AgarSqueezer

PROTOCOL

distributed in the US/Canada by:
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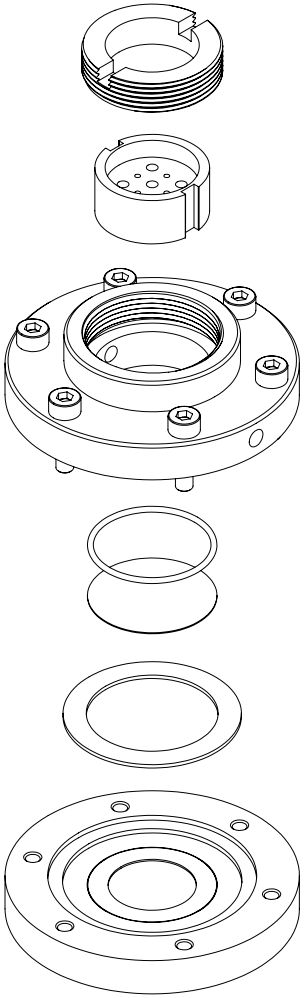


1. The material you need

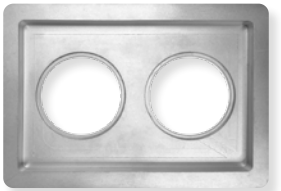
HARDWARE

- 1 AgarSqueezer

- 1 agar holder

- 1 AgarSqueezer white screwdriver (included in the kit)

- An AgarSqueezer holder, to adapt 1 or 2 AgarSqueezer devices on the microscope stage (included in the kit)



- 1 autoclavable screwdriver
- 2 heating plates (80°C and 100°C)
- A UV lamp: at least 12W, 254 nm or 365 nm. Alternatively, the UV lamp of the cell culture hood could be used if you have no other choice.
- A wafer to mold pillars in agarose

CONSUMABLES

- Round coverslips (22 or 30 mm)
- 16G and 20G flat-cut needles (included in the kit)
- Adhesive tape
- Cleanroom paper

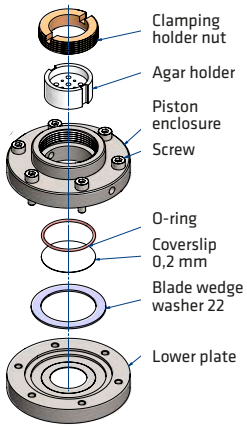
REAGENTS

- Agarose
- Cell culture medium
- Option: fluorescent beads BZ5400, Interchim Fluoprobes
- Option: a drug to be tested on the confined cells

2. Assemble the AgarSqueezer device

You receive the AgarSqueezer assembled. For your first use, you will need to disassemble it and add a coverslip on it. For all forthcoming uses, you will need to assemble it thoroughly.

This step requires 30 min in addition to the sterilization process.



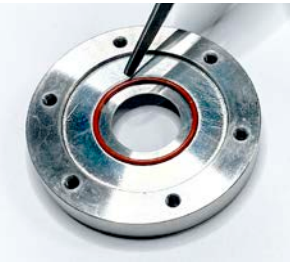
You can assemble the device in advance. It may be useful to stock it already assembled. If you assemble just before performing your experiment, be sure to let the system cool down after autoclaving and before seeding cells.

- First clean with ethanol 70 %, and distilled water the glass coverslips, the O-ring, the inox parts of AgarSqueezer, the tweezers, the screwdriver, the clamping holder nut, the AgarSqueezer white screwdriver and the agar holder. Dry them with a blow gun.
- Put the coverslip on the lower plate.

- Put the blade wedge washer on it.

If you are using a 30 mm coverslip, place the coverslip first and then the blade wedge washer on it. If you are using a 22 mm coverslip, place the blade wedge washer first and then the coverslip on it.

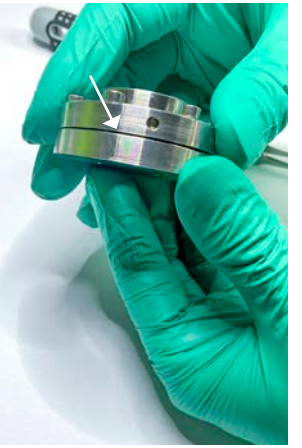
- Center the O-ring.



- Place the piston enclosure on top.

- Start to screw gradually in a cross pattern.

Do not screw completely. Leave a space of 1 mm between both parts to deal with thermal dilation during autoclaving.



- Autoclave 7 min at 134°C (EXH+dry after heating to release pressure quickly) the assembled AgarSqueezer, the tweezers, the screwdriver, the clamping holder nut, the AgarSqueezer white screwdriver.

Do not autoclave the agar holder.

You may either use sterilization bags, inox boxes or glass jam jar for autoclaving and then storage.

3. Prepare the agarose gel

This step requires half a day.

