

Protocol for fabrication of microcompartments for long-term culture and imaging of small *C. elegans* larvae

Henrik Bringmann, March 2011.

Step-by-Step Protocol

Step1 : Preparing a humidity dish (see illustration on page 3)

A) Mill a square opening (18 x 18 mm) into the bottom of a 3.5cm Petri dish.

Note: Use a sharp tool and low speed as the plastic melts when becoming too hot.

B) Place a piece of double-sided sticky tape (20 x 20 mm) symmetrically onto the outside of the opening – thus closing it. Turn the dish 180° and place onto a hard surface.

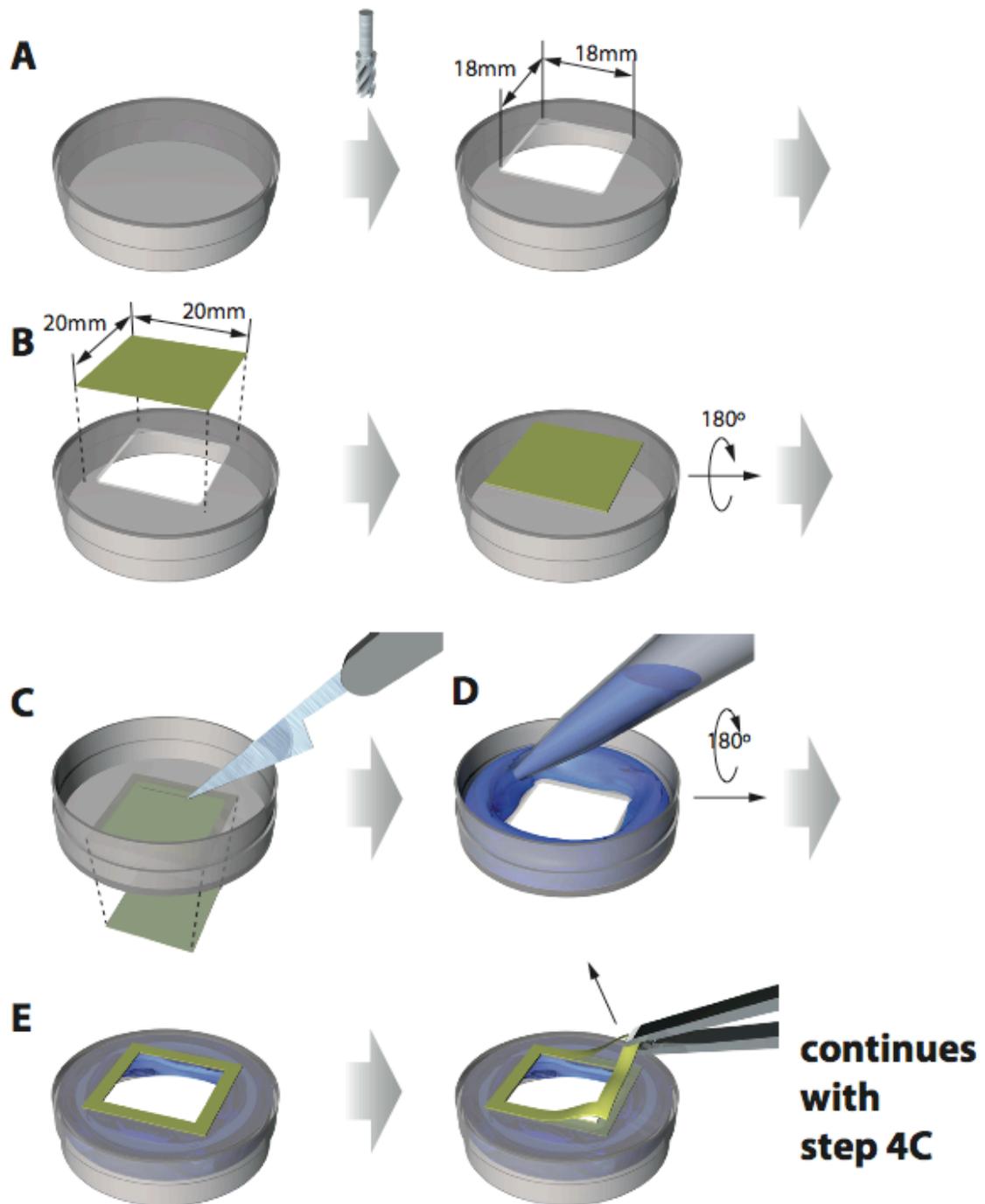
C) Using a scalpel, cut around the inner corners of the square opening and discard the square piece of double-sided sticky tape (18 x 18 mm).

