Chapter 20

Lab-on-a-Chip for Studying Growing Pollen Tubes

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Abstract

A major limitation in the study of pollen tube growth has been the difficulty in providing an in vitro testing microenvironment that physically resembles the in vivo conditions. Here we describe the development of a lab-on-a-chip (LOC) for the manipulation and experimental testing of individual pollen tubes. The design was specifically tailored to pollen tubes from *Camellia japonica*, but it can be easily adapted for any other species. The platform is fabricated from polydimethylsiloxane (PDMS) using a silicon/SU-8 mold and makes use of microfluidics to distribute pollen grains to serially arranged microchannels. The tubes are guided into these channels where they can be tested individually. The microfluidic platform allows for specific testing of a variety of growth behavioral features as demonstrated with a simple mechanical obstacle test, and it permits the straightforward integration of further single-cell test assays.

Key words Pollen tube, *Camellia japonica*, Cell culture, Lab-on-a-chip, Microfluidics, Microstructures, MEMS, Soft lithography, Tip growth

1 Introduction

In order to reach its target, the ovule, the pollen tube needs to invade the pistillar tissues of the receptive flower and follow guidance cues emitted by the sporophytic tissues and the female gametophyte [1–3]. Studying the roles of chemical, proteic, and mechanical cues that direct pollen tube growth and the mechanism by which the tube turns has become an important aspect of pollen tube research [4–7]. Conventionally, experimentation on pollen tubes is performed on cells germinated in bulk samples and growing in essentially homogeneous and isotropic growth matrices, either a liquid medium or an agarose-stiffened substrate. This in vitro environment is in stark contrast with the in vivo growth conditions which present a microstructured environment consisting of the various cell types and tissues the pollen tube encounters on its path through the pistil [6]. To test the behavior of pollen tubes in structured microenvironments featuring complex geometrical challenges or simple or superimposed chemical gradients, we have

Viktor Žárský and Fatima Cvrčková (eds.), *Plant Cell Morphogenesis: Methods and Protocols*, Methods in Molecular Biology, vol. 1080, DOI 10.1007/978-1-62703-643-6_20,

developed an experimental platform based on microfluidics and microelectromechanical systems (MEMS) technology, the TipChip.

The TipChip is a lab-on-a-chip device with planar geometry that allows for high-resolution optical microscopy and fluorescence imaging. It consists of a microfluidic network with limited thickness in order to restrain any interactions between two cells or cell and microstructure to a two-dimensional space, to avoid the accumulation of pollen grains into stacks, and to ensure that all growth activity occurs in one focal plane. The design meets several criteria: (1) several cells can be treated and observed simultaneously; (2) positioning of pollen grains occurs through defined fluid flow; (3) the experimental environment is enclosed from all sides, thus preventing evaporation of the growth medium, while allowing continuous flow of medium to supply fresh nutrients and oxygen to pollen tubes; and (4) optical compatibility must allow monitoring of pollen tube growth in bright-field and fluorescence mode.

The fabrication of the design starts with the planning of its layout to ensure the proper, fluid-flow-mediated positioning of the pollen grains at the entrance of the microchannels and the incorporation of the experimental tests within the microchannel. The design pattern is drawn in a CAD software, reproduced on a photomask, and transferred to a silicon/SU-8 mold through photolithography. Next, the microfluidic network is fabricated from polydimethylsiloxane (PDMS) by creating replicas using the silicon/SU-8 mold [8, 9]. The choice of PDMS as material is motivated by its biocompatibility (nontoxicity), optical transparency, relative low cost, and ease of use. Conventional planar microfabrication techniques and soft lithography make redesign loops straightforward since fabrication is systematically performed. Furthermore, the fabrication procedure can be modified easily to include more sophisticated structures, layers, or features.

