CHAPTER

Methods for Two-Dimensional Cell Confinement

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Abstract

Protocols described in this chapter relate to a method to dynamically confine cells in two dimensions with various microenvironments. It can be used to impose on cells a
given height, with an accuracy of less than 100 nm on large surfaces (cm²). The method is based on the gentle application of a modified glass coverslip onto a standard cell culture. Depending on the preparation, this confinement slide can impose on the cells a given geometry but also an environment of controlled stiffness, controlled adhesion, or a more complex environment. An advantage is that the method is compatible with most optical microscopy technologies and molecular biology protocols allowing advanced analysis of confined cells. In this chapter, we first explain the principle and issues of using these slides to confine cells in a controlled geometry and describe their fabrication. Finally, we discuss how the nature of the confinement slide can vary and provide an alternative method to confine cells with gels of controlled rigidity.

**INTRODUCTION**

Numerous methods have been used to study cells in a confined environment. The most common methods have certainly been three-dimensional (3D) cell cultures, which involve embedding cells in a gel or another 3D material (see Justice, Badr, & Felder, 2009). Although these methods offer environmental conditions for cells that are close to those of intermediate tissues in vivo, the various physical parameters of the cell environment (geometry adhesion, environment elasticity, etc.) are not homogeneous and well defined, making it difficult to decipher their respective roles. Moreover, observation is challenging since cells can migrate in the three dimensions, limiting the possibilities of high-resolution microscopy. To circumvent these limitations, other methods have been developed. Among them, many have been driven to a large extent by technological developments that allow controlling forces or geometries at the micrometer scale, for example, apparatuses such as atomic force microscopes, microplates, or micromanipulators that have been designed by physicists to manipulate single cells and constrain them in various manners (see Mitrossilis et al., 2010). The main advantage of these approaches is the possibility to dynamically measure and control forces on a single cell. On the other hand, statistical and molecular analysis of a single cell is challenging. Microfluidic channels offer a different strategy to answer specific questions using simple ways to confine cells in a given geometry (see Velve-Casquillas et al., 2010). These channels allow the observation of many cells at the same time and offer tools to control specifically the microenvironment of the cell (chemical gradients, temperature, electric fields, etc.). Even though these microchannels are suitable to obtain statistics and build complex environments, the dynamic change of cell confinement and molecular analyses remain challenging in these conditions.

Here we describe an intermediary method that allows confining a population of adherent cells in a culture without the need to detach them from their original culture substrate. Because the confinement is applied on a population of cells, statistical data are easy to obtain, and because confinement can be released, the cell material is usable for further study (immunofluorescence, Western blot, qPCR [quantitative polymerase chain reaction], or other molecular analysis). The method is based on the gentle application of a confining slide on the cultured cell (Fig. 14.1A) using a
suction cup device or a modified multiwell plate. The confinement slides are made of a glass coverslip, which is covered by a layer of material ensuring confinement. We will show how to use the microstructured layer of polydimethylsiloxane (PDMS), which is a relatively stiff biocompatible silicone rubber, to confine cells between two parallel planes (Fig. 14.1B). In this case, cells can move in two dimensions and the height of the cells is precisely imposed (geometric confinement). Alternatively, we will also show how to use a layer of polyacrylamide (PAM) gel to impose on the cell an environment of controlled stiffness (elastic confinement). In this second case, cells are covered by a gel of controlled rigidity and have to push on the gel to define their own space. In both cases, the rigid glass slide provides homogeneous confinement with no long-range deformation and the soft material (PDMS rubber or gel) ensures the robustness of the confinement by absorbing the local substrate inhomogeneities due to the possible presence of dust or particles. Except microfabricated molds, which need photolithography equipment, the fabrication of the confinement slides and handling devices is simple enough to be done in a cell biology lab with a small investment. Thus, this confinement method is well adapted to study of the roles of geometry and cell environment in cell processes such as migration (see Le Berre et al., 2013), division (see Lancaster et al., 2013), or adaptation (see Le Berre, Aubertin, & Piel, 2012).

