MICROFLUIDIC PLATFORM TO STUDY ELECTRIC FIELD BASED ROOT TARGETING BY PATHOGENIC ZOOSPORES

*Debolina Sarkar[1,2], Yiling Sun[1,3], Ayelen Tayagui[1,2,3], Ryan Adams[1], Ashley Garrill[1,2], and Volker Nock[*1,3]*

[1]Biomolecular Interaction Centre, Electrical & Computer Engineering, University of Canterbury, NZ
[2] School of Biological Sciences, University of Canterbury, NZ and
[3]The MacDiarmid Institute for Advanced Materials and Nanotechnology, Wellington, NZ

2022 IEEE 35th International Conference on Micro Electro Mechanical Systems Conference (MEMS) | 978-1-6654-0911-7/22/$31.00 ©2022 IEEE | DOI: 10.1109/MEMS51670.2022.9699559

ABSTRACT

This paper reports the fabrication and application of a microfluidic Lab-on-a-Chip platform to study the electrotactic movements of pathogenic microorganisms. The movement of the pathogens in response to electric fields are one way in which they are thought to locate their hosts. Design and fabrication of the platform, and associated micro-electronics are described. The platform contains arrays of micro-electrodes that generate an electric field of defined strength in a micro-chamber into which feed inlet and outlet channels for entry and exit of media and microorganisms. To demonstrate applicability of the platform, motile zoospores of the pathogenic oomycete *Phytophthora nicotianae* were seeded in the inlet and a voltage was applied to investigate the electrotactic responses of the zoospores. This platform offers a unique opportunity to study electrotactic movements that may be responsible for the ability of the pathogens to locate and invade host tissue.

KEYWORDS

Phytophthora nicotianae, Zoospores, Electrotaxis, Electric Fields, Lab-on-a-Chip.

INTRODUCTION

Oomycetes are organisms that play an essential role in ecosystems through the breakdown of organic material. Certain species are however pathogenic and can significantly impact ecosystems, biodiversity and biosecurity by infecting and causing disease in plants and animals. Perhaps the most well-known is the plant pathogen *Phytophthora*, a genus that contains numerous species that have had significant impact through the disease that they cause [1].

To move between plants, many *Phytophthora* species produce motile asexual reproductive structures called zoospores that are able to swim due to the presence of two flagella [2]. These need to locate the correct host plant for the successful completion of the asexual life cycle (Fig. 1) [3]. They are attracted to the roots by electrical and/or chemical gradients [4-8], and thus demonstrate electrotactic and/or chemotactic swimming behavior.

Earlier studies of electrotaxis were carried out on bulk populations of zoospores, using large observation chambers that contained stainless-steel micro-electrodes [8]. Alternately, an agarose bridge was connected to a chamber containing an electrolyte solution and a platinum wire was used to supply a constant voltage [6]. These can be large and cumbersome, and make it difficult to accurately image individual zoospores. The use of microfluidic Lab-on-a-Chip (LOC) platforms can overcome some of these issues and we have recently demonstrated their use for the study of infective capabilities of individual oomycete and fungal cells [9-12]. The work in this paper describes a new LOC platform and its use as a tool to help to study electrotactic responses via concurrent electrical stimulation and high-resolution microscopy of individual zoospores.

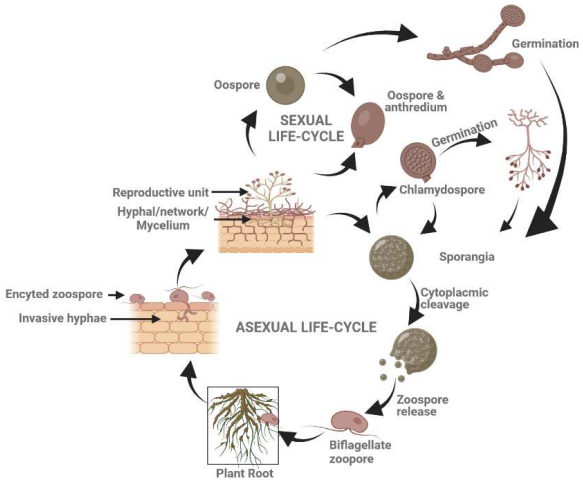

Figure 1: A schematic representation of the invasive life cycle of a Phytophthora spp.