D) Using a P1000 pipette, fill the bottom of the Petri dish with 2 ml of hot liquid agarose agarose (3% high melting point agarose in S-Basal).

Notes: Agarose liquid and hydrogel are colorless but were colored blue in the drawings for better visibility. It is comfortable to use a 2ml tube of agarose from an aliquoted stock. The agarose is melted by placing a 2ml tube into a heating block at 95-98°C 15 min before sample preparation. S Basal: 5.85g NaCl, 1g K₂HPO₄, 6g KH₂PO₄, 1ml cholesterol (5mg/ml in ethanol), H₂O to 1 litre. Sterilize by autoclaving. 3% Agarose in S-Basal: Solve 3g of agarose (Fisher Bioreagents, BP(E)164-1000) in 100 ml S-Basal. Use a hot plate set to 180°C and a magnetic stirrer to solve the agarose. Make 2ml aliquots of the agarose in 2ml Eppendorf tubes and store at room temperature.

E) Wait until the agarose has solidified. Then, turn the dish around and peel off the protective foil of the double-sided sticky tape.

Step 1



**Step 2: Cast an agarose hydrogel slab containing microcompartments
(see illustration on page 5 and 6)**

A) Expose PDMS and a standard microscope glass slide to an air plasma (Harrick plasma cleaner at “medium” settings, 0.5mbar, 1 minute). Then, place the PDMS slab onto a glass slide with the flat surface not containing the microstructures facing the glass surface.

