Video Article

Development of Microfluidic Devices to Study the Elongation Capability of Tip-growing Plant Cells in Extremely Small Spaces

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Abstract

In vivo, tip-growing plant cells need to overcome a series of physical barriers; however, researchers lack the methodology to visualize cellular behavior in such restrictive conditions. To address this issue, we have developed growth chambers for tip-growing plant cells that contain a series of narrow, micro-fabricated gaps (~1 μm) in a poly-dimethylsiloxane (PDMS) substrate. This transparent material allows the user to monitor tip elongation processes in individual cells during microgap penetration by time-lapse imaging. Using this experimental platform, we observed morphological changes in pollen tubes as they penetrated the microgap. We captured the dynamic changes in the shape of a fluorescently labeled vegetative nucleus and sperm cells in a pollen tube during this process. Furthermore, we demonstrated the capability of root hairs and moss protonemata to penetrate the 1 μm gap. This *in vitro* platform can be used to study how individual cells respond to physically constrained spaces and may provide insights into tip-growth mechanisms.

Video Link

The video component of this article can be found at https://www.jove.com/video/57262/

Introduction

After pollen grains germinate on a stigma, each grain produces a single pollen tube that carries sperm cells to the egg cell and the central cell in the ovule for double fertilization. Pollen tubes elongate through the style and eventually reach the ovule by sensing multiple guidance cues along their way[1]. During the elongation, pollen tubes encounter a series of physical barriers; the transmitting track is filled with cells, and pollen tubes must enter the minute micropylar opening of the ovule to reach their target (**Figure 1A**)[2]. Therefore, pollen tubes must have the ability to penetrate physical obstacles, while tolerating the compressive stress from their surroundings. Root hairs are another type of tip-growing plant cell that must withstand physical obstacles in the environment, in the form of packed soil particles (**Figure 1B**).

Various mechanical properties of the pollen tube have been studied, including turgor pressure and stiffness of the cell's apical region, which can be measured using the incipient plasmolysis method[3,4] and cellular force microscopy (CFM)[5,6], respectively. However, these methods alone do not reveal whether pollen tubes are capable of elongating through physical barriers along their growth paths. An alternative technique that allows pollen tube elongation to be monitored *in vivo* is two photon microscopy[7]. However, with this method, it is difficult to observe the morphological changes in individual pollen tubes deep inside the ovule tissue. Additionally, root hair growth in soil can be visualized using X-ray computed tomography (CT) and magnetic resonance imaging (MRI)[8], albeit with low resolution. Here, we present a method that can be used to acquire high-resolution images of a cell's deformation process on a conventional microscope.

The overall goal of the method described here is to visualize the elongation capability of tip-growing plant cells, including pollen tubes, root hairs, and moss protonemata, in extremely small spaces. As the poly-dimethylsiloxane (PDMS) microdevices presented in this manuscript are optically transparent and air permeable, we can culture living cells inside the device and observe their growth behaviors under a microscope. It is also possible to create micro ~ nanometer scale spaces by the soft lithography technique[9] with the use of molds. These features allow us to study the elongation capability of tip-growing plant cells in a physically confined environment.

In this work, we constructed 1 μm wide gaps (4 μm in height) in microfluidic devices and examined the ability of pollen tubes to penetrate these artificial obstacles that are much smaller than the diameter of the cylindrical pollen tube (approximately 8 μm). This experimental platform enables us to visualize the pollen tube's response to microgaps and capture time-lapse images of the response, which track the cell's deformation process. We also developed the microdevices that can be used to investigate the penetration capability of root hairs and moss protonemata. Several microdevices have been reported to date that enable the visualization of plant root[10,11,12,13] and moss protonemata[14] growth at high resolution. In our device, a series of root hair growth channels are perpendicularly connected to a root growth chamber, and individual root hairs (approximately 7 μm in diameter) are guided to fluidic channels with a 1 μm wide gap. We also cultured moss protonemata (approximately 20 μm in diameter) in a microdevice containing microgaps to examine their responses to these physical barriers. The proposed

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microfluidic-based approach allows us to explore the capability of various tip-growing plant cells to elongate through extremely small spaces, which cannot be examined by any other currently available method.