EXPERIMENTAL METHODS

Platform Design

The platform design was created using L-Edit (Mentor Graphics) and primarily consisted of two layers: a polydimethylsiloxane (PDMS) microfluidic observation chamber (OC) bonded onto a glass substrate containing an array of photolithographically-patterned gold thin-film electrodes (Fig. 2(a)). Dimensions of the platform were matched to the diameter of *P. nicotianae* zoospores. A qMicro tunable resistive pulse sensing (TRPS, IZON) system was used to estimate the diameter of zoospores [11]. It should be noted that this is an estimate, given that zoospores are bean shaped and the qMicro will not differentiate between length and width measurements. The mean diameter (± SEM) from 5 replicate experiments was 11.7 ± 0.4 µm. In order to have sufficient volume to observe zoospore swimming behavior, the platforms contained OCs that were 500 µm long, 300 µm wide and 30 µm deep. Zoospores could be confined to the chamber using either a sieve structure (Fig. 2(b)) or pneumatic valves (Fig. 2(c)) [12]. For the sieve-like structure, micro-pillars were spaced 5 µm apart in an attempt to prevent zoospores from escaping the chamber.

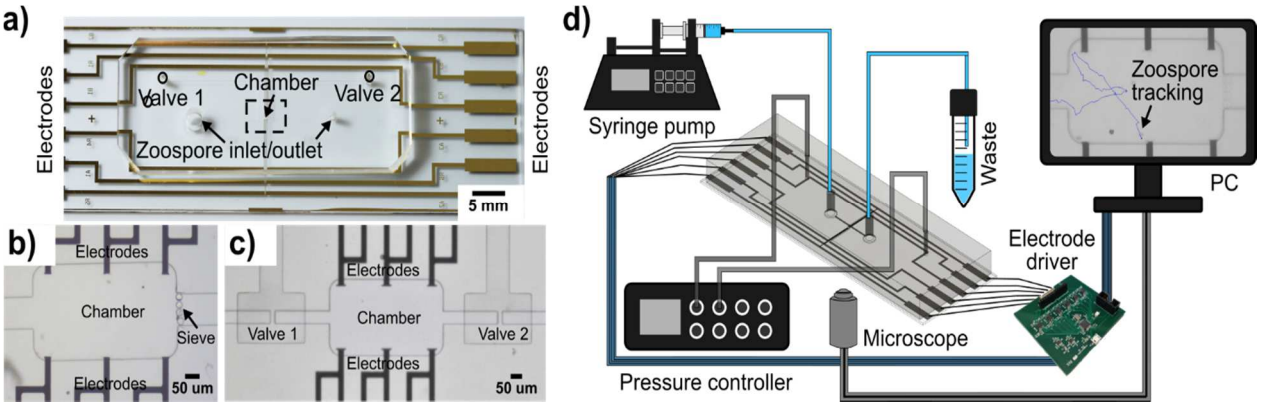

Figure 2: (a) Photograph of the electrotaxis platform with the external electrode, fluidic and gas connections indicated. (b, c) Optical micrographs of the two versions of the chip produced with either sieve or pneumatic valves to confine zoospores to the central OC. (d) Schematic representation of the experimental setup used to operate the electrotaxis platform. A custom electrode driver provided potential to the electrodes. Zoospore motility was recorded using an inverted microscope and tracked in ImageJ. Note: the pressure controller was only used for the pneumatic valve version of the platform.

Chip Fabrication

PDMS microfluidic chips and gold electrode substrates were fabricated using a combination of photo- and soft-lithography (Fig. 3) [9,11]. In brief, a laser mask writer (μPG101, Heidelberg Instruments) was used to produce 4" chrome-on-glass photomasks (Nanofilm) for both the PDMS mold and gold electrode containing substrates. For the mold, negative-tone dry film photoresist (ADEX30, DJMicrolaminates) was laminated onto a 4" silicon wafer, which had been dehydrated in a 185 °C oven for 2 h and then cleaned using O_2 plasma (Tergeo, PIE Scientific) at 100 W for 10 min. Patterns were transferred from the template mask onto the photoresist using UV lithography (MA-6, Suss) in low-vacuum-contact mode and a post-exposure bake. The resist was then developed in cyclohexanone, rinsed with water and dried with N_2, before the post-exposure bake was performed.