Using the TipChip in various implementations [10, 11], we obtained successful pollen germination and properly elongating tubes displaying growth morphology and behavior that are indistinguishable from conventional in vitro setups. Pollen tubes grow along the microchannels in the direction enforced by their shape attaining total lengths over 1 mm. Pollen germination and growth rate within the device are consistent with those observed under conventional in vitro conditions confirming that the spatial confinement and associated limitation of the volume of the surrounding growth medium does not interfere negatively with cellular behavior. The interaction of pollen tubes with the microchannel features can elucidate many aspects of pollen tube behavior as demonstrated here through a simple mechanical obstacle test. More importantly, the presented microdevice allows for the design and easy integration of different kinds of microsensors within the microfluidic network to measure various biological parameters at the level of a single pollen tube. This opens multiple new avenues for experimental assays that have not been possible to conduct in conventional bulk experiments.

2 Materials

1. Computer-aided design (CAD) drawing software (*see* **Note 1**).
2. Fluid-flow simulation software (*see* **Note 2**).
3. A class 1000 cleanroom facility (*see* **Note 3**).
4. Silicon wafers (WRS materials) (*see* **Note 4**).
5. Sulfuric acid (H_2SO_4), peroxide (H_2O_2), and a glass container.
6. Hydrofluoric acid (HF), HF antidote (calcium gluconate gel), sodium bicarbonate, and a Teflon container.
7. Negative photoresist SU-8 2035 (MicroChem).
8. SU-8 developer (MicroChem).
9. Isopropyl alcohol (IPA).
10. Deionized water.
11. Hot plate.
12. UV light exposure system.
13. Trichlorosilane.
14. Polydimethylsiloxane (PDMS) (Sylgard® 184 Silicone Elastomer Kit—base and curing agent).
15. Vacuum desiccator.
16. Cutter, revolving punch, syringes.
17. Plasma cleaner.
18. PVC tubes (peristaltic pump tubing).
19. *Camellia japonica* pollen.
20. Growth medium: 1.62 mM H_3BO_3, 2.54 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.81 mM $MgSO_4{\cdot}7H_2O$, 1 mM KNO_3, 8 % sucrose (w/v), in distilled water.
21. Microscope with image capture.

3 Methods

Carry out all procedures in the cleanroom at room temperature (unless otherwise indicated). Meticulously follow all waste disposal regulations.

3.1 Microfluidic Network Design

1. Design the microfluidic network according to the intended application. Here we develop a microfluidic chip to investigate the instantaneous growth rate of pollen tubes as they encounter a mechanical obstacle consisting of a flat surface oriented at a defined angle relative to the growth direction: 0° (perpendicular to the growth direction), 30°, and 60° (Fig. 1; *see* **Note 5**).
2. Carry out microfluidic simulations to support and validate the platform design. Depending on the simulation result, the

