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A microsystem-based assay for studying pollen tube guidance in plant reproduction

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Abstract

We present a novel microsystem-based assay to assess and quantify pollen tube behavior in response to pistil tissues. During plant reproduction, signals from female tissues (pistils) guide the sperm-carrying pollen tube to the egg cell to achieve fertilization and initiate seed development. Existing pollen tube guidance bioassays are performed in an isotropically diffusive environment (for example, a semi *in vivo* assay in petri dishes) instead of anisotropically diffusive conditions required to characterize guidance signal gradients. Lack of a sensitive pollen tube guidance bioassay has therefore compounded the difficulties of identifying and characterizing the guidance signals that are likely produced in minute quantities by the ovules. We therefore developed a novel microsystem-based assay that mimics the *in vivo* micro-environment of ovule fertilization by pollen tubes in the model research plant *Arabidopsis thaliana*. In this microdevice, the pollen tube growth rate, length and ovule targeting frequencies were similar to those obtained using a semi *in vivo* plate assay. As a direct measure of the microdevice's utility in monitoring pollen tube guidance, we demonstrated that in this device, pollen tubes preferentially enter chambers with unfertilized ovules, suggesting that the pollen tubes sense the concentration gradient and respond to the chemoattractants secreted by unfertilized ovules.

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1. Introduction

Research on pollen tube guidance has received significant attention in the past decade because of its importance in both biological and biotechnological research. During plant reproduction, a pollen grain lands on the surface of the pistil, absorbs water from the stigma and forms a pollen tube; a long polar process that transports the cellular contents, including two sperm cells. Pollen tubes grow through the stigma and style, and travel through the transmitting tract, where they encounter guidance cues that lead them to the ovule micropyle [1]. Typically, only one tube enters the ovule, terminating its journey within a synergid cell by bursting and releasing

the sperm cells to achieve fertilization [1]. The remarkable precision in the process of a pollen tube approaching an ovule micropyle is likely controlled by a series of molecular signals originating from the pistil and ovules [2–5].

These conclusions were based on analysis of fixed tissues, and it has been hypothesized that additional subtle, dynamic interactions between the pollen tube and pistil cells exist to ensure compatible pollination [6]. To identify at least some of the factors in these subtle interactions, a semi *in vivo* assay that facilitates real-time observation of pollen tube–ovule interactions in *A. thaliana* was developed [5]. The semi *in vivo* studies were conducted using a petri dish with pollen growth medium (PGM; [5]). A cut portion of the pistil and a

few ovules are placed on the PGM to recapitulate the *in vivo* environment. Pollen tubes emerge from the cut end of the pistil, and travel across the PGM before entering the excised ovules. The plate assay is performed on the PGM surface without the transmitting tract and the ovary chamber, which confine the path of the guidance cues emitted from the ovules in the *in vivo* environment.

Using the plate assay, ovule-derived attractants were reported to play a critical role in guiding pollen tube entry into the ovule [5]. In another study, applying stochastic mathematical modeling of pollen tube growth in the plate assay, pollen tubes were found to respond to ovule-secreted chemoattractants by following their gradients [7, 8]. Despite these characterizations, the cues that mediate these signaling events remain largely unknown, particularly in *A. thaliana*. The hypothesis that chemotropic signals mediate pollen tube guidance has been around for more than 110 years. For example, in experiments as early as 1893, secretions from *Narcissus* (Daffodil) ovules, when placed on agar plates, attract pollen tubes *in vitro* [9]. Unfortunately, the next logical step in these studies, the identification of the chemotropic substance, was not possible due to technological limitations at that time. However, recent advances in analytical chemistry, instrumentation, and genome sequencing have made it feasible to profile complex tissues, searching for proteins or small molecules, even those that are present in pM–nM concentrations [10, 11]. If these new capabilities could be combined with a sensitive and quantitative pollen tube guidance assay, it might be feasible to identify the elusive pollen tube guidance signals.

Existing bioassays to monitor pollen tube behavior in response to guidance cues involve a complex, biological tissue such as an ovule [5, 12]. Therefore, we set out to develop a sensitive *in vitro* pollen tube guidance assay in *A. thaliana* that reveals changes in pollen tube behavior in the absence of ovules. Such an assay will be useful to: (i) model pollen tube behavior, (ii) purify to homogeneity and identify pollen tube guidance signals in pistil extracts, and (iii) confirm guidance activity of purified signals. Microdevices have been developed for numerous biological applications [13, 14], largely due to the capability of realizing 3D structures with a length scale ranging from 1 to 1000 μm . Indeed, microsystem technology can duplicate attributes such as the complex configurations of the pistil micro-environment and can replicate signal micro-gradients; these cannot be achieved in plate assays. Furthermore, there is a decisive advantage of a microsystem-based assay, compared to any other assay, in the flexibility to manipulate certain control parameters to isolate and highlight separate mechanisms involved in the intricate interaction between pollen tubes and ovules. Here, we report, for the first time, the development and validation of a microsystem-based assay suitable for studying pollen tube–ovule interactions and adaptable for analyzing pollen tube behavior in response to other pistil tissues and signals.