Protocol

1. Fabrication of the PDMS Microdevice to Examine Growing Pollen Tubes and Moss Protonemata

NOTE: We used a maskless photolithography instrument to prepare PDMS molds on silicon wafers. The details regarding the operation of the system are omitted in this manuscript. A standard photolithography technique[9] using a photomask may also be used to create the PDMS molds described in this manuscript.

1. Pour 11 g of pre-polymer PDMS mixture (elastomer base:curing agent at a ratio of 10:1) into each 4-inch mold.
2. Degas the mold prepared in step 1.1 for 20 min in a vacuum chamber.
3. After curing at 65 °C for 90 min in a non-convection oven, peel the PDMS layer off the mold, and punch access holes into the fluidic channels using biopsy punches.
   NOTE: For the pollen tube device, the size of the hole must be adjusted to reflect the diameter of the pistil. This value will therefore vary by species. In this experiment, we punched a 1 mm hole for the pistil inlets and 1.5 mm holes for the liquid medium reservoirs. For the moss protonemata device, a 4 mm hole was used for the sample reservoir.
4. Expose the PDMS layer and a glass bottom dish (3.5 - 5 cm in diameter) to air plasma for 50 s.
5. Press the PDMS layer into the glass bottom dish and heat at 65 °C for 30 min in a non-convection oven to completely seal off the microfluidic network.

2. Fabrication of the PDMS Microdevice for Root Hairs

1. Repeat steps 1.1 - 1.3 using the molds to prepare two PDMS layers for the root and root hair microdevices.
   NOTE: We used a 2 mm hole for the liquid medium reservoirs in the root microdevice.
2. Expose both PDMS layers to air plasma for 50 s.
3. Assemble the two PDMS layers under a stereomicroscope using a custom-made desktop aligner.
   NOTE: The microchannels on these PDMS layers must face each other. Before assembling, make sure that the alignment marks on both layers match up.
4. Heat at 65 °C for 30 min in a non-convection oven to completely seal off the microfluidic network.
5. Remove the cover slip from the constructed microdevice and place the device on a glass bottom dish that is 5 cm in diameter.

3. Preparation of *In Vitro* Cell Culture Medium for Pollen Tubes (*Torenia fournieri*)

1. Prepare modified Nitsch's medium as described previously[15] and autoclave the medium at 121 °C for 20 min.
   NOTE: The composition of the modifed Nitsch's medium is NH_4NO_3 (80 mg/L), KNO_3 (125 mg/L), $Ca(NO_3)_2$#4H_2O (500 mg/L), $MgSO_4$#7H_2O (125 mg/L), KH_2PO_4 (125 mg/L), $MnSO_4$#4H_2O (3 mg/L), $ZnSO_4$#7H_2O (0.5 mg/L), H_3BO_3 (10 mg/L), $CuSO_4$#5H_2O (0.025 mg/L), Na_2MoO_4#2H_2O (0.025 mg/L), sucrose (50,000 mg/L), and casein (500 mg/L). The prepared medium can be stored at 4 °C for 4 months at least.
2. Prepare 26% (w/v) polyethylene glycol using autoclaved deionized water and filter the solution with a 0.3 µm pore filter.
   NOTE: The prepared medium can be stored at 4 °C for 1 month.
3. Mix the reagents prepared in step 3.1 and 3.2 in a 1:1 (v/v) ratio.
   NOTE: This medium should be prepared fresh for each use.

4. Preparation of *In Vitro* Cell Culture Medium for Root Hairs (*Arabidopsis thaliana*)

1. Prepare a solution of 0.215% (w/v) Murashige & Skoog Medium, 0.05% (w/v) MES, 1% (w/v) sucrose, and 1% (w/v) agar in deionized water. Autoclave the solution at 121 °C for 20 min.