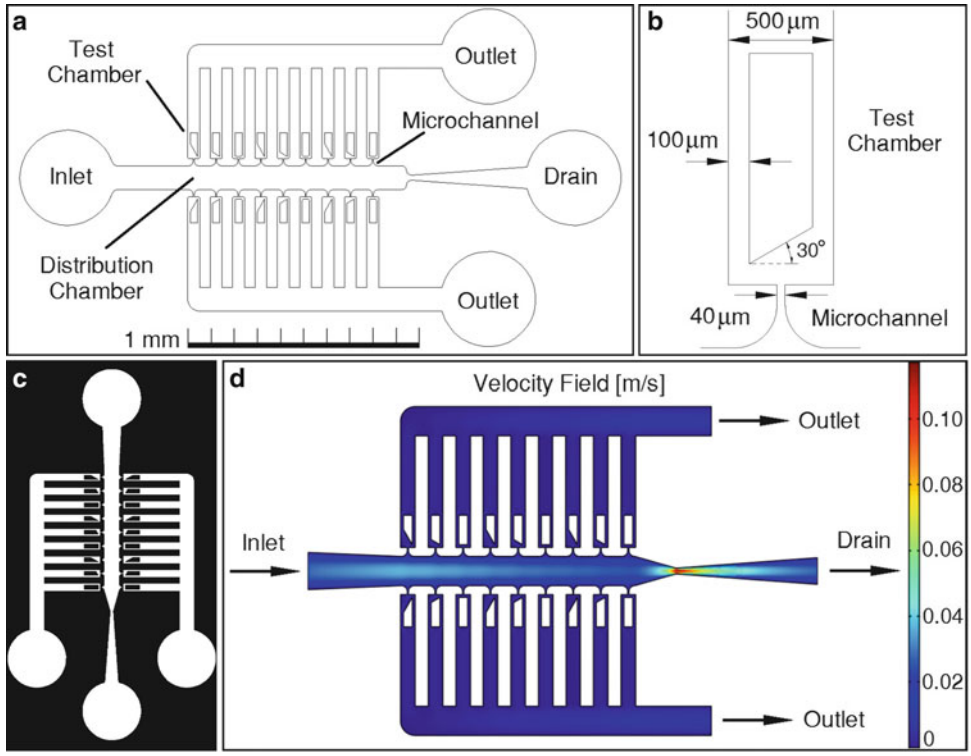

Fig. 1 Overall design of the LOC. (**a**) Schematic. (**b**) Detailed layout of a microchannel and test chamber. (**c**) Photomask. (**d**) Velocity fluid field simulation of the microfluidic platform

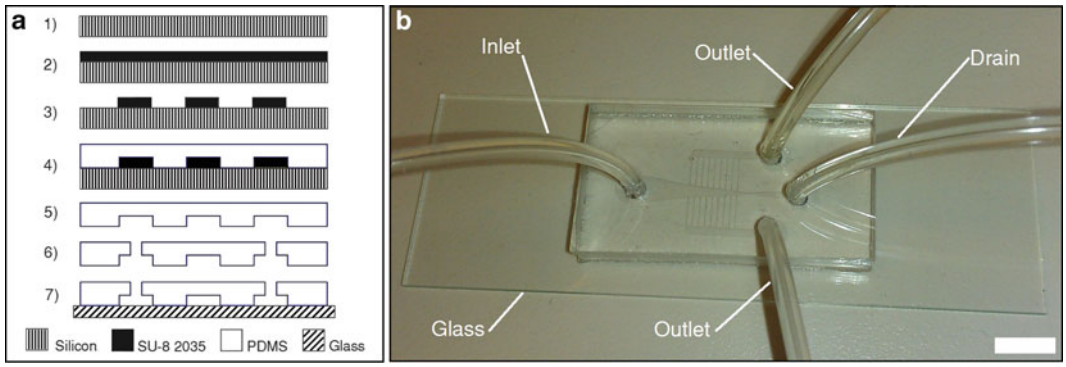

Fig. 2 (**a**) Microfluidic platform fabrication: (*1*) Silicon wafer cleaning, (*2*) SU-8 2035 photoresist spin-coating, (*3*) Photolithographic patterning and photoresist development, (*4*) PDMS pouring and curing, (*5*) PDMS layer detachment, (*6*) Microfluidic access drilling, (*7*) PDMS-glass bonding. (**b**) Fabricated LOC. Scale bar = 1 mm. Reproduced from [11] with permission from IOP Publishing Limited

overall design of the microfluidic network might need to be revisited. Figure 2 shows the microfluidics simulation for the current platform which ensures a uniform distribution of pollen grains over the microchannel entrances (*see* **Note 6**).

3. Draw the microfluidic design in Fig. 1 in the CAD software (*see* **Note 7**).
4. Reproduce the design on a photomask (*see* **Note 8**).