PDMS (10:1 w/w, Sylgard 184, Dow Corning) was replica-cast off the mold after treatment with trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane (Sigma-Aldrich). Next, premixed and degassed PDMS was poured onto the mold, and degassed again. After being baked for 2 hours at 80°C on a hotplate, the PDMS was carefully peeled off and baked for a further 2 hours at 80 °C. Once cured, the PDMS was cut to the appropriate size for the individual devices and inlet and outlet holes were punched with a 1.5 mm hole puncher (ProSciTech) [10]. The electrode-containing substrate was prepared using a glass slide coated with a 5 nm Cr/100 nm Au (DRLI). Positive photoresist (AZ1518, MMRC) was spin-coated at 3000 rpm for 1 min (Laurell WS-650) and the slide was soft-baked at 90°C for 90 s. Patterns were transferred using UV lithography (MA-6, Suss) and development (AZ 326MIF, MMRC), followed by a water rinse, drying with N_2 and a hard-bake for 10 min at 110°C. Gold etchant and chrome etchant were used to transfer patterns into the gold layer [12]. At the end of this process the gold-electrode substrate with the pattern was rinsed using acetone, methanol and isopropanol.

Microfluidic chips with sieve valves were assembled by manually aligning and bonding the PDMS to the glass substrate using an O_2 plasma cleaner (Tergeo, PIE Scientific) at 15 W for 1 minute. This was followed by a bake for 2 hours at 80°C to yield the device shown in Fig. 2(b). For the fabrication of a microfluidic chip with the pneumatic valve, two hybrid membrane micro-valves were included at the inlet and outlet of the observation chamber and a gas layer was used for actuation of the normally closed valves (Fig. 2(c)) [12]. Finally, microfluidic chips were degassed and sealed in food-grade vacuum bags using a vacuum sealer (Sunbeam FoodSaver).

Biological Sample Preparation

Stock cultures of *Phytophthora nicotianae* were grown on 2% V8 Agar plates and incubated at a temperature of 25.5°C and humidity of approximately 60%. To produce zoospores, a week-old culture plate was divided into two halves and transferred to two other 90 mm sterile Petri plates. Each of the halves were further cut into 5 x 5 mm pieces [13]. To each of the plates 20 mL of sterile distilled

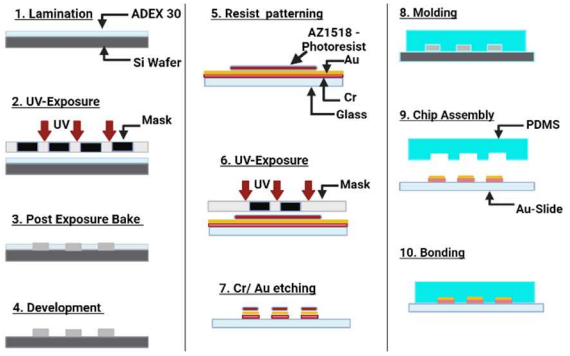

Figure 3: Fabrication process of the electrotaxis platform. (1-4): For the microfluidic mold, negative tone photoresist was laminated onto a silicon wafer and exposed using UV. (5-7): For the gold-electrode substrate positive photoresist and gold etching were used. (8): Devices were assembled by casting PDMS on the mold and (9 & 10): bonding this to the pre-etched gold-coated glass slide.