The first section describes how to design microfabricated molds to produce confinement slides for geometric confinement. The following two sections specify the fabrication of the slides (for geometric confinement or gel confinement, respectively). Finally, two sections explain methods to handle these slides (either dynamically with a suction cup device or in multiwell plates to assay multiple conditions at the same time) followed by a discussion of how these slides can provide more complex configurations of confinement.
14.1 SLIDE DESIGN FOR GEOMETRICAL CONFINEMENT

For geometrical confinement, microspacers have to be molded under the confinement slides in a PDMS layer in order to control the distance between the slide and the culture substrate.

To get a robust confinement, the geometry of these spacers needs to be controlled. If the pillars are too close, many cells will be squashed by the pillars, but if they are too sparse, the glass slide sags in between the pillars, and the thickness of the cells is weakly controlled. In addition, if the surface of the pillars is large, a large proportion of the cells will be squashed, but, if the pillars are too small, they deform under pressure, giving an uncontrolled confinement height.

An optimal geometry of these spacers has been determined by in silico mechanical simulations to get pillars as far as possible from each other while making negligible long-range deformation of the confinement geometry (<100 nm when a 10-kPa pressure is applied on the slide) and a small amount of squashed cells (6%) (see Le Berre et al., 2012). Typically, spacers of 440 μm in diameter spaced by 1 mm in a hexagonal network provide meet these specifications (see Fig. 14.2A). Note that this geometry is adapted to confine cells to thicknesses above 3 μm. Below this height, sagging is more pronounced, and a capillary effect often sticks the PDMS layer on the culture substrate, squashing the cells on the whole surface.

Based on this geometry, we need a mold to imprint the structures on the confinement slides. This mold can be produced by photolithography of a layer of photoresist deposited on a silicon wafer by using standard microfabrication protocols. These protocols require a photomask that is used to shade a layer of photoresist from

![FIGURE 14.2](image_url)

*FIGURE 14.2*

Optimal geometry of the pillars for geometrical confinement. (A) Dimensions of the pillars in a round slide of diameter $D$. Typically, $D = 10$ mm for the suction cup device, and $D = 18$ mm for a 6-well plate, and $D = 12$ mm for a 12-well plate. The number of pillars has to be adapted depending on $D$. (B) Photomask to produce a negative photoresist layer (such as SU8) corresponding to the design in (A).
the applied UV light and define the shape of the spacer after development of the photoresist. With this fabrication mode, the thickness of the photoresist layer corresponds to the height of the pillars and, therefore, the thickness of the cells after confinement. If manipulated carefully, a mold can be used many times. Thus, it is not necessary to have access to a microfabrication facility, and the mold, which is technically easy to fabricate, can be created by a specialized collaborator or a microfluidic foundry company. The mold provider usually needs a drawing of the photomask in DXF (Drawing eXchange Format) or GDSII (Graphic Database System II) format, which can be done easily with CAD software like AutoCAD (Autodesk) or L-Edit (Tanner). But due to the poor resolution required to produce the mold, the mask can also be printed on a plastic sheet with a high-resolution printer by using a simple drawing software like Adobe Illustrator. This way, many academic microfabrication facilities can make such a mold for less than $200. (For more details on mold fabrication, see, for example, Velve-Casquillas et al., 2010.)

14.2 GEOMETRICAL CONFINEMENT SLIDE FABRICATION

This section describes the fabrication of stiff confinement slides for geometrical confinement.

14.2.1 Materials

- A mold to imprint slides (see previous section 14.1).
- 10-mm-diameter standard glass coverslips.
- PDMS (RTV615, General Electrics or Sylgard 184).
- Wash bottle of isopropanol.
- Scalpel blade.

For slide treatment (optional):

- Parafilm.
- Wash bottle of pure water.

Adhesive:

- Fibronectin solution 50 μg/ml in phosphate buffered saline (PBS) (30 μl per slide) (Fibronectin from Sigma).

Nonadhesive:

- Pll-g-PEG solution 500 μg/ml in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer 10 mM, pH 8.6 (30 μl per slide) (pLL-g-PEG (20)-[3.5]-(2) from SuSoS).

For antiadhesive coating of the mold (optional):

- Trimethylchlorosilane (TMCS) (Sigma).
- Fume hood.
14.2.2 Equipment

- Plasma cleaner (for example, PDC-32G, Harrick).
- Pressurized, filtered, and oil-less air stream.
- Hot plate.