3.2 Silicon/SU-8 Mold

1. The fabrication process of the silicon/SU-8 mold is illustrated in Fig. 2a. Start by cleaning a silicon wafer with a piranha bath. The solution is dangerously aggressive and corrosive; use protective gear. Carefully mix three parts of sulfuric acid (H_2SO_4) and one part of peroxide (H_2O_2) in a glass container. A total volume of 200 ml is enough for cleaning one or two wafers. Use tweezers to place the silicon wafer slowly inside the solution. The exothermic reaction of the solution is good for cleaning only for about one hour. Afterwards, move the wafer to deionized water and air-dry with filtered, pressurized air or ideally with a N_2 gun (*see* **Note 9**).
2. Perform an HF cleaning. Hydrofluoric acid is a lethal solution; handle with extreme care. Use protective gear: butyl rubber gloves, face shield, safety glasses, leather closed shoes, lab coat, and chemical apron. Perform the cleaning inside a fume hood. Have a safety shower and HF antidote (calcium gluconate gel) nearby in case of skin contact. Mix 10 ml of HF with 200 ml of deionized water in a Teflon container. Place the silicon wafer slowly inside the solution with tweezers and leave for 3 min. Next move the wafer to deionized water and air-dry. Neutralize the HF with copious amounts of diluted sodium bicarbonate. HF should be disposed as a corrosive hazardous waste (*see* **Note 10**).
3. Spin-coat 4 ml of SU-8 on the 10 cm silicon wafer at 1,500 rpm for 30 s. Soft-bake for 5 min at 65 °C and then 10 min at 95 °C on a hot plate to harden the photoresist (by evaporating the photoresist solvent) and to increase adhesion to the substrate. Next, leave the wafer to cool down at room temperature (*see* **Note 11**).
4. Expose the negative photoresist SU-8 to UV light using the photomask (*see* **Note 12**).
5. Perform a postexposure bake (PEB) directly after exposure to enhance the chemical linking induced by the UV light. Bake for 5 min at 65 °C and then 10 min at 95 °C on a hot plate.
6. Develop the photoresist layer to obtain the final SU-8 pattern. Pour enough developer in a glass container to fully cover the silicon/SU-8 mold. Immerse the silicon/SU-8 mold in the SU-8 developer to dissolve the areas not exposed to UV light. Agitate gently. The development time depends directly on the thickness of the SU-8 layer. For an 80 μm thick SU-8 layer, the development time is about 8 min. Next, rinse with IPA and again with fresh developer. Air-dry. Monitor the state of

development by microscope and continue development if necessary (*see* **Note 13**).

7. Hard-bake at 150 °C for 10 min to solidify the remaining photoresist and reduce mechanical stresses in the structure (*see* **Note 14**).
8. Verify the mold under the microscope (*see* **Note 15**).
9. Silanize the silicon/SU-8 mold. The mold is exposed to trichlorosilane (or simply silane) vapors to prevent the PDMS replica from sticking to the mold. Use protective gear and handle with care. Silane is highly flammable (flash point of 87 °C), highly corrosive, and reacts violently with water. Using a plastic dropper (or syringe), place four drops of silane in a glass dish close to the silicon wafer. Close the glass dish and place it on a hot plate at 70 °C to evaporate the silane. Leave for at least 4 h. Cool at room temperature before opening the glass dish to allow the vapors to settle (*see* **Note 16**).

3.3 PDMS Microfluidic Chip

1. Thoroughly mix ten parts of PDMS polymer base with one part of PDMS curing agent in a disposable container (*see* **Note 17**).
2. Pour the PDMS mix onto the silicon/SU-8 mold. Place the mold/PDMS ensemble in a vacuum desiccator for 15 min to degas the PDMS and next cure in an oven at 80 °C for 2 h.
3. Carefully excise each PDMS replica from the mold (*see* **Note 18**).
4. Punch inlet and outlet ports of the PDMS replica. Rinse with IPA and air-dry to clean any dirt particle (*see* **Note 19**).
5. Bond the PDMS replica to a glass slide to seal the microfluidic chip (*see* **Note 20**).
6. Insert inlet and outlet PVC tubes from the top of the structure to obtain the microfluidic platform shown in Fig. 2b (*see* **Note 21**).

3.4 Microfluidic Platform Testing

1. Collect, dehydrate, and store *Camellia japonica* pollen on silica gel at –20 °C for later use (*see* **Note 22**).
2. Prior to experimentation, thaw and rehydrate a few milligrams of the pollen in humid atmosphere for 1 h (*see* **Note 23**).
3. Prepare liquid growth medium (*see* **Note 24**).
4. Place the microfluidic platform under the microscope (or any other imaging setup).
5. Immerse the pollen grains in 1 ml liquid growth medium. Agitate gently to mix the suspension; pollen grains are very sensitive to excess mechanical stress.
6. Using a syringe, carefully inject the pollen suspension through the PVC tube into the microfluidic device. Monitor the injection through the microscope. Figure 3a shows a typical pollen grain distribution.