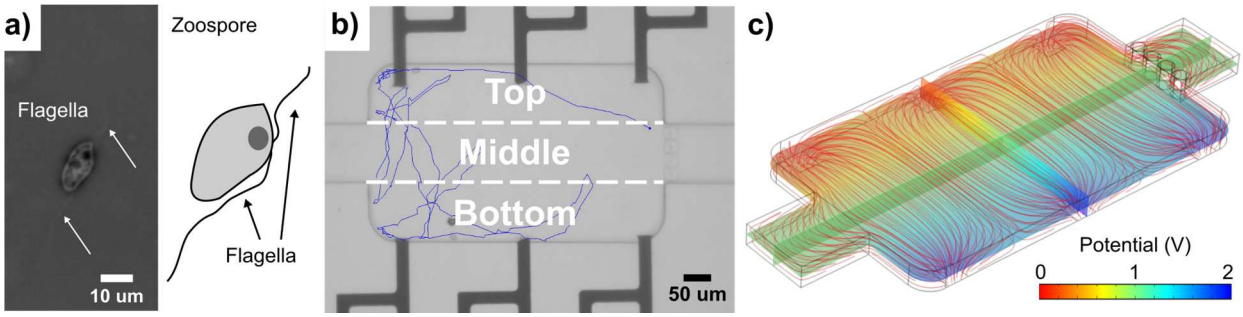

Figure 4: (a) Optical micrograph of an individual P. nicotianae *zoospore on the chip and schematic highlighting two flagella which provide motility. (b) An example of zoospore tracking with the blue line representing where the zoospore had swum. Also indicated are the OC top, middle and bottom zones that were used for residence time analysis (Fig. 5). (c) Finite element simulation (Comsol Multiphysics V5.5) of the electric potential and current density (streamlines) in the OC when the top electrodes were grounded, and 2 V DC applied to the bottom electrodes.*

water was added and left to incubate for 1 hour in a laminar hood. After 1 hour, the distilled water was exchanged with 20 ml of sterile distilled water containing ampicillin (100 µg/ml), vancomycin (20 µg/ml), amphotericin B1 (250 µg/ml). These plates were kept for 4 days in a dark chamber at a temperature of 22.5 ± 2.5 °C. Following this step, culture plates were given a cold shock for 1½ hour. Then 10 mL of sterile tap water was added to flood the plate, into which zoospores were released over 30 min [14].

Experimental Setup

Solutions containing the zoospore were introduced to chips with the help of syringe pump (NE-1000, New Era) at a speed of 1 µL/ min [10]. The syringe pump was used to limit flow of the zoospore solution, and to start with a fresh batch of zoospore at the beginning of every experiment. To determine the effect of solution conductance on the electric-field, the conductance of solutions was measured using an external conductivity meter (PL-700AL, GOnDO). The entire chip was connected to a PC controlling a custom-built Tiva TM4C123GXL microcontroller board with two digital-to-analog converters. Once a zoospore entered the OC, its swimming was monitored and recorded using an inverted bright-field microscope (Nikon Eclipse TS100) with attached digital camera. Movement was manually tracked in ImageJ. Finite-element simulations (Comsol Multiphysics V5.5) were used to model electric potential and current densities within the OC (Fig. 4(c)).

EXPERIMENTAL RESULTS

We have designed and fabricated a new LOC platform consisting of a central OC, an array of gold electrodes on a glass slide, and inlet and outlet channels incorporating either a sieve or pneumatic valve to study the electrotactic behavior of individual zoospores. The zoospores and their flagella were able to be imaged in the OC (Fig. 4(a)) and their movements over time were able to be tracked (Fig. 4(b)). The presence of the sieve/valve retained most of the zoospores within the OC, enabling observations to be made over several seconds/minutes. Integrated electrodes enabled the generation of electric fields within the OC (Fig. 4(c)).

For proof of concept experiments, the OC was divided into three zones, which we designated top, middle and bottom (Fig. 4(b)), relative to their respective positions to the upper and lower electrodes in the field of view. Data are presented as the percentage of time spent in each of these zones relative to the total time spent in the OC (Fig. 5).

