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# Growth assay of individual pollen tubes arrayed by microchannel device

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ABSTRACT

To restrict pollen tube growth to a single focal plane is an important subject to enable their accurate growth analysis under microscopic observation. In the conventional method to assay pollen tube growth, the pollen tubes grow in a disorderly manner on solid medium, rendering it impossible to observe their growth in detail. Here, we present a new method to assay pollen tube growth using poly-dimethylsiloxane microchannel device to isolate individual pollen tubes. The growth of the pollen tube is confined to the microchannel and to the same focal plane, allowing accurate microscopic observations. This methodology has the potential for analyses of pollen tube growth in microfluidic environments in response to chemical products and signaling molecules, which paves the way for various experiments on plant reproduction.



Sperm-carrying pollen tubes (PTs) penetrate surrounding tissues in a style to deliver gametes to egg cells in female tissues to achieve fertilization and initiate seed development. PT growth is achieved by finger shaped cellular protrusion that grows at its tip and is able to orient its growth direction following guidance cues. A pushing force for this invasive growth is generated by the internal turgor pressure [1]. Communication among female tissues and pollen tubes (PTs) also plays an essential role in plant reproduction because signals from female tissues guide the PTs to the egg cells to achieve fertilization. Recently, various secreted polypeptides have been reported to be involved in communication during PT–female tissue interactions [2]. For example, the defensin-like polypeptides, LUREs, were shown to be the PT attractants secreted from synergid cells [3]. However, there have been few quantitative studies on the mechanisms of PT growth and PT–female tissue communication. One of the major obstacles has been the difficulty in quantitatively analyzing PT growth. Conventional *in vitro* assays monitor PT growth on agar gel medium in Petri dishes; however, PTs grow in a disorderly manner in these conditions (Fig. 1A) [4]. In addition, the chemical environment that develops on agar plates allows spatial control only in the order of millimeters [5,6], which is far below the resolution required cellular-level studies. A new and widely applicable method that allows precise handling of both individual PTs and signaling molecules is required for quantitative studies on PT–female tissue communications during plant reproduction.

Microfluidic-systems, typically fabricated using MEMS (Micro-Electro-Mechanical-Systems) technology [7], have been used for various biological experiments at the cellular [8,9] and molecular level [10,11] taking advantage of miniaturization for handling small volume of liquids. A microfluidic system has much potential to further research on plant reproduction, since it allows precise handling of the PT itself, and also allows the development of a chemical environment at a high spatial resolution on a micrometer scale. Such a system also can be used to develop an environment that mimics the internal micro-environment of the pistil during *in vivo* PT growth. Considering those advantages, a microsystem-based assay was developed to study the attraction of PT to ovules in *Arabidopsis thaliana* [12]. In another study, a microfluidic network analysis was used to examine the growth response of PTs to various mechanical challenges [13] and the device was designed for experimentation and phenotyping of PTs [14]. However, those studies were conducted on scales of a few tens to hundreds of micrometers, which are far larger than the size of the PT itself. To observe the growth of PTs continuously and to precisely measure their growth rate, PTs must be isolated and confined to the same focal plane during microscopic observations.

In this report, we describe a method to analyze growth of individual PTs confined to the same focal plane using a microchannel device. We developed a poly-dimethylsiloxane (PDMS) microchannel

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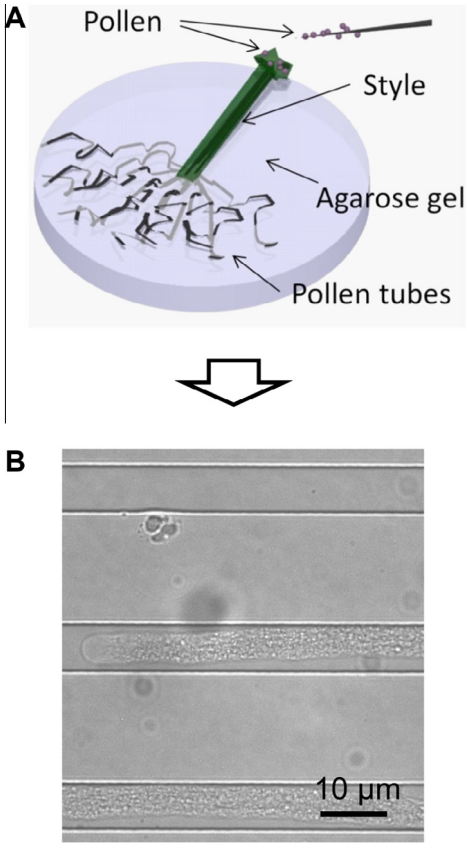


**Fig. 1.** (A) Schematic diagrams of conventional pollen tube assay on agar plate. Pollen tubes grow in a disorderly manner on and in agarose gel medium in a Petri dish. This prevents accurate quantitative analysis of pollen tube growth. (B) Microscopic image of the growing pollen tubes in the microchannel device. Individual pollen tubes are isolated and grow straight along each channel, enabling precise measurement of their growth rates.

device to isolate individual PTs and restrict the meandering in z-direction (Fig. 1B). We have adopted *Torenia fournieri* as a model plant because it enables us to perform live-imaging of the interaction between PT and female tissue. The design and dimensions of the microchannel device were optimized for handling individual PTs of *T. fournieri,* whose diameter is 8.6 ± 0.8 μm in our condition.