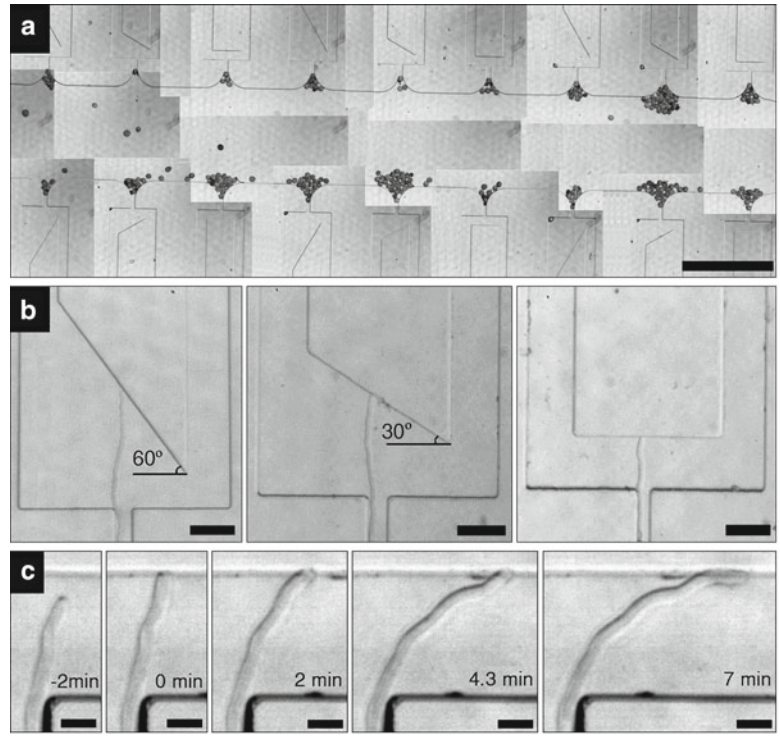

Fig. 3 Mechanical obstacle test. (**a**) Initial pollen grain distribution (image is stitched from several high-magnification micrographs. Scale bar = 1 mm. (**b**) Pollen tubes colliding with flat wall oriented at 60°, 30°, and 0° relative to the growth direction. Scale bars = 100 μm. (**c**) Time-lapse sequence of a type 0° collision. Scale bars = 25 μm. Reproduced from [11] with permission from IOP Publishing Limited

7. Leave the pollen tubes to grow. *Camellia* pollen tubes usually germinate after 30 min imbibition in growth medium, elongate at average growth rates of 12 μm/min, and easily attain more than 1 mm in total length after 2 h. Figure 3b, c shows pollen tubes encountering mechanical obstacles at defined angles [11].

4 Notes

1. The current design is drawn in AutoCAD, but any CAD software can be used as long as the output format is compatible with the photomask creation process.
2. The current design uses COMSOL multiphysics, a finite element analysis (FEA) solver of partial differential equations for various coupled phenomena.
3. Although a class 1000 cleanroom or better (maximum of 1,000 particles of size of 0.5 μm or larger in a cubic foot of air)

is ideal, a class 10000 cleanroom is sufficient to properly fabricate a microdevice with feature sizes in the order of a few micrometers. A less clean environment would often result in fabrication defects.