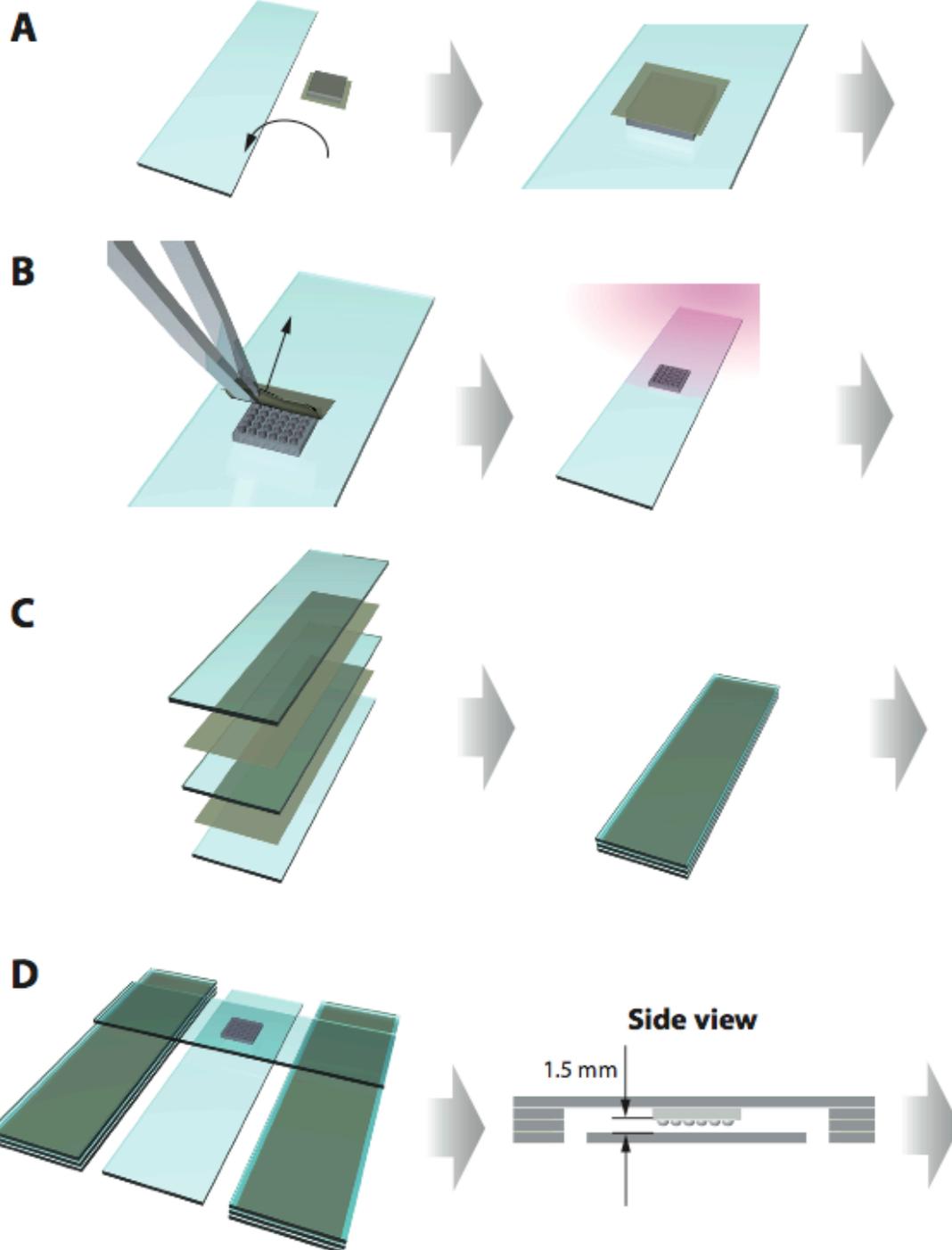
Notes: PDMS is clear and colorless. It is colored grey in the drawings for better visibility. The shape of the microstructures in these images is only symbolic. Structures are not drawn to scale. PDMS stamps can be made by standard procedures. We have our PDMS stamps made in the Stanford Microfluidics Foundry. PDMS slabs are shipped to the lab with a protective piece of sticky film covering the microstructured surface.

B) Remove the protective film and expose the PDMS stamp to air plasma for 60 seconds as above.

C) Build two spacers from glass slides held together by double-sided sticky tape.

D) Place the PDMS stamp orthogonally across the two spacers with the microstructured PDMS surface facing down. The stamp should be placed towards one side of the spacers. Place one additional glass slide underneath the stamp. There should be a gap between the microstructured PDMS surface and the glass slide. Adjust the size of the gap to approximately 1.5mm by changing the number of glass slides of the stacks used as spacers.

Step 2



E) Place a drop of 2ml liquid 3% high melting point agarose in S Basal onto the glass coverslip. Slide the PDMS stamp horizontally into the liquid agarose.

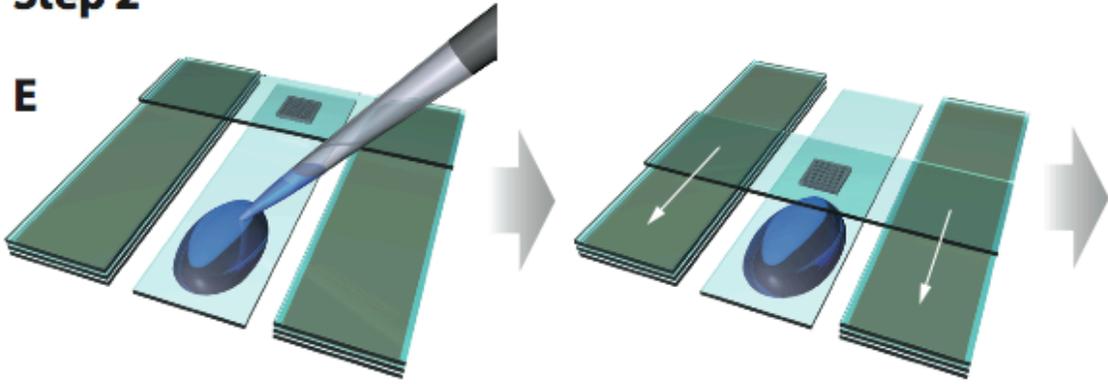
Note: Moving the stamp in vertically will cause air bubbles to get trapped.