2. Microdevice design and fabrication

Attempts to utilize a plate assay for the investigation of pollen tube guidance were not successful as discussed in

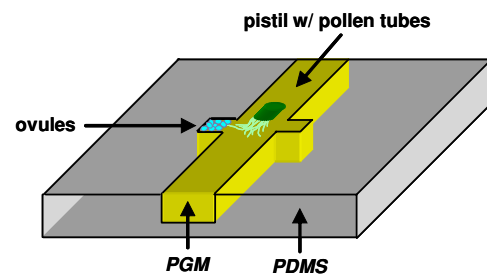


Figure 1. A schematic layout of a typical microdevice designed to support pollen tube growth featuring a microchannel for pistil placement and side chambers for ovules.

appendix A.1. We therefore developed a microdevice to mimic the micro-environment of *in vivo* ovule fertilization by pollen tubes [15]. The microdevice design features a main groove with side chambers. While the groove is used for placement of the pollinated pistil, the chambers on each side of the groove are used for placement of ovules; moreover, guidance substances can also be delivered to these chambers to generate respective chemical gradients. The groove width is set at about 1000 μm to accommodate a cut pistil, whereas the planar area of the side chambers varies from $250 \times 250 \mu\text{m}^2$ to $1000 \times 1000 \mu\text{m}^2$ to allow delivery of guidance molecules or placement of a test tissue of choice such as ovules. The groove total length is about 5 mm allowing pistil placement at different locations for various tests. Based on test results from the plate assay, summarized in appendix A.2, the depth of the groove and chambers was selected to be about 500 μm . A schematic layout of a typical device, consisting of a pistil groove and two side-chambers, is shown in figure 1.

Transparent microdevices are highly desirable to allow the use of microscopy to monitor the pollen tube interaction with ovules. Therefore, silicon-based organic polydimethylsiloxane (PDMS) (Dow Corning Corp.) was chosen as the substrate material for the microdevices due to its transparency and bio-compatibility. Furthermore, device fabrication using PDMS is simple, fast and relatively inexpensive. Cross-sectional schematics for the major steps of the fabrication process are illustrated in figure 2. The fabrication starts with the formation of a mold using a photosensitive polymer SU-8 (MicroChem Corp.). To achieve a thickness of 500 μm , SU-8 2100 is spin coated on a silicon wafer using a two-layer coating technique [16]. After two soft-baking steps at 65 °C and 95 °C, the SU-8 film is exposed to UV light using an ABM aligner with a photo mask of the device patterns (figure 2(a)). The SU-8 film is next developed, transferring the device pattern from the mask to the film (figure 2(b)), and completing the construction of the mold. For device fabrication, a PDMS mixture of base and curing agent with a weight ratio of 10:1 is poured onto the mold and cured overnight at room temperature as shown in figure 2(c). Microdevices are then obtained by peeling off the PDMS replicas from the mold.

The PGM is routinely prepared in the liquid phase under elevated temperatures before cooling off and turning into gel. It is clearly advantageous to load microdevices with PGM in the liquid phase rather than the gel phase and, since the

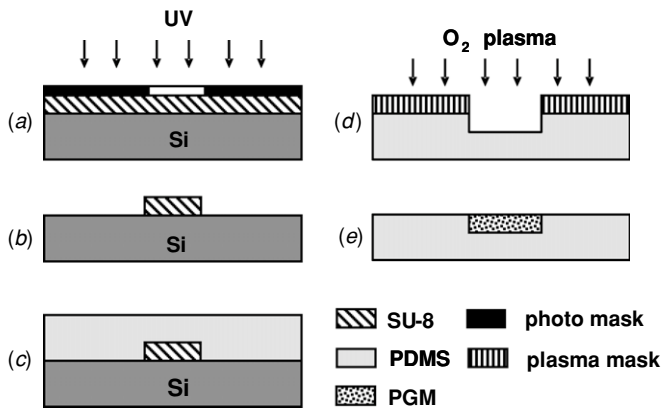


Figure 2. Cross-sectional schematics of the major steps in the fabrication process of the devices used in the microsystem-based assays.

microgrooves are essentially open channels, it is simplest to use capillary forces to drive liquids into the fabricated devices. However, to prevent the PGM from spilling out of the designated features onto adjoining surfaces, the PDMS hydrophobic surface is selectively treated with O_2 plasma as shown in figure 2(d). The device is masked during the treatment, covering it with a patterned PDMS film leaving only the surfaces of the groove and chambers uncovered. Exposure to oxygen-plasma treatment for 3 min renders the PDMS surface hydrophilic [17], facilitating the action of capillary forces in drawing working liquids exclusively into the hydrophilic microgroove and chambers. After peeling off the masking PDMS film, a needle syringe is used to inject the hot liquid PGM into one end of the groove. The PGM fills up the groove and chambers evenly due to capillary forces, as shown in figure 2(e), and solidifies upon cooling down to room temperature. An image of a fabricated PGM-filled microdevice, with placed pistil and ovules ready for experiments, is shown in figure 3.