14.2.3 Fabrication (Fig. 14.3A–F)

1. Prepare 10 g of PDMS mix with a ratio of PDMS/cross-linker (A/B) of 8/1 (w/w), avoiding introducing too many bubbles during mixing. PDMS can be mixed either manually with a simple swizzle stick or with an automatic planetary mixer (e.g., ARE-250, Thinky). If the PDMS is mixed manually, bubbles can be removed by centrifugation (2 min at 1000 rpm). Be careful in

![Diagram of the fabrication process]

**FIGURE 14.3**

Confinement slide fabrication. The schemes and corresponding photos summarize the fabrication process: (A) Start with a microfabricated mold (Mo) made of a silicon wafer (Si) covered by a patterned layer of photoresist (Res). (B) A drop of PDMS (Pd) is placed on the mold, for instance, with the help of a glass rod (Gr). (C) A plasma-activated glass coverslip (Glc) is pressed onto the drop of PDMS with the help of tweezers in order to keep only a residual layer of PDMS under the coverslip. The excess PDMS should make rims around the coverslips (Pr). (D) After baking, the PDMS rims (Pr) are removed. (D2) No residual PDMS should be visible on the mold at this stage. (E) To unmold the confinement slide (Cs), the slide is wet in isopropanol (Ipa) before a razor blade (Rb) is introduced between the mold and the slide. (F) After unmolding, the confinement slide (Cs) is rinsed with isopropanol and air dried.
In this case that PDMS is well mixed because unreticulated PDMS is very difficult to remove and can make your mold unusable.

2. Place the 10-mm glass coverslips on a big glass slide and treat it in the plasma cleaner for 2 min at maximum power in order to activate one side of the slide (when the plasma is on, the plasma may produce a dense pink glow). This step allows the glass slide to stick onto the PDMS layer in the next step.

3. With a glass rod or equivalent, distribute drops of PDMS on the mold in the places you want to put the plasma-treated coverslips (Fig. 14.3B).

4. Place the coverslips on top of the drop and push gently with tweezers everywhere on top of the coverslips to keep only a very thin layer of PDMS under the coverslip. If you push too hard on the coverslip, it will break, and, if you push too gently, the residual layer of PDMS will be too thick, and the pillars will collapse. To gauge the right pressure, notice that it corresponds to the minimum pressure where you see a small white star appearing under the tweezers. Do not use sharp tweezers: You will break your coverslips! The ejected PDMS should form a rim partially covering the slide (Fig. 14.3C).

5. Bake the mold with the coverslips on the hot plate at 95 °C for 15 min. At this step, if the PDMS is baked too long or not long enough, it will stick too strongly to the mold, and it will be very difficult to unstick confining slides without breaking them.

6. Remove the PDMS rim with tweezers (Fig. 14.3D).

7. Place a droplet of isopropanol on the mold; it will help to unmold the slide.

8. Use a razor blade to gently unstick the coverslip from the mold. To avoid scratching the mold (molds made of photoresist are very easy to scratch) or breaking the coverslip, use the following method: At the beginning, place the razor blade on the edge between the mold and the coverslip at an angle of 20°, and then carefully slide the blade under the confining coverslip parallel to the mold surface (Fig. 14.3E).

9. Rinse the coverslip with isopropanol.

10. Dry the confining coverslip with an air gun (otherwise it will leave stains and inhomogeneities on the confining slide surface) (Fig. 14.3F).

11. Gently clean the mold with a clean wipe soaked with isopropanol.

12. Optional: If the mold becomes too sticky and coverslips are difficult to detach, use an antiadhesive agent. For this step, work under a fume hood: TMCS is very toxic. Carefully clean the mold with isopropanol and a soft cleaning paper wipe (e.g., Kimtech, Kimberly-Clark), and put it in a petri dish. Seed two or three drops of TMCS around the mold, and close the petri dish. The drops will evaporate and react with the surface. After 5 min, open the petri dish, and vent the remaining TMCS vapors for 5 min, then bake the mold for 10 min at 70 °C to stabilize the surface.

### 14.2.3.1 Slide treatment

The coverslip can be used either without any treatment (it is not toxic to the cells) or with a chemical treatment to promote or avoid cell adhesion. However, we advise treating the confining slide systematically in order to avoid trapping air bubbles.
between the slide and the substrate, which can kill the cells (PDMS is very hydrophobic and often retains air bubbles on its surface).