F) Wait until agarose is solidified. Then, lift off the PDMS stamp vertically. Proceed immediately with filling worms and bacteria into the microcompartments.

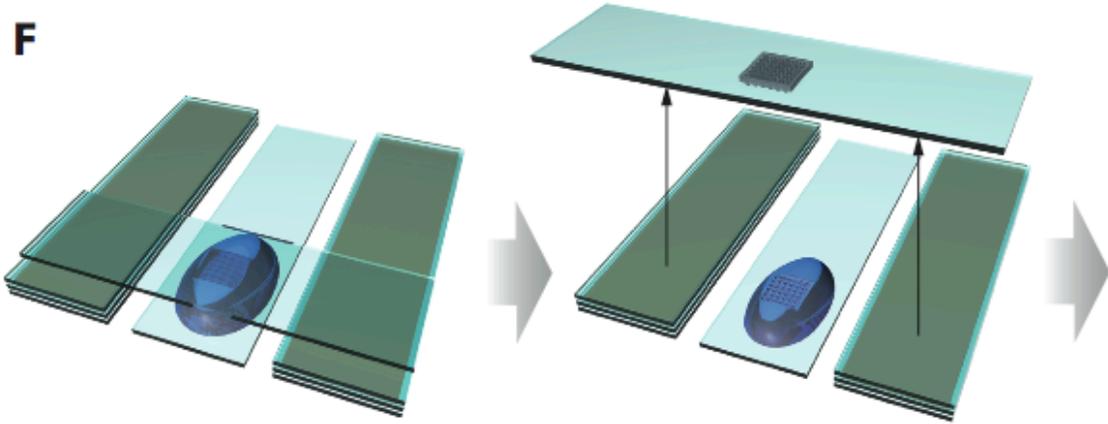
Note: Liquid agarose is clear, solid agarose hydrogel is slightly opaque. Microcompartment may be damaged if the stamp is not pulled off vertically.

Step 2

E



F



Step 3: Filling worms and bacteria into the microcompartments (see micrograph on page 9)

A) Transfer eggs at the pretzel stage onto a fresh NGM/OP50 plate using a platinum wire pick.