It has been observed that the experiment is more successful when the agarose gel is freshly prepared, no more than 1 day before the confinement experiment.

- At this step, it is highly important to be sure that the agarose gel

is well prepared to ensure a homogeneous confinement on cells.

- Dissolve standard agarose in distilled water (2% w/v) to get a 150 kPa rigidity.

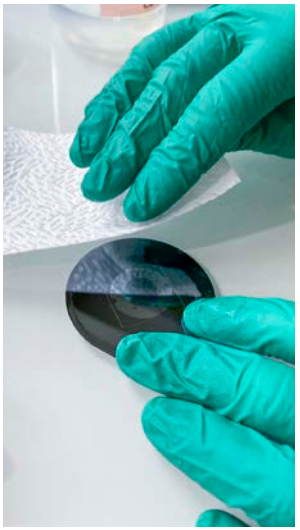
You can use different agarose and different ratios to reach lower rigidities.

- Autoclave 15 min at 121°C or use a microwave.

Avoid bubbles that lead to an inhomogeneous pressure on cells. If you use a microwave and observe bubbles, put the bottle on a heating plate, at 100°C, with a stirrer.

- Once the agarose gel is dissolved, put the bottle on a 100°C heating plate.
- Clean the wafer with distilled water and cleanroom paper.

Avoid paper that could leave lints, that would lead to deleterious roughness.



- Put P1000 tips, the piston, and the wafer on the 80°C heating plate.

You may cut the end of the P1000 tip to facilitate the manipulation of the agarose gel.

- Place some adhesive tape on one face of the agar holder to cover the holes in order to avoid the leak of liquid agarose.

The agarose gel is prepared in 2 steps.

3.1 PREPARATION OF THE FIRST LAYER OF AGAROSE

- On the 80°C hot plate: put 500 µL of liquid agarose in the agar holder, on the side that has not been taped.
- On the benchtop: remove the agar holder from the hot plate and let agarose jellify at room temperature for exactly 5 minutes.

It is recommended to use a timer at this step of jellification.

- Remove the tape with the tweezers.
- Drill the small holes only, with the 20 G flat-cut needle. Go well through twice. These holes are necessary to remove the excess of agarose at the next step.

- Turn the agar holder upside down. Make the holes on the side that will face the cells on the coverslip.

Respecting this sequence will ensure you to obtain a plane surface toward cells, and thus a homogeneous pressure.

3.2 PREPARATION OF THE SECOND LAYER OF AGAROSE THAT INCLUDES PILLARS MOLDING

Before the first use of the wafer

- Handle in the cleanest possible atmosphere, with nitrile or polyvinyl gloves.
- Open the box, the wafer is attached on the back side by a green adhesive tape, named REVALPHA 120.
- Detach the REVALPHA 120 tape from the transparent box held together with kapton tape or stickers.
- Remove the clips (tape or stickers) from the REVALPHA 120.
- Place the REVALPHA 120 and the wafer on a hot plate at 125 - 130° for 2 minutes.The green ribbon must be in contact with the hot plate. It turns white.
- The back side is detached thanks to the temperature. Recover the adhesive and the wafer assembly by keeping it horizontal. Do not peel off on the hot plate, because of the risk of burns.
- Detach the wafer from the adhesive. Hold the wafer with gloved fingers by the edge if it does not slide off by itself.

- Put the agar holder and the wafer on the 80°C hot plate, the agarose-containing side facing up.
- On the 80°C hot plate: deposit 500 µl of liquid agarose with or without beads on the cleaned wafer.

It is better if the agarose gel forms a dome.

If there are bubbles, try to remove them with a needle.

Do not hesitate to put more agarose than indicated: it can be easier to put some more and to let it gel before removing it.

- Remove the whole from the hot plate. Put it on the bench, and rapidly place the agar holder on it, the agarose filled side facing the liquid agarose. Try to superimpose

the agar holder as much as possible to the shape drawn on the wafer.

You know that it is OK when the agarose gel rises through the holes.

- Keep it 3 min in place with hands.

- Let it jellify at room temperature for an additional 10 minutes.

It is highly recommended to use a timer in order to get a good jellification of the agarose.

Add some distilled water to remove the agar holder by gently sliding it.

- Remove the excess of agarose around the agar holder with cleanroom paper.
- Use 16G and 20G needles to drill the 9 holes in the agarose gel.

To make nice holes: punch first on the side that will be in contact with the glass slide, then the side that will be upside.

- Clean the wafer with water and cleanroom paper.
- Wrap the wafer in cleanroom paper to prevent it from breaking. Keep it in a clean plastic box wrapped in parafilm.
- Put the agar holder containing the agarose gel in a sterile dish containing sterile PBS.

- Sterilize it with a UV lamp: 20 min each side under culture hood.

If you do not have a dedicated UV lamp, you can use the culture hood UV lamp. In this case, we recommend an overnight exposure.

Try to put the dish as close as possible to the UV lamp.