1. For nonadhesive treatment only: Place the confinement slide on a big glass slide with the PDMS side facing upward, and treat it in the plasma cleaner for 30 s maximum at maximum power in order to activate the PDMS surface.
2. On a piece of parafilm, drop 30 µl of the fibronectin solution (for adhesive treatment) or of the pLL-g-PEG solution (for nonadhesive treatment).
3. Place the confinement slide on the drops, the structured slide in contact with the solution, and wait for 30 min.
4. Rinse extensively with pure water.
5. Dry the slide with an air jet.

### 14.3 SOFT CONFINEMENT SLIDE FABRICATION

This section describes the fabrication of soft confinement slides for elastic confinement.

#### 14.3.1 Materials

- a. Coverslips (any glass coverslip size can be used).
- b. Ethanol 70%.
- c. Deionized water.
- d. Acrylamide 40% (Biorad).
- e. Bis-acrylamide 2% (Biorad).
- f. 10 mM HEPES buffer pH 7.6.
- g. Irgacure 0.5% in 10 mM HEPES pH 7.6 (Irgacure 2959, CIBA). The Irgacure stock solution has to be protected from light.
- h. Aminopropyltrimethoxysilane (APTMS) (Sigma) 4% solution in acetone.
- i. Clean and smooth PMMA (polymethyl methacrylate) plate (Good Fellow) (Also available under the commercial name of “plexiglas”).
- j. PDMS (RTV615, GE).
- k. Wash bottle of isopropanol.

#### 14.3.2 Equipment

- l. Ultrasonic bath.
- m. Plasma cleaner (e.g., PDC-32G, Harrick).
- n. UV lamp (e.g., Delolux 03 S).
- o. Fume hood.
14.3.3 Method

1. Coverslip surface preparation
   a. Wash the glass coverslip with ethanol 70% and dry it.
   b. Expose the dry coverslips to air plasma for 30 s, maximum power.
   c. Incubate clean slides in a solution of APTMS (4% in acetone) for 30 min under sonication.
   d. Wash with distilled water, and air dry (slides can be stored for several weeks at room temperature).
   e. Place the slide on a drop of glutaraldehyde solution (0.5% in PBS) for 30 min. It is convenient to do it on a laboratory parafilm. Drops of 100 µl are sufficient for coverslips of 18 mm. Keep track of the side of the coverslip that was treated. Coverslips should be used right after this step. (Glutaraldehyde is toxic and an irritant, and it has to be manipulated under a fume hood.)
   f. Wash coverslips with distilled water, and air dry.

2. Gel polymerization (Fig. 14.4A–E)
   a. Prepare the PAM mix in a stock solution of Irgacure (0.5% in 10 mM HEPES pH 7.6). The concentration of acrylamide and bis-acrylamide depends on the gel rigidity you want to obtain (see Table 14.1 and Boudou, Ohayon, Picart, & Tracqui, 2006).

**FIGURE 14.4**

Fabrication of confinement slides with gels of controlled rigidities. (A) First a droplet of the gel precursor mix (Gel) is deposited on a PMMA plate and covered by a pretreated glass coverslip (Glc). (B) The gel is polymerized under UV. (C) After polymerization, the confinement slide (Cs) can be detached from the PMMA plate with a razor blade (Rb). (D) The gel is incubated in PBS to remove any remaining diffusible molecules. (E) Before the experiment, the gel is incubated in the appropriate culture medium (Cm) upside down on the surface of the medium to avoid getting the back side wet in the medium. (F) When positioned on the confinement apparatus, the dry side of the slide sticks naturally to the PDMS surface of the slide holder (Sh).
b. Put drops of the PAM solution on the PMMA plate, and gently place the coverslips on them with the treated surface toward the PAM solution (the coverslip should float on the drop). The volume of the drops can be adapted according to the thickness of the gel you want to obtain (200 μl for an 18-mm coverslip will result in gels of ~0.8 mm during the fabrication, but note that the gel will swell to ~2 mm after incubation) (Fig. 14.4A).

c. Place the setup under a UV lamp for 10 min (Fig. 14.4B).

d. Carefully detach the coverslips with gel from the plastic using a razor blade or a scalpel (Fig. 14.4C), and quickly place it in sterile PBS for incubation overnight (Fig. 14.4D).

e. Before experiments with cells, gels have to be incubated at least 2 h in the cell culture medium that will be used during the experiment. If drugs will be used in the experiment, the gel has to be incubated in the medium containing the appropriate concentration of the given drug. To avoid wetting the back side of the confinement slide, it can be deposited upside down on the medium surface. This way, the slide will be incubated floating on the surface (Fig. 14.4E).