5. Preparation of *In Vitro* Cell Culture Medium for Moss Protonemata (*Physcomitrella patens*)

1. Pre-incubate the moss protonemata in BCDAT medium[16] in a Petri dish.
   NOTE: The composition of BCDAT medium is 1 mM $MgSO_4$, 10 mM KNO_3, 45 µM $FeSO_4$, 1.8 mM KH_2PO_4 (pH adjusted to 6.5 with KOH), trace element solution (0.22 µM $CuSO_4$, 0.19 µM $ZnSO_4$, 10 µM H_3BO_3, 0.1 µM Na_2MoO_4, 2 µM $MnCl_2$, 0.23 µM $CoCl_2$, and 0.17 µM KI), 1 mM $CaCl_2$, and 5 mM diammonium(+)-tartrate.
2. Culture the moss in the BCDATG medium in the microdevice.
   NOTE: The BCDATG medium is BCDAT medium with 0.5 % (w/v) glucose. The prepared medium can be stored at 4 °C for 1 month.

6. *In Vitro* Culturing of *T. fournieri* Pollen Tubes in the Microdevice

1. Place the pollen tube microdevice in a vacuum chamber and degas for 20 min.

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2. Remove the microdevice from the vacuum chamber and introduce the growth medium to the pistil inlet using a micropipette with a very fine tip. Fill the remaining wells to their tops with the same medium. Wait for a few minutes until all the microchannels are filled with the medium by the vacuum created inside of the microchannels.
3. Place some wet paper towel in the glass bottom dish to maintain the humidity in the dish.
4. Transfer the pollen grains from a wild-type *T. fournieri* 'blue and white' flower to its stigma using a dissection needle.
   NOTE: We also conducted this experiment with a transgenic *T. fournieri* 'Crown violet' flower (the *RPS5Ap::H2B-tdTomato* line[17]), with pollen tubes containing fluorescently labeled sperm cells and vegetative nuclei.
5. Cut the pollinated style (1 cm long) using a blade.
6. Insert the cut style into the inlet of the device as shown in **Figure 2**.
7. Put a lid on the dish and seal it with tape.
8. Place the device in an incubator at 28 °C for 5 - 6 h in darkness.

7. *In Vitro* Culturing of *A. thaliana* Root Hairs in the Microdevice

NOTE: Steps 7.1 - 7.9 (except for 7.3 and 7.5) should be performed in a laminar flow hood.

1. Sterilize seeds from *A. thaliana* Columbia (Col-0) and the transgenic line *UBQ10pro::H2B-mClover* (containing the fluorescent nuclear marker) by soaking them in sterile liquid (5% (v/v) household bleach and 0.02 % (v/v) Triton X-100) for 5 min.
2. Thoroughly rinse the sterilized seeds with autoclaved water.
3. Store the rinsed seeds in water for 2 days at 4 °C in darkness.
4. Sterilize the microdevice under UV light overnight.
5. Place the microdevice in a vacuum chamber and degas for 20 min.
6. Introduce the growth medium to the wells in the device using a micropipette. Wait a few minutes until the vacuum fills all the microchannels with the medium.
7. Place autoclaved wet paper towel in the glass bottom dish to maintain the humidity in the dish.
8. Transfer a sterilized seed into the inlet of the device.
9. Put a lid on the dish and seal it with tape.
10. Place the device vertically in an incubator at 22 °C under continuous white light.
11. Check root hair growth after 4 - 10 days of incubation. Obtain both bright field and fluorescent images using an inverted microscope.

8. *In Vitro* Culturing of *P. patens* (moss) Protonemata in the Microfluidic Device

NOTE: Steps 8.2 - 8.6 (except for 8.3) should be performed in a laminar flow hood.

1. Preculture *P. patens* strain Gransden 2004[18] on BCDAT medium covered with cellophane in a Petri dish for one week under continuous white light at 25 °C.
2. Sterilize the microdevice under UV light overnight in a laminar flow hood.
3. Place the microdevice in a vacuum chamber and degas for 20 min.
4. Introduce the growth medium to the wells using a micropipette. Wait a few minutes until the vacuum fills all the microchannels with the medium.
5. Add autoclaved water into the dish surrounding the microdevice to maintain the humidity in the dish.
6. Transfer a small piece of moss protonemata tissue to the inlet of the microdevice and culture the tissue in an incubator at 25 °C under continuous white light.
7. Check the moss protonemata growth after 2 - 3 weeks of incubation. Obtain bright field images using a microscope.