3. Microsystem-based pollen tube growth and guidance assay

Pistils and ovules are staged and isolated for the assay as described previously [5]. For the microsystem-based assay, a freshly cut pistil is placed on the groove, which is filled with the PGM. To promote the growth of pollen tubes toward the cut end, the pollinated pistil is first placed vertically in the groove with its cut end in contact with the PGM surface for 2 h. Then, the pistil is laid down horizontally on the PGM groove with its transmitting tract parallel to the groove's axial direction. In this approach, random growth of pollen tubes is suppressed; instead, the tubes are directed to grow from the pollinated stigma into the pistil, toward the cut end, equivalent to the *in vivo* confinement imposed by the transmitting tract. Similar results can be achieved by selective pollination of the 'forehead' of the stigma, immediately followed by 'face-up' placement of the pistil in the device to prevent direct contact between the pollen grains and the PGM. Finally, carefully placing the prepared pistil at identical distances from

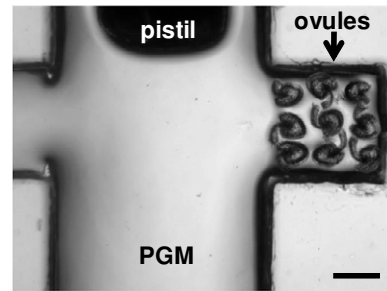


Figure 3. A micrograph of a fabricated microdevice filled with pollen growth medium (PGM); a pollinated pistil and ovules are placed in preparation for pollen tube guidance assay. Scale bar, 200 μm .

the groove sidewalls is critical to avoid introducing initial directional bias in pollen tube growth.

Space is a significant limiting factor in the microdevice, and therefore a population of 10–60 pollen tubes has been found to be optimal; it offers a population size sufficient for statistical analysis and also enables reliable tracking of individual tubes. Additionally, this number of pollen tubes within a 1000 μm wide groove prevents over-crowding and minimizes physical contact between tubes. Thus, the effects of tube–tube interaction on pollen tube growth and guidance can be minimized. In order to obtain a population of 10–60 pollen tubes, pollen from a dehiscing anther is diluted by pollinating two dummy virgin *male sterile 1 (ms1)* pistils before pollinating the stigma of the pistil to be used in the assay [5].

Placement of ovules inside a chamber is similar to that in the plate assay [5], and care must be exercised to place the ovules in the chamber without injuring them. In all experiments involving ovules, for consistent signal-gradient generation, nine ovules are placed next to each other within the chamber and, whenever possible, with their micropyles facing the chamber opening to the main groove. In this study, for the chamber configuration used in the microdevices, no more than nine ovules could be individually placed in the chamber.

Following proper placement of a cut pistil and ovules in the PGM-filled groove and chamber, the device is placed on a 2 mm thick layer of PGM within a petri dish to prevent drying out of the PGM in the microdevice. Consequently, microsystem-based assays are conducted following the same incubation conditions as those used in plate assays [5]. Digital images of pollen tube growth are captured with a Zeiss Axiovert 100 (Carl Zeiss, Germany) using Metamorph software (Molecular Devices, <http://www.moleculardevices.com/>).

4. Pollen tube growth rate and final length

It has been observed that pollen tube growth in a microsystem-based assay is qualitatively similar to that in a plate assay if the microdevices are placed on a 2 mm thick layer PGM in a petri dish, as shown in figure 4(a). Tubes fail to grow, however, if the microdevices are placed on the bottom surface of a bare petri

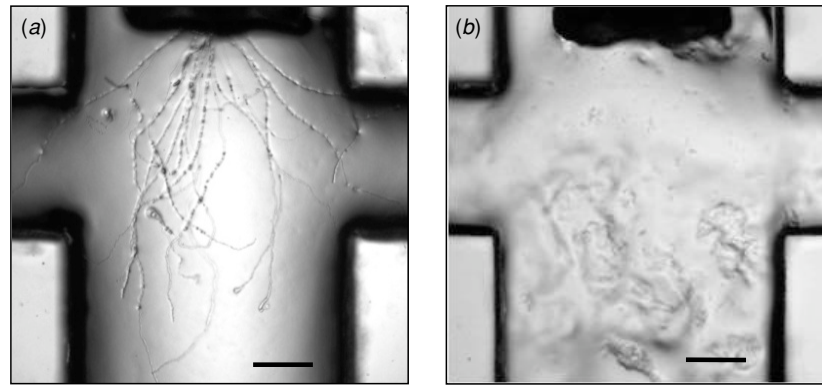


Figure 4. Micrographs of pollen tube growth in microsystem-based assays with the device placed under (a) humid and (b) dry conditions. Scale bar 200 μm .

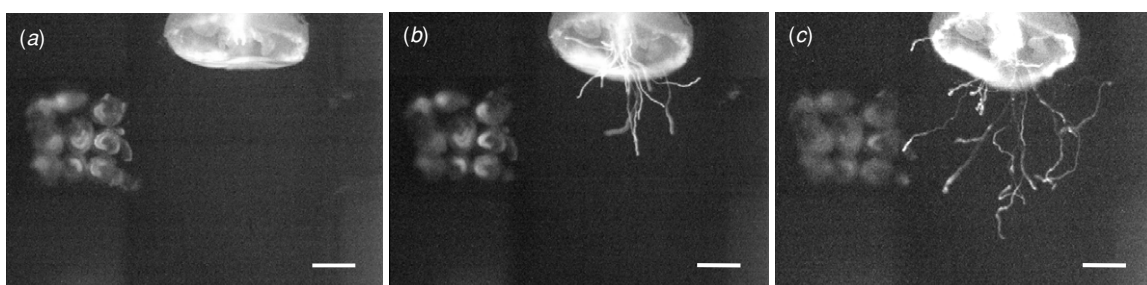


Figure 5. A time-lapse image sequence of pollen tube growth in a microdevice, with ovules in the left chamber and an empty right chamber, following incubation time of (a) $t = 0$, (b) $t = 4.5$ h, and (c) $t = 18$ h. Scale bar, 200 μm . An MPEG movie comprising the entire time-lapse series shown in this figure is available online at stacks.iop.org/JMM/21/054018/mmedia.