- Remove the PBS and add culture medium in the dish.

If you prepare the agarose gel in advance, keep it sterile in PBS at 4°C. Remove PBS, add culture medium at 37°C at least 12h before assembling the system.

If you want to visualize pillars when imaging, you can add fluorescent beads as follows:

- Put fluorescent beads in an ultrasound bath for 30s.
- Add them in dissolved agarose: for 400 nm diameter beads, add 250 µL in 4 mL of agarose and for 200 nm diameter beads, add 200 µL in 1 mL of agarose
- Keep the agarose + beads on the 80°C hot plate.

4. Seed the cells

- Put the autoclaved AgarSqueezer under the hood.
- Finish to screw the lower plate together with the piston enclosure of the AgarSqueezer.

Tighten gradually and simultaneously in a cross pattern.

If necessary, you can coat the coverslip with adhesive proteins. As an example, you can use 250 µL of a Fibronectin solution (50 µg/mL in PBS) to coat the coverslip surface. In this case, incubate for 1 hour in the AgarSqueezer device used as a dish at room temperature. Remove the excess of Fibronectin with 3 PBS washes. Then proceed to cell seeding.

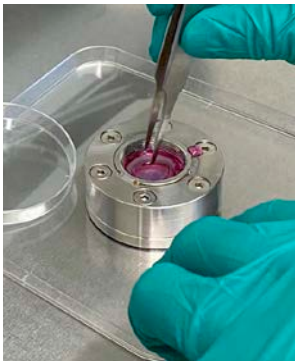
- Prepare a solution of cells so that you put 500 µL resulting in 100 000 cells/coverslip.

Example for MCF10A cells: prepare a 200 000 cells/mL solution (see table below)

- Add 500 µL on the coverslip.
- Let cells adhere at 37°C during at least 12h and then wash three times with pre-warm culture medium.

5. Confine the cells

- Insert the agar holder on the glass coverslip, the agarose side facing the coverslip.



- Screw with the clamping holder nut.

- Finish to screw with the AgarSqueezer white screwdriver.

This moment is starting the confinement experiment.

- Place the system in a sterile dish.

You can put up to 4 AgarSqueezer devices in a 245 * 245 square dish.

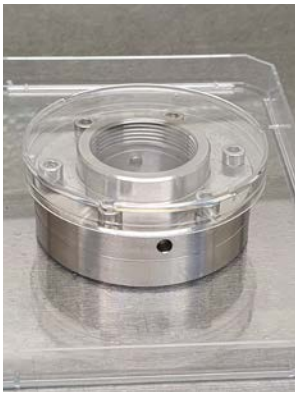
Cell lines	Coating	Number of cells / coverslip	Cell density (cells/mL)	Duration of the experiment
MCF10A	No	100 000	2E+05	Up to 8 days
HT29	Fibronectin 50µg/ml 1h 37°C	90 000	1,8E+05	2h - 3 days
HCT116	Fibronectin 50µg/ml 1h 37°C	90 000	1,8E+05	2h - 3 days
TF1-GFP	Fibronectin 50µg/ml 30 min 37°C	Only adhesive cells after 2h are kept	5,6E+05	2 days
TF1-BA	Fibronectin 50µg/ml 30 min 37°C	Only adhesive cells after 2h are kept	1,02E+06	2 days
HS27A	No	125 000	2,5E+05	3 days

Do not forget to write on the dish the height of the pillars if you test multiple heights in parallel.

- Wash the chamber 3 times, 5 min each with 500 µL of pre-warmed culture medium.

It is possible to remove culture medium and add fresh culture medium directly with a pipetman above the agar holder.

- Finish by adding 500 µL of culture medium above the agar holder.
- Put a 35 mm petri dish cover on the top of the system to keep it sterile and put it in the incubator.



For long-term culture: change the culture medium every day. Keep the same volume of culture medium.

Diffusion experiments show that 3 hours are required for the diffusion of small molecules such as BSA or FITC in the system.

- Incubate at 37°C as long as needed.

6. Subsequent analyses

IMAGING

- You can start to image on the microscope.
- Use the AgarSqueezer holder to place up to 2 AgarSqueezers under the microscope stage.

IN SITU IMMUNOSTAINING

You can use the AgarSqueezer as a dish.

- **After confinement, you can fix cells in situ with 4% paraformaldehyde (PFA):**
  - Remove the cell culture medium.
  - Wash the samples twice, 10 min each with PBS.
  - Add 750 µL of 4% PFA.
  - Incubate for 40 min at RT.
- **After incubation:**
  - Wash the samples three times, 20 min each with 500µL of 3% Bovine Serum Albumin (BSA) in PBS.
  - Unmount the agar holder.
  - Perform standard immunostaining procedure used for 2D cells.

Check videos of protocol, examples
of results and much more on:
idylle-labs.com/agarsqueezer-by-softconfiner

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