9. Time-lapse Imaging of *T. fournieri* Pollen Tube Growth

1. Place the microdevice on an inverted fluorescence microscope equipped with image acquisition hardware (*e.g.*, a CCD camera) and software. Find the microgap locations.
   NOTE: For the image acquisition software, we used a commercially available product (**Table of Materials**). Open source microscopy software such as μManager is also available online (https://micro-manager.org/wiki/Micro-Manager). We recommend installing a microscope condenser on the microscope to obtain higher resolution images.
2. Capture bright field images every 10 s using microscope image acquisition software.
3. To observe the fluorescently labeled sperm cells and vegetative nucleus in the pollen tube of the *RPS5Ap::H2B-tdTomato* line, irradiate the specimen with 561 nm laser and use a bandpass optical filter (578/105 nm).
   NOTE: Although the time-lapse images of pollen tube growth were captured frequently, imaging did not appear to affect growth. However, it is always recommended to minimize the laser intensity, exposure time, and time-lapse interval, to minimize the phototoxicity and photobleaching of the fluorophore.
4. Adjust the brightness and contrast of the images and prepare a video file using ImageJ software (https://imagej.nih.gov/ij/).

Representative Results

As illustrated in **Figure 1**, tip-growing plant cells encounter a series of physical barriers along their growth paths *in vivo*. The microfluidic *in vitro* cell culture platforms presented in this study enabled the examination the of tip-growing process in three types of plant cells (pollen tubes, root hairs, and moss protonemata) through 1 μm artificial gaps (**Figure 3**, **Figure 4**, **Figure 5**). For the pollen tube study, live-cell imaging was used to monitor morphological changes in the apical region of pollen tubes as well as the vegetative nucleus and sperm cells in response to encountering an extremely small space (**Supplemental Movie 1 and 2**).

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Although most of the microgaps were successfully fabricated by employing the method described here, we noticed that a few of them were completely closed (**Figure 6**). Because the 1 µm wide channels created on the silicon mold are fragile, repeated use of this mold might damage the gaps, leading to gap blocking on the PDMS layer. Therefore, before performing the experiment, it is important to check that the PDMS microgaps are intact.

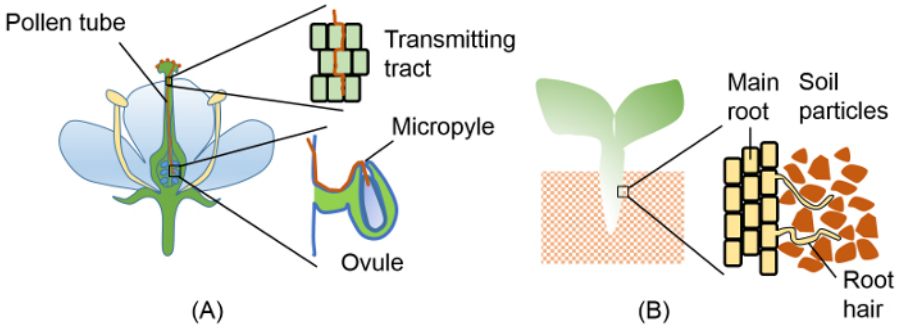
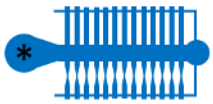
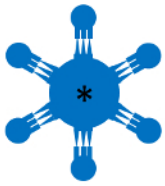
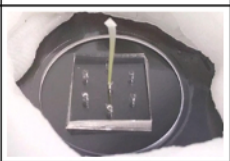
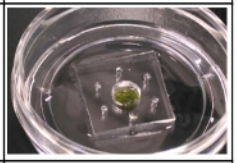
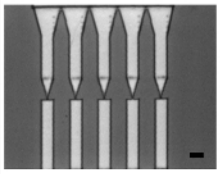
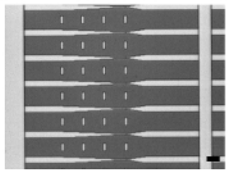
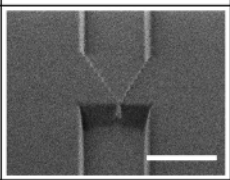
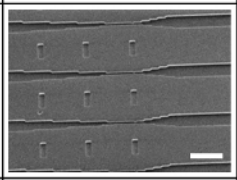