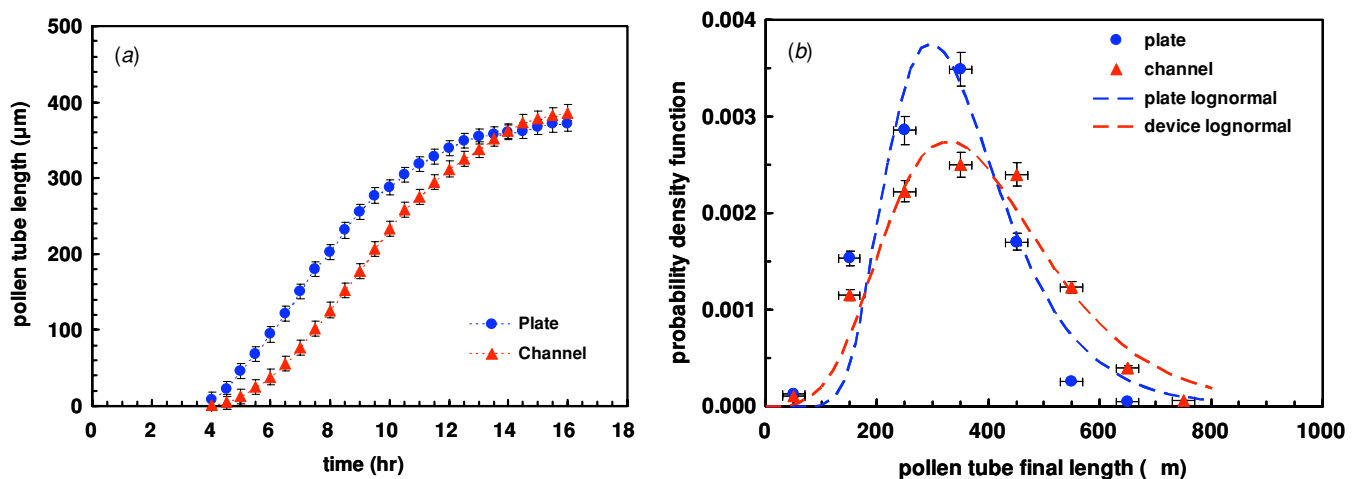


Figure 6. A comparison between pollen tube growth in a microsystem and in a plate assay: (a) time-dependent pollen tube length and (b) probability density function of pollen tube final length.

dish without the PGM layer as shown in figure 4(b); moreover, the PGM surface is severely warped. The PGM layer on the petri dish does not have direct contact with the pollen tubes and ovules in the microdevices. The role of the layer is limited to maintaining proper humidity around the microdevices and preventing the PGM in the microdevices from drying out. This demonstrates that humidity is an important factor for pollen tube growth during incubation. Therefore, the incubations in all microsystem-based assays described in this study were

conducted with the devices placed in a petri dish containing a 2 mm thick layer of PGM.

Fresh cut pistils from *A. thaliana* plants were used to investigate whether microdevices could support proper growth of pollen tubes. As shown in figure 4(a), pollen tubes emerge from the cut end of the pistil and grow on the PGM microgroove of the PDMS device, qualitatively similarly to the growth on plates. However, a more rigorous quantitative analysis is required to establish the suitability of a microsystem-based assay. Therefore, time-lapse images of

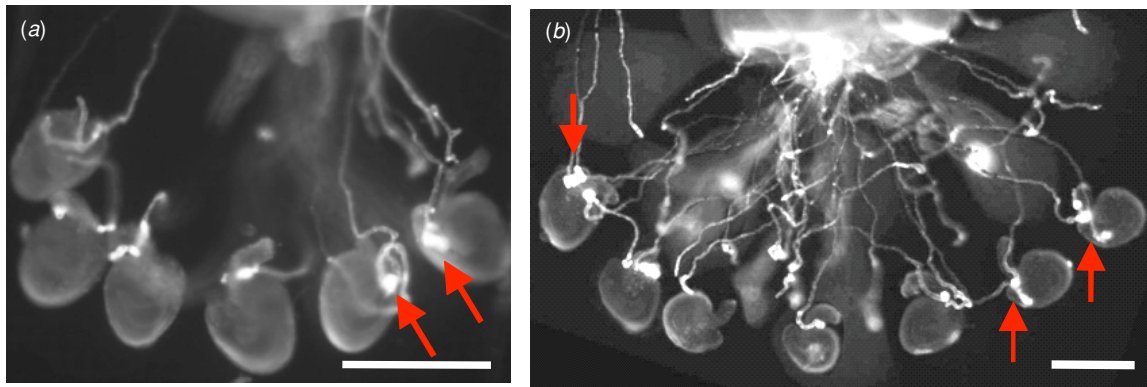


Figure 7. Ovule targeting by green fluorescent protein-tagged pollen tubes in (a) plate and (b) microsystem assays; in both images, each red arrow points to a pollen tube burst used as a marker for successful targeting of an ovule by a pollen tube. Scale bars, 200 μm .