4. The current design uses 100 mm diameter standard type silicon wafers in particular. For the current application the dopant and orientation are not an issue. The size of the silicon wafer is highly dependent on the UV exposure system at hand. Silicon is preferred because of the good adhesion between silicon and the SU-8 photoresist, but the process can be performed on other substrates as well.
5. The current design of the microfluidic network is based on an inlet, a linear distribution chamber, two series of symmetrical microchannels and test chambers (top and bottom), two outlets, and a drain outlet at the end of the distribution chamber. Only straight-shaped microchannels are used in order to impose an initial direction of growth on the pollen tubes and to obtain a homogeneous fluid flow among the microchannels [11]. In the setup shown here, the sizes of structural features are chosen to fit the dimensions of *Camellia japonica* pollen grains and tubes. However, the design can be easily adapted to serve different applications. The test chamber, for example, can be modified to allow for the integration of microelectrodes. Examples for designs can be downloaded from the Optical-Bio Microsystems Laboratory website (http://users.encs.concordia.ca/~mpackir/index.html) and from the Geitmann Lab website (*see* Publications at http://www.geitmannlab.org).
6. In order to predict the fluid-flow behavior within the microfluidic network, and particularly the movement of pollen grains along the streamlines, a 2D Finite Element Method (FEM) fluid analysis implementing the incompressible Navier–Stokes and continuity equations was carried out using COMSOL. A velocity of 0.02 m/s was selected as the boundary condition at the inlet to reflect typical medium injection by syringe, and the outlets were set to atmospheric pressure. Since the liquid medium used here consists mostly of water [7], the density (ρ) and the dynamic viscosity (μ) are set to 10^3 kg/m^3 and 10^{-3} Pa s, respectively.
7. Be careful to use simple curves. Lines and arcs alone can be used to draw most designs. Keep the amount of vertices to a minimum and do not overlap any. Make sure the design is composed of closed curves; by definition, no single curve should be open. Once the skeleton of the drawing is done, shade every region as necessary. In the current design we use a negative photoresist (SU-8) to fabricate the mold; hence, those regions that are not to be permanent in the mold must be dark (*see* Fig. 1c).

8. Low-resolution (low cost) photomasks can be easily obtained by using high-resolution digital printing on a transparent film at 3,600 dpi. However, this method results in rough walls for feature sizes in the range of 10–50 μm and completely misses features below 10 μm. For smoother, well-defined features down to 1 μm, a more precise (and expensive) alternative is Direct Writing Laser Lithography on a glass/chrome mask.
9. The piranha bath is advised but optional depending on the cleanliness state of the silicon wafer and the cleanroom. However, we found that it is a must if the silicon wafer is being recycled.
10. The HF cleaning is used to eliminate any native oxide layer on the silicon wafer. We have noticed that this native oxide layer often prevents the SU-8 from adhering firmly to the wafer during the developing process. It is advised to keep the silicon wafers always inside a hermetic box in the cleanroom to avoid contact with the ambient air as much as possible. HF cleaning should be carried out if and only if adhesion problems between silicon and SU-8 arise since HF needs to be handled with extreme care due to its potentially lethal effects. Although not advised, the Teflon container can be replaced with a regular plastic container if necessary.
11. We found that it is best to dispense the SU-8 directly from the bottle to avoid the formation of air bubbles in the SU-8 layer. Be particularly careful at the end of the dispensing when pulling out the bottle since a narrow stream of photoresist might easily create bubbles in the already dispensed SU-8. Stop the dispensing with tissue if necessary. The spin speed is set to obtain a thickness of 80 μm since *Camellia* pollen grains vary from 50 to 60 μm in diameter. However, the thickness can be changed to accommodate different-sized specimens. A hot plate is preferred over a convective oven to ensure uniform temperature across the wafer during soft-bake. Thicker layers require longer soft-bake times (see manufacturer's SU-8 datasheet for a complete table of suggested soft-bake times). After proper soft-bake the SU-8 layer must be smooth; extend the duration of the soft-bake should wrinkles, bumps, or bubbles appear on the surface (an expired resist can also produce similar issues).
12. UV light exposure is critical in the mold fabrication. Make sure the SU-8 layer is completely flat and hardened before exposure. Any air bubble or dirt in the SU-8 layer will result in a loss of features in the area. Although exact exposure energies can be found in the SU-8 datasheet, an exposure matrix must be carried out in order to determine the optimal exposure parameters for the current setup of UV exposure system, substrate, and SU-8 thickness. Enough exposure should produce a latent image on the SU-8 layer within the first minute of postexposure bake. It is useful to realize that since SU-8 is a

negative photoresist, the areas exposed to UV light are meant to remain on the silicon wafer, whereas the areas covered by the photomask will be dissolved during development. Therefore, it is generally preferred to overexpose (ensure the exposed SU-8 will remain at the expense of enlarging the features due to light scattering) than to underexpose (lose parts of the design).