Figure 1: Penetration of tip-growing plant cells into physically constrained spaces. (A) Pollen tube elongating through several physical barriers on its way to an ovule. Barriers include transmitting tract tissue in the style and the integuments surrounding the micropyle. **(B)** Root hairs penetrating through dense soil. Insets in **(A)** and **(B)** show enlargements of the boxed regions. Please click here to view a larger version of this figure.

	T. fournieri pollen tubes	*A. thaliana* root hairs	*P. patens* protonemata
Schematic drawings of microchannels			
Photographs of microdevice			
Microscopy images of microgap area			Same as the microdevice used for the pollen tube study
SEM images of microgaps			Same as the microdevice used for the pollen tube study
Typical cell culturing periods in microdevice	6 - 12 hours	4 - 10 days	14 - 21 days

Scale bar, 20 µm

Figure 2: Characteristics of the microdevices used to study *T. fournieri* pollen tubes, *A. thaliana* root hairs, and *P. patens* protonemata. In the schematic drawings of microchannels, a sample is placed at the location indicated by an asterisk mark (*). Microchannel designs, photographs of each device, channel structures, and cell culturing periods are summarized. This figure has been modified with permission from Yanagisawa *et al*. 2017[19]. Scale bar, 20 µm. Please click here to view a larger version of this figure.

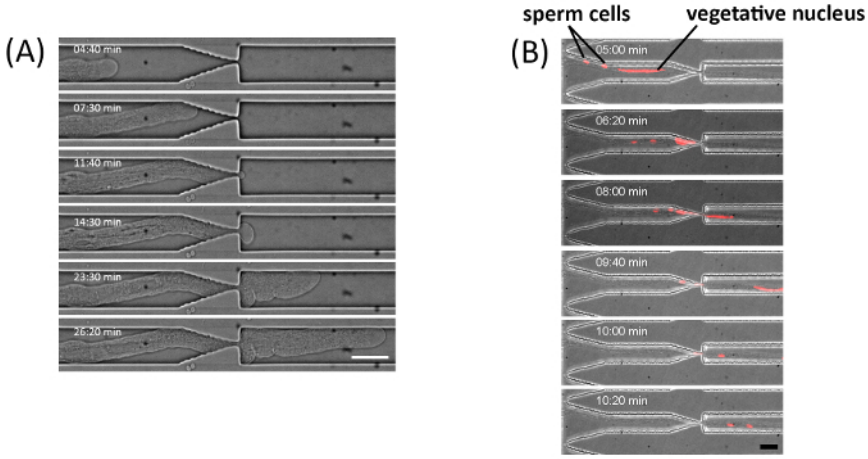


Figure 3: *T. fournieri* pollen tube elongation through a 1 µm gap. (A) Pollen tube passing through a 1 µm wide and 4 µm high gap. The time-lapse bright field images were captured using an inverted microscope equipped with a spinning-disk confocal system. Scale bar, 20 µm. **(B)** Time lapse images of a fluorescently labeled vegetative nucleus and sperm cells in a pollen tube of the *RPS5Ap::H2B-tdTomato* line crossing the microgap. Scale bar, 20 µm. Figure **(A)** and **(B)** have been modified with permission from Yanagisawa *et al*. 2017[19]. Please click here to view a larger version of this figure.