Individual zoospores showed strong bias towards the cathode, relative to when there was no electric field present (Fig. 5a - d). Thus, in Fig. 5(b) and (c) the zoospores spent more time in the top zone compared to Fig. 5(a), where there was no electric field present. This response was field strength dependent, with zoospores spending less time in the middle and bottom zones at 2 V compared to 0.5 V. When the cathode was switched from the top to the bottom electrodes, zoospores spent more time in the bottom zone of the OC (Fig. 5(d)). Attraction to the cathode has previously been reported for *Pythium aphanidermatum*, although in contrast *Phytophthora palmivora* will swim towards the anode, and so it seems that directionality may be species specific [6].

We measured the conductance of media and currents at the applied voltages in the OC, and using the relationship of field strength = current density/conductivity, have calculated field strengths of 4.3 and 9.3 mV/cm at 0.5 and 2 V, respectively. These are in the range of the thresholds of 2 - 5 mV/cm previously reported for zoospore electrotaxis for *P. palmivora* and *P. aphanidermatum* using larger non-LOC OCs [6,8]. In nature, an electric current can be generated around growing or wounded areas of roots, due to the flow of protons and other ions. These currents have been estimated to produce field strengths in the same range as those that induce electrotaxis [6-8], and thus the response we report on the current LOC platform is likely replicating the response of zoospores in nature. The versatility of the device, which in future versions could incorporate channels to add chemo-attractants, as well as an electric field, means that it could be an important experimental tool to further our knowledge of the mechanisms that pathogenic oomycetes use to locate and invade their hosts.

CONCLUSIONS

In this paper we report the first demonstration of a microfluidic platform to study electrotaxis of motile zoospores of pathogenic oomycetes that enable them to target plant roots. This research will help address the

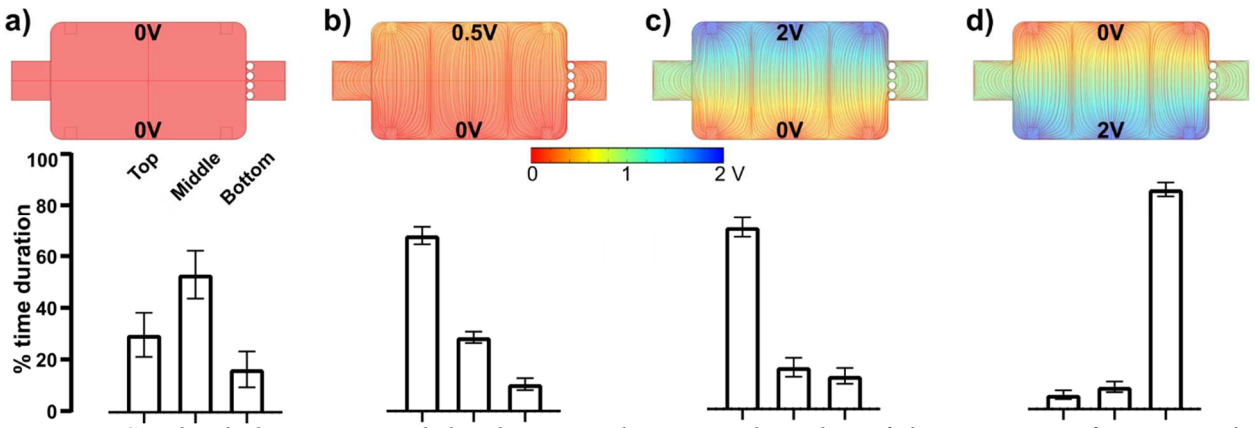

Figure 5: Simulated electric potential distributions and corresponding plots of the percentage of time spent by zoospores in the OC zones labelled "top", "middle" and "bottom", as a function of applied DC potential. (a) Bottom and top electrodes grounded (no potential), (b) bottom electrode grounded, top electrode 0.5 V, (c) bottom electrode grounded, top electrode 2 V, and (d) top electrode grounded, bottom electrode 2 V. Error bars represent standard error. Results show that zoospore position could be influenced by electrode biasing, indicating electrotactic stimulation of zoospore swimming by the electrode array.

question of what the primary attractant directs infectious zoospores towards the roots of a plant. It will help in developing techniques to prevent infection. We present the platform and show experimental results demonstrating that electrotaxis of zoospores of *Phytophthora nicotianae*, a pathogenic oomycete with a broad host range, can be studied at single cell level.