The PDMS microchannel devices prepared in this study consisted of a buffer and style inlet, buffer outlets, micro-slits, and microchannels with widths ranging from 5 to 20 μm on the same device (see Fig. S1 in Supplementary material). Thus, the device allows for growth assays of multiple PTs, even from the same sample, in parallel channels with various width. The heights of the channels varied from 5 to 20 μm. The distance between the center of the style inlet and the entrance of the microchannels was 2 mm. The microchannel devices were developed using the fabrication process described elsewhere [15]. Namely, the negative master for the molding of PDMS was fabricated on a silicon wafer with an ultrathick photoresist (SU-8 3005 and SU-8 3010, Microchem Corp., Newton, MA, USA). The prepolymer of PDMS (Sylgard® 184, Dow Corning, Midland, MI) was cast onto the master, which had a frame to hold the prepolymer in place. The prepolymer was degassed in the vacuum chamber for more than 20 min. After 90 min incubation at 65 °C, the cured PDMS was peeled off from the master, and the through-holes for style and buffer inlets (1.5 mm in diameter) were punched out manually.

The experimental protocols were as follows: First, the PDMS microchannel device was placed in the middle of a glass-bottomed dish. The dish was kept moist at room temperature throughout the assay. Second, after degassing the device in a vacuum chamber at 10 kPa for at least 40 min, 20 μL modified Nitsch's Medium [16] was injected from the style inlet to fill all the channels via the power-free pumping mechanism of the PDMS microchannel device [15]. Third, a hand-pollinated style of *T. fournieri* Lind. 'Crown Violet', cut to a length of 1 cm, was directly set in the style inlet of the device. The PTs grew and passed through the style after the pollination, and appears from the other side of the style in the microchannel device. The growth experiments were conducted under no-flow condition. Finally, at approximately 4 h after pollination, the PTs entered the microchannels and were observed by bright field microscopy on an inverted platform. To determine the measured rates in this experimental condition, an image of the growing PTs was captured every 30 s using a CCD camera mounted on the microscope.

The protocol using liquid media allowed for successful assays of PT growth in the channels with dimensions comparable to those of a single PT. PTs were isolated and straightened while they grew in the microchannels (Supplementary material 2). We also observed PTs entering channels less than 8-μm high, even though the diameter of PT tip is 8.6 ± 0.8 μm. The PTs were able to squeeze into the channels less than 8-μm wide. In those conditions, even though PTs were deformed and stressed by the channel to some extent, they did not stop growing, nor did their tips rupture. Similar phenomena were observed with *Camellia japonica* PTs that they passed through the gap narrower than their diameter by changing cell shape [17]. Thus, this protocol using liquid medium allows for growth assays of individual PTs by separating them among PDMS microchannels. The appropriate range of channel dimensions to assay growth of a single PT was defined by the height and width allowing entry of one PT. We investigated PT behavior depending on channel height, and then PT behavior depending on channel width. When the channel was less than 5-μm high, a single PT could not enter it. More than two PTs entered the channel higher than 12 μm (Fig. 2A). When the channel width was less than 5 μm, a PT could not enter the channel, while more than two PTs could enter the channel wider than 12 μm (Fig. 2B). Therefore, the appropriate channel height, and also the width, for a single PT observation were determined to be between 5 and 12 μm.

For more detailed investigations, PTs must be tightly confined vertically so that the growth proceeds in the same focal plane. Even a slight vertical displacement at the micrometer scale causes defocusing during microscopic observation. Within the range of appropriate channel heights, measured value of PT growth rates (hereinafter measured rates) were plotted against channel height to determine the "ideal height" of the channel for further quantitative growth analysis (Fig. 3A). The measured rates were highest, around 50 μm/min, in channels between 8 and 10-μm high. The measured rates decrease with channel height at the range higher than 10 μm. Our previous study reported measured rate of 16.3 ± 2.5 μm/min ($n$ = 10) in microchannels of 100-μm high and 100–1000-μm wide [18]. These results might be explained by both reduction of growth rate and the underestimation of the growth rates in the liquid environment caused by the meandering of PTs in z-direction. In channels less than 8-μm high, the measured rates were also lower. This may be caused by the spatial confinement as well as the lack of the nutrients since smaller PT's surface area contacts with the surrounding medium. Thus, the "ideal height" the microchannel to assay growth of a single PT was determined to be between 8 and 10 μm. Measured rates of pollen tubes did not appear to depend on microchannel width (Fig. 3B). This is because their horizontal meandering restricted on the focal plane is relatively easy to trace during microscopic observation.

We also compared the newly developed "on-chip" method with a conventional "off-chip" method. The "off-chip" condition was prepared by placing the modified Nitsch's Medium on the glass