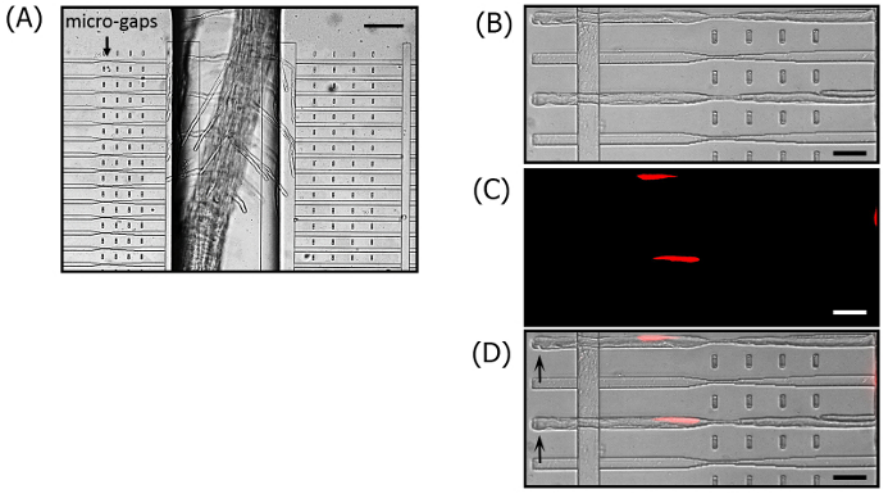


Figure 4. *A. thaliana* root hair elongation through a 1 µm gap. (A) Root and root hair growth in the microdevice. The microgaps (1 µm in width and 4 µm in height) are located on the left side of the root growth channel. The microchannels on the right side do not contain microgaps and can be used as a control. Scale bar, 100 µm. **(B)** Bright field, **(C)** fluorescence, and **(D)** merged images of root hairs passing through the microgaps. The nuclei in root hairs are fluorescently labeled in the *UBQ10pro::H2B-mClover* line. The tip of each root hair is indicated by an arrow. Scale bar, 30 µm. These figures have been modified with permission from Yanagisawa *et al*. 2017[19]. Please click here to view a larger version of this figure.