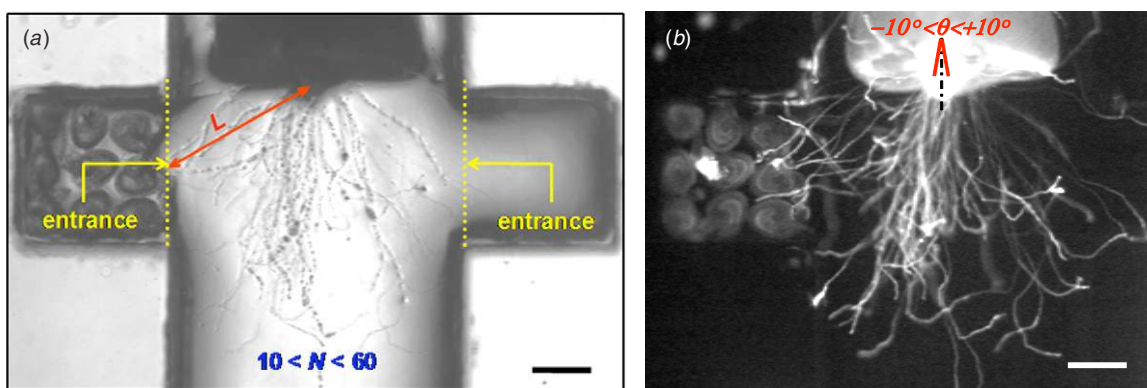


Figure 8. An illustration of the conditional-sampling criteria applied for quantitative measurement of pollen tube responses to guidance signals from ovules. The assay uses both (a) a bright-field image and (b) a fluorescent image of the same experiment. Assays are included in the statistical analysis only if (i) the number of pollen tubes is between 10 and 60, (ii) the average length of pollen tubes is larger than the distance between the ovule-chamber entrance and the point of pollen tube emergence from the pistil, L , as indicated in panel (a), and (iii) the transmitting tract is oriented along the channel axial direction within $\pm 10^\circ$ as indicated in panel (b). Scale bars, 200 μm .

the pollen tube growth were collected, as shown in figure 5, allowing length measurements of the growing pollen tubes. The average pollen tube length is plotted in figure 6(a) as a function of time for both plate and microsystem assays (12 samples for plate assay and 13 samples for microdevice assay). The results indicate that the evolution of pollen tubes in both assays is about the same. Pollen tubes emerge from the pistil cut end about 4 h after placement of a pollinated pistil in the PGM groove. For the next 8 h, the emerging tubes grow almost linearly with time at a rate of about $30 \mu\text{m h}^{-1}$. The growth rate gradually diminishes during the next 4 h as the tubes reach their final length.

All pollen tubes are obviously not identical; they grow and meander in different directions until they cease to grow. The final length of numerous pollen tubes was measured from their emergence point from the cut pistils, 475 tubes for the plate assay and 506 tubes for the microsystem-based assay, and summarized as a statistical distribution for each assay in figure 6(b). The results indicate that the majority of the tubes, in both plate and microsystem-based assays, grow to a maximum length ranging between 400 and 450 μm . Longer tubes up to 1000 μm are observed in the microsystem-based assay; these are rarely seen in plate assays performed under

similar conditions. This suggests that perhaps the microgroove physically confines the orientation of pollen tubes, reducing the number of turns they take and, thus, allowing some of them to stretch over longer distances. Indeed, pollen tubes grown in microdevices do not fan out widely and also do not often take a winding path; these growth attributes are different from what is typically observed in plate assays. In general, though, the measured tube growth rate and final length both validate that the behavior of pollen tubes in microdevices is similar to that in plates, and microsystems can be used to assay pollen tube guidance.

5. Pollen tube targeting of virgin ovules

A critical aspect of a semi *in vivo* pollen tube guidance assay is the interaction between pollen tubes and ovules. Therefore, to investigate targeting of ovules by pollen tubes in microdevices, unfertilized *A. thaliana* ovules were excised and distributed on the PGM in both microsystem and plate assays essentially as described in [5]. Since the measured average final length of pollen tubes in a microsystem-based assay is about 450 μm , the pistils are placed with their cut edge within a distance of 300 μm from the ovules ensuring that the majority of the pollen