### 14.4 DYNAMIC CELL CONFINER

The application of the confinement slide on the cultured cells can be done in several ways. In all cases, a slight pressure (~10 kPa) has to be applied on the slide to get a robust and uniform contact with the substrate of the cultured cells, and tangential movement must be eliminated to avoid shearing the cells. A practical way to proceed is to use the so-called cell confiner that is described in this section.

The cell confiner is a device that acts as a suction cup, as shown in Figure 14.5. It is composed of a soft empty part that forms a sealed cavity when it is placed on a surface. When a vacuum is created in the sealed cavity, the device is stuck on the substrate and cannot move. In the center of the empty part, a piston holds the confinement slide, which can be displaced vertically by changing the strength of the vacuum in the device. After the threshold strength of the vacuum is applied, the confinement slide comes into contact with the cultured cells.
By using this device, confinement slides can be applied onto any substrate provided that it is flat. The culture substrate just has to be large enough to accept the device (the schematic shown in Fig. 14.6A is adapted to 35-mm-diameter petri dishes).

### 14.4.1 Materials

**For fabrication:**
- Machined confiner mold: This mold, intended to mold the PDMS device, can be fabricated in stainless steel in a standard workshop. In the simplest version, the mold is composed of two stainless rings, a silicon wafer (which can be replaced by a glass slide), and a 0.5-mm-thick, round glass coverslip of 16 mm diameter (which can be created by gluing several thinner glass coverslips together), according to the layout shown in Figure 14.6B.
- PDMS (RTV615, GE).
- A standard glass slide (typically 1 × 3 in., 1 mm thick).
- A hot plate.
- A 0.75-mm puncher to make the vacuum inlet hole in the device (Elveflow).

**For handling:**
- The cell confiner.
- A confinement slide.
- 70% alcohol.
- Absorbing paper.
Removable adhesive tape (e.g., Scotch Magic Tape, 3 M).

A precision vacuum generator or controller (e.g., Elveflow VG1006). Check before the experiment that you have all the connections plugged into the device. Sometimes, the culture medium is aspirated into the controller by the vacuum, which can cause severe damage to the controller. To avoid this, we strongly recommend placing a security reservoir between the device and the vacuum controller.

FIGURE 14.6
Cell confiner fabrication. (A) Dimensions of the cell confiner. Typically, for a cell confiner adapted to 35-mm dishes: \(a = 22\) mm; \(b = 7\) mm; \(c = 16\) mm; \(d = 8\) mm; \(e = 5\) mm. (A) A mold can be easily fabricated by assembling on a silicon wafer (Si) a 0.5-mm glass coverslip (Glc) and two rings forming the outer part of the mold (Opm) and the inner part of the mold (Ipm). (B) After the mold has been filled with PDMS (Pd), a glass slide (Gs) is pressed on the top of the structure to flatten the device surface and produce its final shape. (C) The device is baked for 30 min at 85°C. (D) After cooling down, the structure can be disassembled to retrieve the molded cell confiner (Cc). (F) A 0.75-mm hole is punched into the wall of the device with a puncher (Pn) by inserting a blade (Bl) below the drilling zone for protection. (G) This hole is used to insert a fluid connector (Cn) plugged into the vacuum tube (Vt).
• Adapted tubing to plug into the vacuum generator, including a 0.51-mm ID tygon tube (Saint-Gobain) and Stainless Steel Tubing 0.025” OD (see, e.g., microfluidic kits, Elveflow) as a connector to plug the tube into the device (see Fig. 14.6G). It is best to bend the tube to avoid it taking up too much space in the petri dish.

• A sharp pair of tweezers.

### 14.4.2 Design and Fabrication

The design shown in Figure 14.6A is for a 35-mm petri dish. This particular geometry has been optimized to get a working pressure of $-10 \text{ kPa} \ (100 \text{ mbar})$, which is sufficient to handle the device easily, but does not notably change the pressure around the cells. For other formats of cell cultures, the device can be scaled up or down: The working pressure of the device is independent of the device size if the proportions are respected.