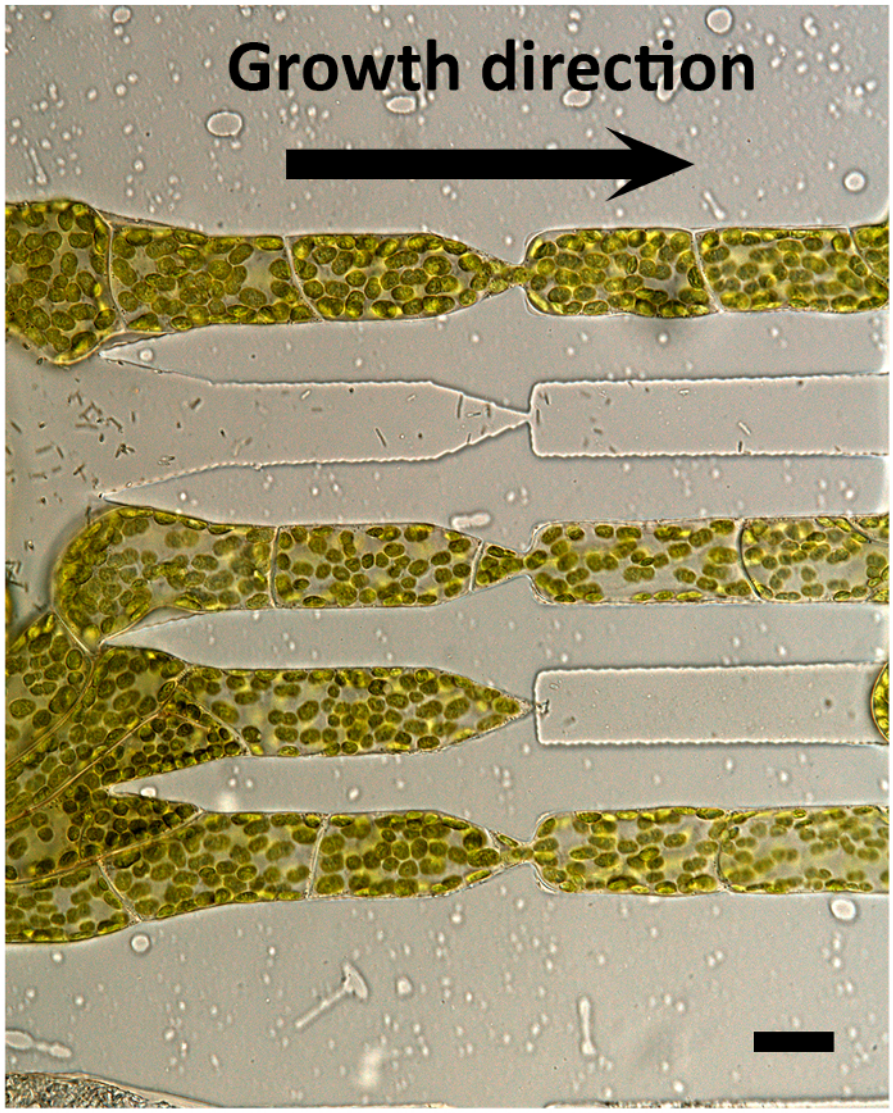


Figure 5. *P. patens* protonemata elongation through a 1 µm gap. Moss protonemata cells crossing through a 1 µm gap (4 µm in height), which was widened due to turgor pressure of the protonemata. Scale bar, 20 µm. The figure has been modified with permission from Yanagisawa *et al*. 2017[19]. Please click here to view a larger version of this figure.

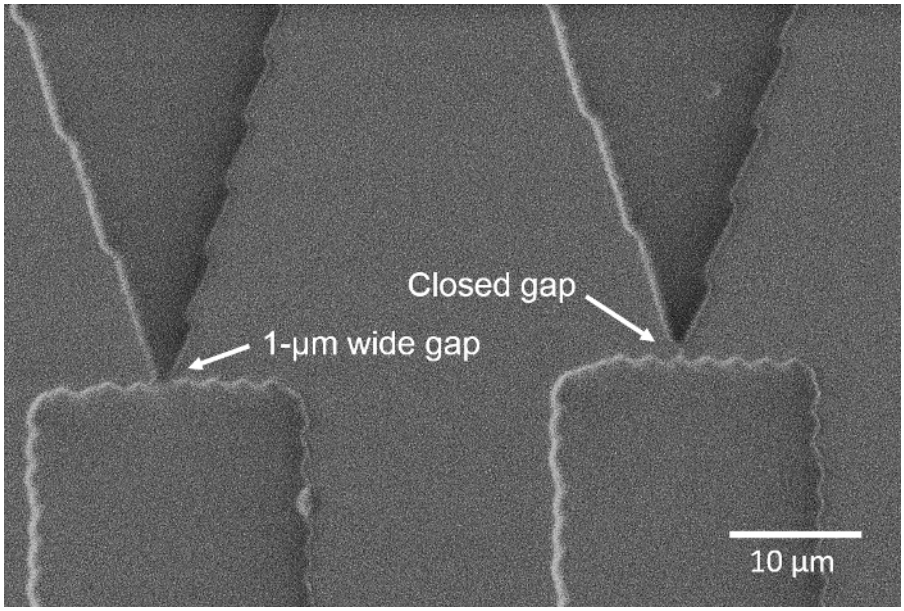


Figure 6. Closed microgaps. SEM image of the 1 μm gap region. The microgap on the right is completely closed. Please click here to view a larger version of this figure.

Supplementary Movie 1. Pollen tube growth through a 1 μm gap.Time-lapse bright field images were captured every 10 s using an inverted microscope equipped with a spinning disk confocal microscope. Scale bar, 20 μm. Please click here to download this file.

Supplementary Movie 2. Penetration of the vegetative nucleus and sperm cells through a 1 μm gap. Time-lapse bright field and fluorescence images were captured every 20 s using an inverted fluorescence microscope. Scale bar, 20 μm. Please click here to download this file.