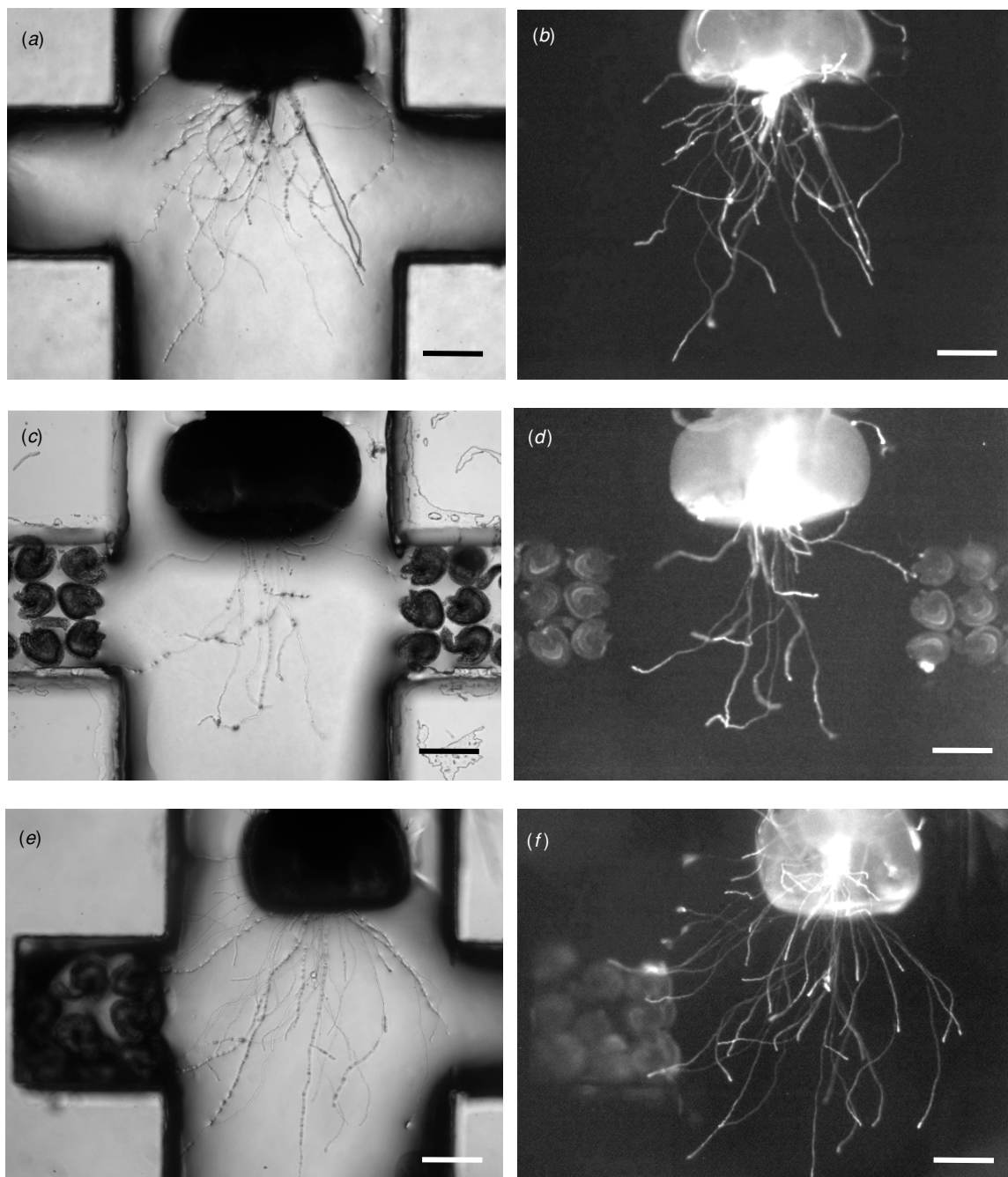


Figure 9. Bright-field (a), (c), (e) and fluorescent (b), (d), (f) images of pollen tube growth in microsystem-based assays with three combinations of ovule placement in the side chambers: (a), (b) empty–empty, (c), (d) unfertilized–unfertilized ovules, and (e), (f) empty–unfertilized ovules. Scale bars, 200 μm .

tubes are able to reach the ovules. Since ovule integument cells are opaque, a pollen tube is usually obscured after entering a micropyle. To facilitate continuous pollen tube observation and confirm targeting events with certainty, pollen carrying a *green fluorescent protein (GFP)* reporter under the control of the pollen-specific *LATS2* promoter is used in these assays [18]. Upon reaching synergid cells, the pollen tubes arrest growth and burst; the released GFP from the pollen tube rapidly diffuses within an ovule, conveniently marking successful targeting of the ovule. Representative images pertaining to ovule targeting by pollen tubes in plate and microsystem-based

assays are shown in figures 7(a) and (b), respectively, where the red arrows point to successful targeting events. Of the tubes that sufficiently grew in length to reach the ovules, 71% (127 out of 178 tubes) successfully entered the micropyles in the microsystem-based assay compared to 70% (135 out of 192 tubes) in the plate assay. These findings validate the utility of microsystem technology in recreating an *in vivo* micro-environment for studying *A. thaliana* pollen tube interactions with ovules; this technology will likely be highly effective for other species with thin pollen tubes similar to those of *A. thaliana*.

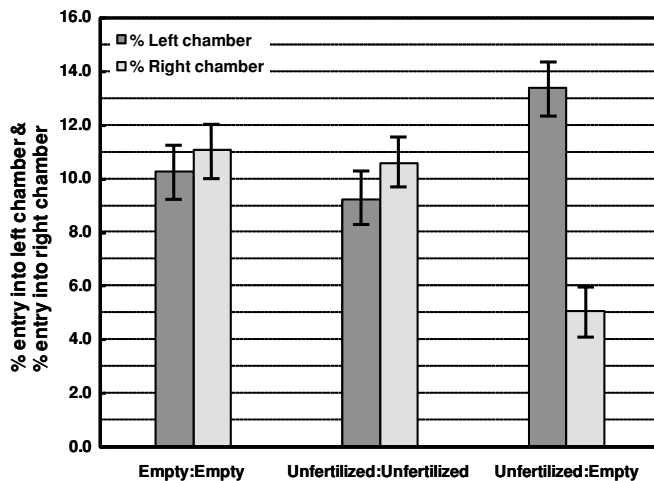


Figure 10. Quantification of pollen tube entries into chambers with and without unfertilized ovules in the microsystem-based assays corresponding to the three experimental conditions described in figure 9.