The suction cup device can be molded with PDMS in a simple mold provided the top and bottom surfaces of the mold are flat enough to get a good optical transparency. Here, we describe a simple method to make a mold based on the simplest pieces, which can be done in any workshop.

1. Prepare a mix of PDMS/cross-linker (10/1 w/w) with no bubbles. (If you do not have a planetary mixer, you can remove bubbles either with a vacuum bell cloche for 2 h or by centrifuging PDMS for 2 min at 1000 rpm.)
2. Stack the different parts of the mold directly on a (cold) hot plate (Fig. 14.6B).
3. Pour PDMS very slowly into the mold, avoiding bubble formation (Fig. 14.6C).
4. Cover the filled mold with a glass slide, and press on it until there remains only a residual layer of PDMS between the mold and the slide (Fig. 14.6D).
5. Heat up the hot plate to $85 \ ^\circ C$ for 30 min (Fig. 14.6D).
6. Cool down the molded part before unmolding. To make unmolding easier, use a razor blade and isopropanol to reduce adhesion of the PDMS on the mold.
7. Punch the inlet hole with the 0.75-mm puncher as shown in Figure 14.6 F.

### 14.4.3 Handling

1. At least 1 h before the experiment, incubate the confinement slide in the cell culture medium that will be used for the experiment to equilibrate the PDMS with the medium. The structured side (PDMS side) of the slide should face upward. Be careful not to lose the orientation of the slide during manipulation; it can be difficult to recover. Note that PDMS absorbs small hydrophobic molecules from the medium. So, if drugs are used in the experiment, these drugs have to be present during this incubation step.
2. Before the experiment, clean the cell confiner with 70% alcohol, and dry it carefully with absorbing paper. Be careful not to leave the device in contact with the alcohol for a long time, or it will absorb the alcohol and become toxic to cells.
3. Clean the bottom face of the device with removable adhesive tape to remove dust particles.
4. Plug the device into the controllable vacuum source, and tune the controller to $-3 \text{ kPa} (-30 \text{ mbar})$. At this pressure, and with a good seal, the device sticks to the substrate, but the confinement slide does not yet touch the cells.

5. Prepare an absorbing paper to dry the back side of the confinement slide.

6. Pick up the confinement slide with tweezers, and dry its back side on the absorbing paper by placing it onto the paper. (If the back side is not totally dry, it will not stick onto the device and can be lost during handling.)

7. Place the confinement slide onto the piston of the cell confiner, structured side up. The slide should stick naturally onto the piston.

8. Put the device in contact with the cell culture substrate. Take care not to deform the device to achieve a good seal of the device cavity. Also, be careful not to push on the center of the device, or cells could be crushed by the device. After the device is well sealed, the membrane sustaining the piston should be slightly deformed. The step of placing the device must be carried out quickly, otherwise the medium will fill the device and enter the vacuum outlet. If the medium enters the vacuum tube, the device may not work properly due to the capillary pressure of the meniscus in the tube. If the medium has entered the tube by accident, the device and the tube must be cleaned and dried to avoid the formation of a meniscus in the tube.

9. At this point, cells can be observed before confinement. The petri dish will not move during the confinement operation, and several cells can be observed simultaneously.

10. To confine cells, slowly decrease the pressure down to $-10 \text{ kPa} (-100 \text{ mbar})$. By observing the cells during this time, one should see the confinement slide touching cells at approximately $-5 \text{ kPa}$.

11. Confinement can be released if the pressure is increased back to $-3 \text{ kPa}$.

### 14.5 MULTIWELL CONFINER

If a large amount of confined cells or multiple conditions are required, a modified multiwell plate can be used to apply confinement slides on cells (Fig. 14.7). In this case, it is not the force induced by a vacuum that holds the slide on the cells, but the pressure induced by the deformation of a soft pillar. In this case, big PDMS pillars are stuck on the lid of the multiwell plate and hold the confinement slides. When the lid is closed, the pillars push the confinement slides onto the culture substrate and confine the cells. Many wells can be processed simultaneously, and confinement slides of larger surfaces can be used; however, the speed of confinement is not controlled, and it is not convenient to observe what happens during the confinement application. For this reason, this method is not applicable to the study of short-time events. In contrast, it is well adapted to long-term experiments and molecular analysis (e.g., overnight time-lapse imaging, immunofluorescence).