Discussion

Several critical steps in the protocol need to be followed precisely to obtain the results presented above. First, the PDMS layer and glass bottom dish surfaces must both be treated with plasma for a sufficient amount of time before bonding. Otherwise, the PDMS layer may locally detach from the glass surface while tip-growing cells are crossing the microgaps. Another crucial step in the root hair and moss protonemata protocol is the sterilization of the microdevice. Normally, root hairs and moss protonemata cells need to be cultured in the microdevice for a couple of weeks. Without sterilizing the device, microorganisms may flourish, which could affect the growth of these tip-growing cells. Sterilization of the pollen tube device is not as critical, since the experiment is concluded within half a day. We have regularly used the pollen tube device without sterilization and did not observe any undesirable effects during the experiment.

The microchannel design should be modified according to the cells of interest. In our work, the channel design used for the *A. thaliana* root hair experiment was different from the one used for the *T. fournieri* pollen tube and the *P. patens* protonemata experiments. Different devices are required because the samples have different dimensions; for instance, root hairs are much shorter than pollen tubes and moss protonemata. Therefore, our root hair device was designed such that the microgap regions were adjacent to the main root channel. In addition to the channel design, the procedure for preparing the root hair device also varies from the procedures used for pollen tubes or moss protonemata. For the pollen tube and moss protonemata studies, we used a microdevice containing a single PDMS layer with the microchannels sealed against a glass bottom dish. However, the device used to study root hairs comprises two PDMS layers: the top layer with a microchannel (depth: 200 μm) for the main root is sealed against the bottom layer, containing microchannels (depth: 4 μm) for root hairs. The standard photolithography technique that was employed to fabricate the silicon mold is limited to an aspect ratio of around 5:1. Therefore, to create a 1 μm wide gap, the channel height will be limited to approximately 5 μm. It is also technically challenging to precisely align a deep channel (*e.g.*, 200 μm) near the 1 μm wide gap on a silicon mold. Therefore, we prepared separate PDMS layers for the main root and the root hairs, and constructed the device by sealing them together.

While this protocol was successfully used to visualize the tip growing process of pollen tubes through the microgaps (**Supplementary Movie 1 and 2**), time-lapse imaging of root hair and moss protonemata elongation at the microgap region would be more challenging. In the microdevice used for culturing *A. thaliana* root hairs, the height of the side channels where the microgaps are located (4 μm) is much shallower than that of the root growth chamber (200 μm), so most root hairs are unable to proceed into the side channels. Even if the root hairs do find the side channel entrance, it is hard to predict when they will enter it. To capture time-lapse images of root hair growth at the microgap, we recommend using a microscope equipped with an automated stage that can be programmed to be situated at multiple positions for image acquisition. When such instrumentation is unavailable, we suggest searching for the root hairs that have already entered the side channels but have not yet crossed the microgaps. Since the growth rate of *A. thaliana* root hairs is much slower (typically 1 - 2.5 μm/min[20]) than that of *T. fournieri* pollen tubes (typically 22 - 23 μm/min[19]), it may be possible to capture time-lapse images of those root hairs that are about to cross the microgaps. On the other hand, moss protonemata observations require a time-lapse system that can be exclusively used for this study, because a prolonged observation period is required due to the slow growth rate.

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The microfluidic approach presented here is the first method that allows us to study the capability of tip-growing plant cells to elongate through extremely narrow spaces. These microdevices may be useful for screening assays that investigate the functions of genes associated with cell wall biosynthesis and the integrity of tip-growing cells through their penetration capability. In addition, Denais *et al.*[21] recently demonstrated a nuclear envelope rupture and repair mechanism by squeezing a cancer cell through a confined space prepared in a microfluidic device, and it may be possible to study whether a similar mechanism exists in pollen tubes using our device. This newly developed experimental platform can be used to investigate how individual cells respond to physically constrained spaces and may thus enhance our understanding of tip-growth mechanisms.

Disclosures

The authors declare that they have no competing financial interests.

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