13. If there are adhesion problems between the silicon wafer and the SU-8 layer, the photoresist will peel off from the wafer during development. Should this detachment occur, an HF cleaning may be required.
14. Since the photoresist is a thermal resin, the hard-bake temperature should be carried out at a temperature slightly higher than the expected microdevice operating temperature in order to ensure the mechanical properties of the photoresist. The visual effect of the hard-bake is to "smooth" the SU-8 layer.
15. Direct visual inspection of the mold is required to determine what step of the process went wrong or can be improved. Verify the thickness of the mold to adjust the SU-8 spin-coating speed accordingly. Verify the dimensions of the mold to adjust the exposure time and possibly the PEB temperature. Pay particular attention to the smoothness of the SU-8 walls and any lost feature since it may indicate a photomask with a resolution that is too low. Any undeveloped SU-8 can be removed by extending the development time. Any cracks in the mold bulk can be reduced by increasing the hard-bake temperature or duration.
16. We found that a minimum of 65 °C is needed for proper silanization. The level of silanization can be varied by dispensing more or less drops or by using shorter or longer times. Adjust if necessary. We found that glass can also be silanized in the same way.
17. The total amount of poured PDMS depends on the size of the container of the mold. In order to not waste PDMS, place the silicon/SU-8 mold in a glass container of approximately the same size. Aluminum foil can also be used as a container with folded "walls." The PDMS on top of the mold will determine the thickness of the PDMS layer. This thickness of the PDMS layer is not critical, usually 2–3 mm, as long as the inlet and outlet drilling can be done properly. However, excessive thickness may compromise the optical properties when using high-resolution microscopy. Since the PDMS volume for a 100 mm diameter mold is in the range of a few tens of milliliters (usually between 40 and 60 ml), it is recommended to use syringes to measure the PDMS base and curing agent quantities. Use separate syringes for base and curing agent to avoid cross-contamination.

18. Handle with care. Use a clean, sharp cutter to dice the cured PDMS replicas before extracting them. The cutter should touch the silicon wafer as the dicing takes place. If silanization was properly performed, the PDMS clearly detaches from the mold as the PDMS is being cut. Removing the replicas from the mold should be done effortlessly. If PDMS is stuck to the mold, the most likely reason is a problem with the silanization. Unfortunately, the mold is almost inevitably lost if the PDMS is stuck since it is next to impossible to remove the PDMS without destroying the SU-8 layer. However, should this happen, a complete wafer cleanup can be performed (piranha bath and HF cleaning) and the silicon wafer can be recovered to restart mold fabrication.
19. Punching of inlets and outlets can be easily done with a revolving punch. The size of the round holes must match the PVC tubes used.
20. Oxygen plasma bonding is recommended for fast and reliable results. A matrix test must be carried out to determine the optimal parameters for bonding glass and PDMS. In our setup (Harrick Plasma PDC-001), the bonding time for a glass/PDMS interface is 40 s with a high voltage application. Another (low-cost) alternative is to spin-coat a thin layer of PDMS on the glass slide (2,000 rpm for 30 s), semi-cure the PDMS layer, make the bond, and then completely cure. The duration of the semi-curing depends on many factors; however, a good starting point is 3 min at 90 °C. We found that it is preferable to leave it longer since if the bonding fails, another thin layer of PDMS can be added on top, whereas if less time is used then the features on the PDMS replica will be filled by the PDMS gel.
21. Since the fluid pressures inside the microfluidic platform are relatively low, the PVC tubes do not need to be glued to the PDMS replica. Friction is sufficient to keep the tubes in place (given the PDMS is at least 1 mm thick). Inlets and outlets on the side of the chip are discouraged since this requires more complex connections.
22. Although fresh *Camellia japonica* pollen is ideal, this may be difficult to obtain as this species flowers only once a year for a few weeks.
23. An easy way to hydrate pollen grains is to wet a piece of paper towel with hot water and place both pollen and tissue in an enclosed glass container (Petri dish). Very importantly, avoid any direct contact between pollen and water to prevent the pollen grains from absorbing liquid water at this point.
24. Liquid growth medium has already been optimized for *Camellia japonica* pollen [7, 12]. Usually, we prepare 10 ml of medium and use 1 ml plastic capsules for testing.

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