6. Pollen tube attraction in a microsystem-based assay

It has been hypothesized that in the semi *in vivo* pollen tube guidance assay, unfertilized ovules release chemoattractants that diffuse via the PGM [5, 8]. Accordingly, if pollen tubes can sense the concentration gradient of the diffused ovule chemoattractants, they might orient their growth toward the ovule. We next investigated if pollen tubes would respond to attractants secreted by ovules in the microdevice described here. For this, a pollinated cut pistil was placed in the microgroove near the two chambers, and several wild-type virgin ovules were placed in one chamber only (leaving the opposite chamber empty) to generate an asymmetric concentration field and gradient; devices with both chambers left empty or filled symmetrically with unfertilized ovules were used as controls.

Qualitative analysis of pollen tube guidance, based on observations of recorded images of pollen tube evolution in microdevices, is subjective and error-prone. Hence, a statistically quantitative criterion is required to obtain a robust and un-biased measure of pollen tubes response to guidance signals. The length scale of signals emanating from ovules is expected to be short in comparison with the distance traveled by pollen tubes [5, 7, 8]; therefore, random orientation of pollen tubes at various stages of their growth could mask subtle pollen tube responses to guidance signals. To minimize this 'noise', conditional sampling was adopted for the statistical analysis. Samples were considered to be valid only if they meet the following three conditions, as illustrated in figure 8: (i) the number of pollen tubes emerging from the cut pistil ranged from 10 to 60, (ii) the average pollen tube length was larger than the distance between the pistil cut edge and the ovule chamber, L , and (iii) the transmitting tract was oriented along the channel axial direction within $\pm 10^\circ$. In such samples, any tube crossing the entrance line of a chamber, as marked in figure 8(a), was included in the population used for examining pollen tube guidance.

Pollen tubes typically emerge from the pistil cut edge and grow along the microgroove. In the control experiments with both chambers empty, no biased turning toward either chamber was observed as qualitatively seen in figures 9(a) and (b). Similarly, no turning bias was observed if the number of unfertilized ovules placed in each of the two chambers was equal, as shown in figures 9(c) and (d). With ovules in only one of the chambers, in contrast, preferential turning of pollen tubes toward the ovule-containing chamber was qualitatively observed (figures 9(e) and (f)). Quantitative analysis based on the conditional sampling approach is summarized in figure 10. From a total of 25 assays totaling 793 pollen tubes, the ratio between the number of tubes entering the ovule chamber and the number of tubes entering the empty chamber was 2.7:1, whereas the ratio in both control configurations was about 1:1 (26 assays with 849 tubes for the empty–empty case, and 21 assays with 793 tubes for the unfertilized–unfertilized configuration). The difference between the ratio for the 'empty-unfertilized' configuration and the ratios for the two control configurations is statistically significant, indicating that ovules affect the pollen tube growth orientation.

These results suggest that experimental investigation of pollen tube growth guidance can be conducted in a controlled manner utilizing PGM-filled PDMS microdevices. Indeed, a microsystem-based assay offers greater promise than current assays in attempting to identify specific attractants and quantifying their effects on pollen tube guidance; this newly-developed assay enables reliable monitoring and quantification of pollen tube responses without having to rely on pollen tube burst inside an ovule.

7. Conclusions

In this paper, we present the design, fabrication and development of a PDMS microdevice capable of supporting pollen tube growth. The measured growth rate and final length of pollen tubes in microdevices were about the same as in plates. The targeting efficiency of unfertilized ovules by pollen tubes in microdevices was also similar to that in plates. Importantly, in these microdevices, preferential turning of pollen tubes toward attractants secreted by unfertilized ovules was achieved consistently. *In vivo* pollen tube guidance to excised *A. thaliana* ovules has clearly been recapitulated in fabricated microdevices and, thus, micro-environments for studying *A. thaliana* pollen tube growth and guidance can be recapitulated *in vitro*. Moreover, these results confirm that microdevices are an excellent tool, far superior to a plate assay, for generating microgradients of guidance cues.

This microsystem-based assay removes a significant barrier to obtaining a comprehensive understanding of pollen tube–ovule interactions and will facilitate key experiments such as measurement of attractant micro-gradients secreted by ovules, as well as detection of repulsion signals, which are impossible to conduct *in vivo* and difficult to control using semi *in vivo* plate assays. Additionally, the assay is robust and flexible enough that it can be adapted to characterize, analyze and quantify subtle behavioral changes of thin pollen tubes in species other than *A. thaliana*.