#### 14.5.1 Materials

For fabrication:
- A 12 (or 6) well multiwell plate (TPP).
- A 48 (or 12) well multiwell plate.
14.5 Multiwell Confiner

14.5.2 Fabrication

1. Prepare the PDMS mix with a ratio of 35:1 w:w PDMS:cross-linker. Due to the small concentration of cross-linker, the PDMS has to be mixed extensively to get a good reticulation.

2. Pour the PDMS mix into the mold, which can be either a smaller multiwell plate or a custom-made mold. For example, for pillars to be used in 12-well plates, use

- PDMS (RTV 615, GE).
- Wash bottle of isopropanol.
- Removable adhesive tape (Scotch Magic Tape, 3 M).
- One sheet of gel-pack film (DGL Film X8 17 mils, Gel-Pack).

For handling:
- Adhesive tape.
a 24-well plate as mold and fill them with PDMS. Pour the PDMS in excess, leave it in the vacuum bell cloche to get rid of bubbles, and carefully close with the lid of the plate avoiding bubble creation. For confinement slides including PAM gels, which have a higher thickness, spacers of appropriate thickness can be stuck on the plate lid to reduce the thickness of the final pillars.

3. A small amount of isopropanol will help get the pillars out of the mold. After unmolding and cleaning, the pillars should be soft and sticky.

4. Clean the pillars carefully with isopropanol.

5. On the multiwell plate lid, place a gel-pack sheet on each well position in order to raise the pillars slightly.

6. Place the pillars on the gel-pack sheet at the center of each well position. Clean pillars will stick naturally. You can clean the PDMS and gel-pack sheet before use with the adhesive tape to remove dust.

7. Now the lid is ready to be used.

14.5.3 Handling

1. Place one confinement slide on each pillar of the modified lid.

2. Optional: sterilize the lid under UV (365 nm).

3. Optional: incubate the pillars in culture medium to equilibrate the PDMS.

4. Gently close the multiwell plate with the modified lid.

5. While pushing on the lid, fix the lid to the multiwell plate with adhesive tape. During this step, avoid moving the lid laterally: This will shear cells and kill them. Pull on the adhesive tape while applying it to the closed plate to put the plate under tension.

6. Verify under a microscope that the cells are well confined.

14.6 DISCUSSION

In this chapter, we have discussed how to confine cells in between two parallel surfaces separated by a defined gap in a reversible manner. Alternatively, we explained an adaptation of the method to confine cells below a soft PAM gel of controlled stiffness. However, since the two surfaces can be treated independently, and because the treatment of the confinement slides can be very versatile, this method is not limited to these two configurations in its principles; an infinity of confinement configuration can be imagined. The method can be easily combined with others to build more complex environments for specific studies. For the geometric confinement, for example, the flat roof can be replaced by more complex geometric features by using advanced photolithography methods (see del Campo & Greiner, 2007). Thus, it might be possible to introduce various textures or restrictions into the geometric landscape of the cell environment. Another parameter that can be varied is the level of adhesion. This can be done by mixing in a radiometric manner pLL-g-PEG with pLL-g-PEG-RGD, which is a modified version of pLL-g-PEG modified with the RGD motif and is responsible for integrin-mediated adhesion (see Barnhart, Lee, Keren, Mogilner, & Theriot, 2011). In this case, the
adhesion level of cells can be modulated independently on both sides of the cell. Confinement can also be combined with other methods. For example, it is possible to print adhesive micropatterns on the bottoms of the wells to restrict geometric cell adhesion. This allows a more reproducible organization of the cell under confinement (see Azioune, Carpi, Tseng, Théry, & Piel, 2010).

**GENERAL CONCLUSION**

Confinement parameters are taking on increasing importance in cell biology studies, and tools that allow the control of the different confinement parameters independently while remaining simple enough to be used in a biological routine are crucial. We described in this chapter a versatile way to confine a population of cells in a controlled environment (geometry, stiffness, adhesion, etc.), which is easily combinable with specific observation techniques and molecular biology protocols. We hope that these protocols will help the community answer certain questions and will lead to new and innovative ideas about how to study cells in complex environments.

**References**