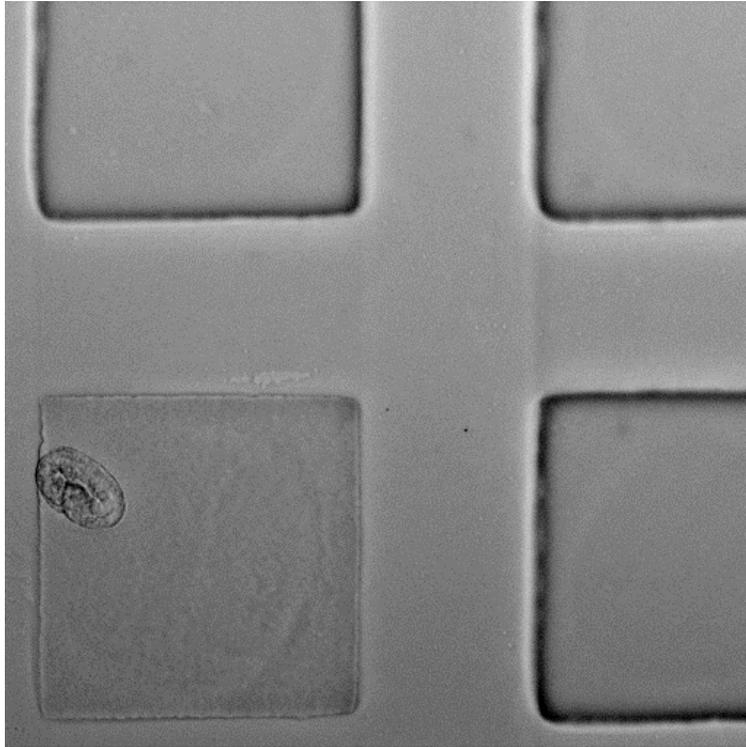
Notes: The eggs should have moving worms inside. Start with a mixed population of worms grown on NGM seeded with *E. coli* OP50. NGM plates with growing populations often contain debris such as shed cuticles that can decrease image quality, especially when using fluorescence microscopy. The additional “cleaning” step reduces such debris.

B) Place the agarose hydrogel microcompartment array and the NGM/OP50 plate under a stereomicroscope so that you can quickly move between the NGM plate and the microchambers into the field of view. Pick up bacterial food and one egg and transfer some of the bacteria and the egg into one microcompartment each.

Notes:

- If the NGM plate is rather large and it takes too much time to find the area containing the eggs you can draw two or three concentric “rings” around the egg area by scraping the bacteria with a platinum wire worm pick.
- It is important that the agarose hydrogel microcompartments are not too dry. Ideal is a microcompartment array if the surface appears dry but with liquid standing inside the microcompartments. The xy borders of the chamber appear dark in brightfield illumination if they are empty. If filled with liquid, the borders appear light. The liquid inside the compartments helps transfer the eggs and bacteria from the pick to the agarose surface.
- The agarose surface is fragile and can be damaged with the pick. Ideally the agarose surface is not touched with the actual platinum wire. Only the bacterial paste should contact the wet surface.
- If the agarose slab containing the microcompartments appears too dry or dries out during filling procedure and the bacteria and eggs do not transfer easily the agarose slab can be rehydrated with a small (2µl) drop of S-Basal placed onto the microcompartment surface. The drop should be placed into one of the corners of the microchamber array. Now you can “pull” some of the liquid over the dry part of the surface containing the microcompartment array and thus fill the microcompartments with liquid. Alternatively, you can use the drop to briefly dip the pick containing the bacteria-egg-paste into it. Now the bacteria egg paste is much more liquid and it is much more easy to transfer eggs and bacteria into the microcompartments in a controlled way.
- A proper worm pick is essential. Normal worm picks used to transfer adult worms are too big. The size of the “picking surface” should be in the range of the microcompartment. If the picking surface is too large, bacteria are placed not only inside the microcompartment but also around. It is useful to reduce the amount of bacteria outside the microwell, as the agarose surface around the microcompartment needs to form a seal with the glass coverslip.
- The amount of bacterial food inside the microcompartment is critical. If too little food is placed inside the compartment, the worms may run out of food before the developmental phase of interest is reached. Starved worms also change their behavior. If too much food is placed inside the microcompartment, it becomes very compact and animals can sometimes escape. Probably by pushing themselves off the solid bacteria.

The micrograph below shows a top view onto open agarose hydrogel microcompartments before sealing with a glass coverslip. The compartment bottom left was filled with OP50 *E. coli* and one wildtype *C. elegans* N2 egg at the pretzel stage. Note that only few bacteria are outside the compartment. Three empty compartments are partially visible. It takes about 20 to 30 seconds to fill one compartment with bacteria and one egg. We usually fill between four and 25 compartments. Below you can see a brightfield image of $190\mu\text{m} \times 190\mu\text{m} \times 10\mu\text{m}$ agarose hydrogel microcompartments viewed at 200 x magnification. The boundaries of empty compartments appear dark in brightfield illumination.