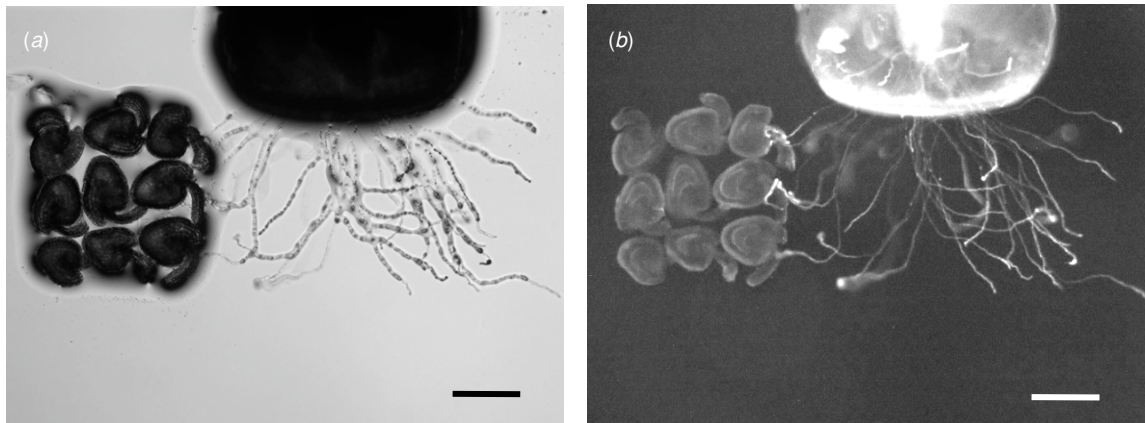


Figure A1. Pollen tube growth in a plate assay with asymmetrically placed ovules: (a) bright-field and (b) fluorescent images of red fluorescent protein-tagged pollen tubes. Scale bars, 200 μm .

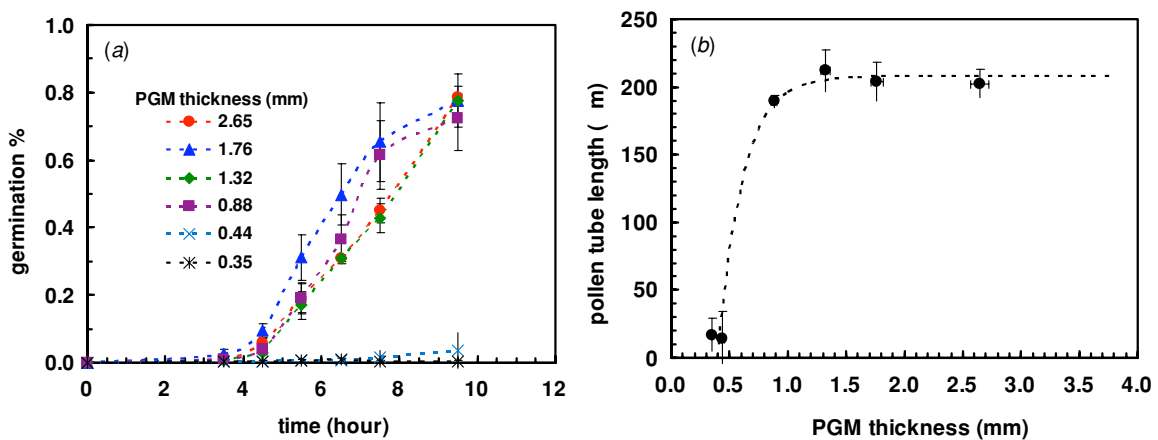


Figure A2. Effect of pollen germination medium (PGM) thickness on pollen tube evolution in a plate assay: (a) time-dependent germination rate and (b) pollen tube length dependence on PGM thickness after 10 h incubation time.

Acknowledgments

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Appendices

A.1. Evaluation of a plate assay for pollen tube guidance studies

A semi *in vivo* assay that facilitates real-time observation of pollen tube–ovule interactions in *A. thaliana* has been developed [5]. However, attempts to utilize this assay for studying pollen tube guidance with or without ovules have not been successful. For example, as demonstrated in figure A1, no preferential turning of pollen tubes toward the ovules is observed when several unfertilized ovules are placed on one side of a cut pistil. One reason the assay has not been successful could be the absence of transmitting tract and ovary chamber, which physically confine the path of pollen tubes *in vivo*. Additionally, the guidance signal diffusion *in vivo* is likely directional [8]; thus, an assay to monitor pollen

tube response to such a signal gradient should be anisotropic rather than isotropic. The isotropic model of the plate assay is therefore not suitable for quantitative investigation of the response of *A. thaliana* pollen tubes to the pistil- or ovule-derived guidance signals.

A.2. Effect of PGM thickness on pollen tube growth

One obvious difference between the microsystem-based assay and the plate assay is the limited amount of PGM present within the confined groove and chambers compared to the amount of PGM in a plate. In conventional plate assays, a layer of up to 2 mm thick PGM is used to support proper pollen tube growth and ovule targeting. Technologically, it is challenging to realize grooves of about 2 mm in depth with a planar scale on the order of several hundred micrometers. Therefore, plate assays with PGM thicknesses ranging from 100 μm to 3 mm were carried out to determine the minimum PGM thickness required to support successful pollen tube growth. The effect of PGM thickness on pollen tube germination rate and growth rate, collected from 18 samples, is summarized in figure A2. The results show that pollen tube germination starts to increase almost linearly with time about 4 h after pistil placement on PGM plates thicker than 880 μm , figure A2(a), but no pollen

tube germination is observed from pistils placed on PGM plates thinner than 440 μm . The average length of germinated pollen tubes is about 200 μm after 10 h incubation time, figure A2(b), practically independent of the PGM thickness for plates thicker than 880 μm . This set of experiments suggests that the depth of the microgroove and chambers should be between 440 and 880 μm ; for ease of fabrication, we selected a depth of 500 μm to provide a proper environment to support pollen tube growth.

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