ACKNOWLEDGEMENTS

The authors would like to thank Helen Devereux and Gary Turner of the University of Canterbury Nanofabrication Laboratory for technical support. Financial support was provided by the Biomolecular Interaction Centre, the MacDiarmid Institute for Advanced Materials and Nanotechnology and Rutherford Discovery Fellowship RDF-19-UOC-019

REFERENCES

[1] S. Kamoun et al., "The Top 10 oomycete pathogens in molecular plant pathology," *Mol. Plant Pathol.*, vol. 16, no. 4, pp. 413-434, 2015.

[2] Y. Sun, A. Tayagui, S. Sale, D. Sarkar, V. Nock, and A. Garrill, "Platforms for High-Throughput Screening and Force Measurements on Fungi and Oomycetes," *Micromachines*, vol. 12, no. 6, p. 639, 2021.

[3] D. Longman and J. A. Callow, "Specific saccharide residues are involved in the recognition of plant root surfaces by zoospores of *Pythium aphanidermatum*," *Physiol. Mol. Plant Pathol.*, vol. 30, no. 1, pp. 139-150, 1987.

[4] K. L. Khew and G. A. Zentmyer, "Electrotactic Response of Zoospores of Seven Species of *Phytophthora*," *Phytopathology*, vol. 64, no. 4, 1974.

[5] K. L. Khew and G. A. Zentmyer, "Chemotactic response of zoospores of five species of *Phytophthora*," *Phytopathology*, vol. 63, no. 151, pp. 1-17, 1973.

[6] B. M. Morris, B. Reid, and N. A. R. Gow, "Electrotaxis of zoospores of *Phytophthora palmivora* at physiologically relevant field strengths," *Plant, Cell Environ.*, vol. 15, no. 6, pp. 645-653, 1992.

[7] B. M. Morris and N. A. R. Gow, "Mechanism of electrotaxis of zoospores of phytopathogenic fungi," *Phytopathology*, vol. 83, no. 8, pp. 877-882, 1993.

[8] P. van West et al., "Oomycete Plant Pathogens Use Electric Fields to Target Roots," *Mol. Plant-Microbe Interact.*, vol. 15, no. 8, pp. 790-798, 2002.

[9] A. Tayagui, Y. Sun, D. A. Collings, A. Garrill, and V. Nock, "An elastomeric micropillar platform for the study of protrusive forces in hyphal invasion," *Lab Chip*, vol. 17, no. 21, pp. 3643-3653, 2017.

[10] Y. Sun, A. Tayagui, A. Garrill, and V. Nock, "Fabrication of In-Channel High-Aspect Ratio Sensing Pillars for Protrusive Force Measurements on Fungi and Oomycetes," *J. Microelectromech. Syst.*, vol. 27, no. 5, pp. 827-835, 2018.

[11] Y. Sun, A. Tayagui, A. Garrill, and V. Nock, "Microfluidic platform for integrated compartmentalization of single zoospores, germination and measurement of protrusive force generated by germ tubes," *Lab Chip*, vol. 20, pp. 4141 - 4151, 2020.

[12] Y. Sun, D. Sarkar, R. Adams, A. Tayagui, A. Garrill, and V. Nock, "Hybrid normally closed dry-film/elastomer valves for integrated electrotaxis studies," in *Digest Tech. Papers MicroTAS'21 Conference*, Palm Springs, Oct 10-14, 2021, pp. 385-386.

[13] N. van den Berg, J. B. Christie, T. A. S. Aveling, and J. Engelbrecht, "Callose and β-1,3-glucanase inhibit *Phytophthora cinnamomi* in a resistant avocado rootstock," *Plant Pathol.*, vol. 67, no. 5, pp. 1150-1160, 2018.

[14] R.C. Kaitany, "Virulence Composition of *Phytophthora sojae* in Michigan," *Plant Dis.*, vol. 85, pp. 1103-1106, 2001.

CONTACT

*V. Nock, tel: + 64 3 3694303;
volker.nock@canterbury.ac.nz