Step 4: Closing chambers with a glass coverslip and assembly of the humidity dish (see illustration on page 11)

A) Cut out the flat part of the agarose slab containing the microcompartments using a scalpel.

B) Use forceps to place the agarose slab containing the microcompartments onto a glass coverslip with the microcompartment surface towards the glass surface, thus closing the compartments.

Note: The best way to do this is to place the agarose hydrogel slab with one corner in contact with the glass surface and then slowly lower the agarose hydrogel slab so that no big air bubbles will be trapped underneath the agarose slab. A big air bubble can prevent the contact between the agarose and the glass surface. Small air bubbles (smaller than the size of the microcompartment) are regularly trapped inside the microcompartments. These are not a problem as the air gets absorbed by the agarose quickly.

C) Using forceps or your fingers place the coverslip containing the agarose slab onto the sticky tape so that the agarose is inside the dish. Use the side of the tip of a pair of forceps to gently press down the coverslip onto the tape.

Notes:

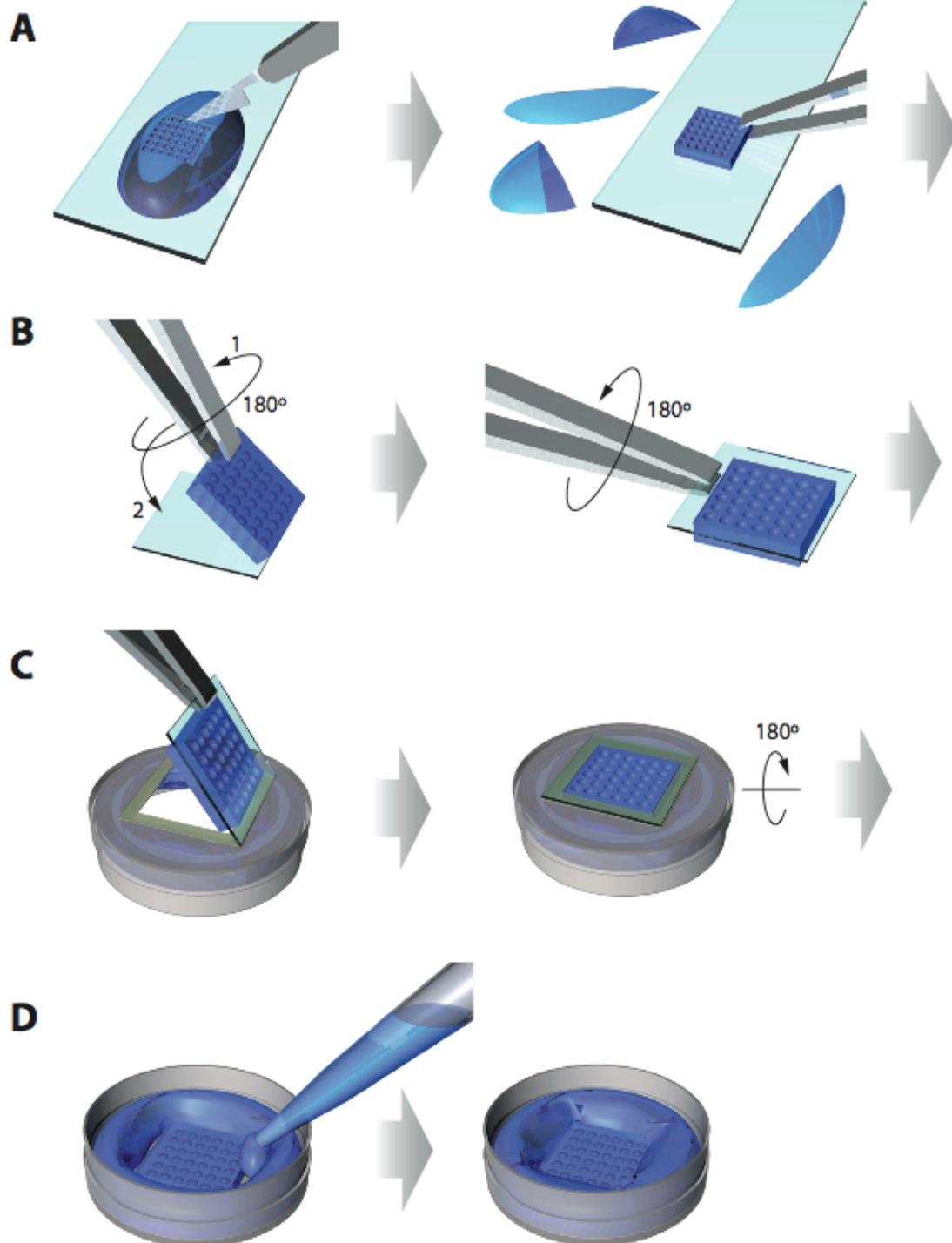
- A gap in the seal may cause drying out of the sample. Gaps can be seen because of reflections at the glass-air-interface. If in doubt, use a stereomicroscope.
- The coverslip glass must be flat and not distorted. This can be seen when looking at the reflection at the cover glass surface.
- Pressing down the coverslip too strongly onto the sticky tape can break the glass.

