the BFI device

This movie shows the movement of *B. subtilis* NCIB 3610 clusters. The time interval between successive frames is 30 min. The .mov was recorded at 2 fps. Scale bar, 50 μm.

Supplementary Movie 6 | Interaction of *B. subtilis* NCIB 3610 with *C. cinerea* hyphae in the BFI device 1

This movie shows the interaction of *B. subtilis* NCIB 3610 with *C. cinerea* hyphae in the BFI device. Time interval between successive frames is 500 ms. The .mov file was recorded at 20 fps. Scale bar, 50 μm.

Supplementary Movie 7 | Interaction of *B. subtilis* NCIB 3610 with *C. cinerea* hyphae in the BFI device 2

This movie shows the interaction of *B. subtilis* NCIB 3610 with *C. cinerea* pMA412 hyphae that express dTomato constitutively in the BFI device. Specifically, emptying of certain hyphal cells (i.e. the loss of the cellular content to the surrounding environment) can be observed over time. The time interval between successive frames is 1 minute. The .mov file was recorded at 20 fps. Scale bar, 25 μm.

Supplementary Movie 8 | Addition of *B. subtilis* NICB 3610 cell free supernatant to *C. cinerea* pMA412 hyphae in the fluid exchange device

This movie supports Figure 6a from the main text. It shows the addition of conditioned medium from *B. subtilis* NCIB 3610 to *C. cinerea* pMA412 hyphae, expressing dTomato constitutively, in the fluid exchange device. Emptying of an apical cell was observed. The time interval between successive frames is 1 minute. The .mov file was recorded at 10 fps. Scale bar, 50 μm. Brightfield and fluorescence channels are merged.

Supplementary Data files

Supplementary Data File 1 | AutoCAD design for the BFI device

This file contains the AutoCAD drawing used to create the BFI device.

Supplementary Data File 2 | AutoCAD design for the fluid exchange device

This file contains the AutoCAD drawing used to create the fluid exchange device.

References

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