D) Turn around the dish and fill the cleft between the agarose slab containing the microcompartments and the dish agarose with 3% low melting point agarose in S Basal. After solidification of the low melting point agarose, the sample dish is closed with a lid heater (see below).

Note:

- The low melting point agarose can also be prepared in advance in 2ml tubes. After short heating to 95-98°C, the agarose melts and the liquid agarose is kept in a 37°C heat block until use.

Step 4



Step 4: Microcopy of the sample (no illustration)

Let the sample equilibrate for at least 2 h on the microscope before you start movie acquisition.

Notes:

-During the first 2 h, some small movements in the agarose may occur that may disturb imaging. Also, air bubbles are absorbed during this time.

- If this protocol is followed precisely, the moisture of the agarose should be in the appropriate range. However, different air humidity or temperature may require small adjustments to the sample wetness because the wetness of the hydrogel is critical for the technique. When establishing the technique, check the sample regularly during the equilibration phase. If the hydrogel is too moist, the seal between the hydrogel and the glass will loosen and worms and bacteria flow out of the compartment. To dry the hydrogel and reseal the microcompartments, open the lid for a few minutes and observe the reformation of the seal. If the sample is too dry the agarose chambers will collapse and compact the bacteria. This may lead to escape of the worms. Open the lid and place a small (5 μ l) drop of S Basal onto the agarose, which will rehydrate quickly. The seal is properly formed if the bacteria that are trapped between the glass and the agarose are not showing Brownian motion. The degree of food is right when the entire chamber appears to be filled with a homogenous suspension of bacteria, which move due to Brownian motion.

-Some of the components can be reused. To reuse the plastic dish, remove the agarose hydrogel from the dish. If oil immersion objectives were used, cut out the coverslip with a diamond pen. Let the dish soak in ethanol for a few days to solve the sticky tape. Remove coverslip and sticky tape. Let dry and reuse. We use dishes approximately 30 times. To reuse the PDMS stamp, wash with deionized water, let dry. Treat with air plasma and reuse. We use stamps approximately 30 times.

- Lid heater: We set the air conditioning of the room to 20°C and the lid temperature to 25.5°C. The temperature inside the agar slab then is 24.5°C. A lid heater is only necessary for inverted microscopes. The lid heater provides a temperature gradient inside the dish with a cool bottom and a hot lid. It prevents fogging of the glass window and drying out of the sample. For upright microscopes, there automatically is a temperature gradient. Because the agarose ‘hangs’ on the slightly warmer lid no condensation on the bottom is observed. Inverted microscopes currently offer the highest precision autofocus options.