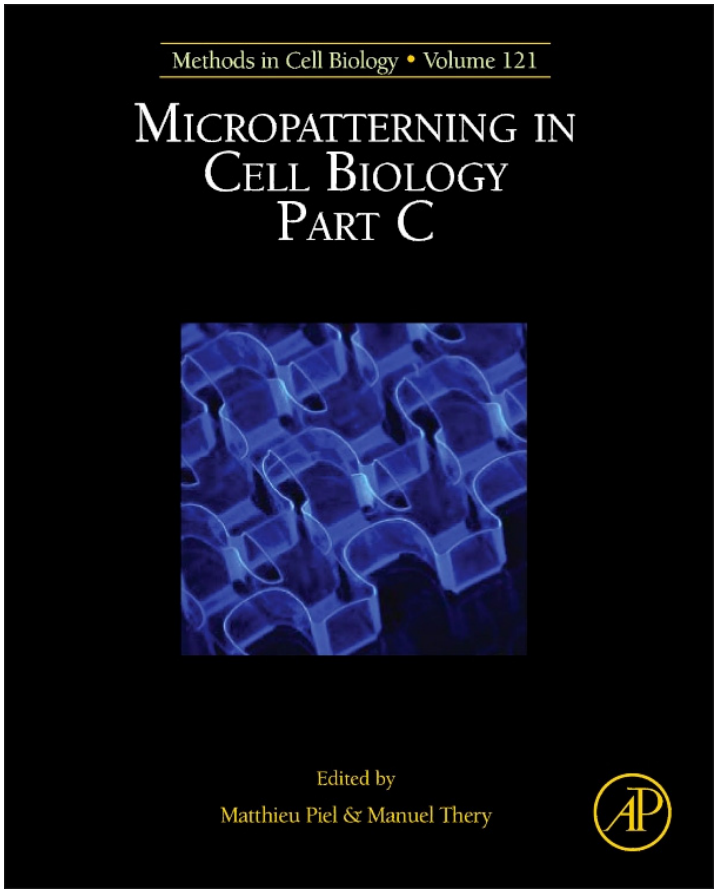

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CHAPTER

Micropatterning on Micropost Arrays

5

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CHAPTER OUTLINE

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Abstract

Micropatterning of cells can be used in combination with microposts to control cell shape or cell-to-cell interaction while measuring cellular forces. The protocols in this chapter describe how to make SU8 masters for stamps and microposts, how to use soft lithography to replicate these structures in polydimethylsiloxane, and how to functionalize the surface of the microposts for cell attachment.

INTRODUCTION

The interplay between the mechanical properties of cells and the forces that they produce internally or applied to them externally play an important role in maintaining the normal function of cells. These forces also have a significant effect on the progression of mechanically related diseases. The tools and methods used to study the interplay between cell mechanics and cell forces have come from recent innovations in microfabrication and micropatterning.

Micropost arrays, also known as *microfabricated post array detectors* or micropillars, are arrays of vertical cantilevers, which are used to spatially measure the traction forces of cells attached to their tips via microscopy (Fig. 5.1). These tools have helped to elucidate the mechanical behavior of cells, the nature of cellular forces, and mechanotransduction (Bhadriraju et al., 2007; Cai et al., 2006; du Roure et al., 2005; Fu et al., 2010; Ganz et al., 2006; Ghibaudo et al., 2008; Grashoff et al., 2010; Han, Bielawski, Ting, Rodriguez, & Sniadecki, 2012; Lemmon, Chen, & Romer, 2009;

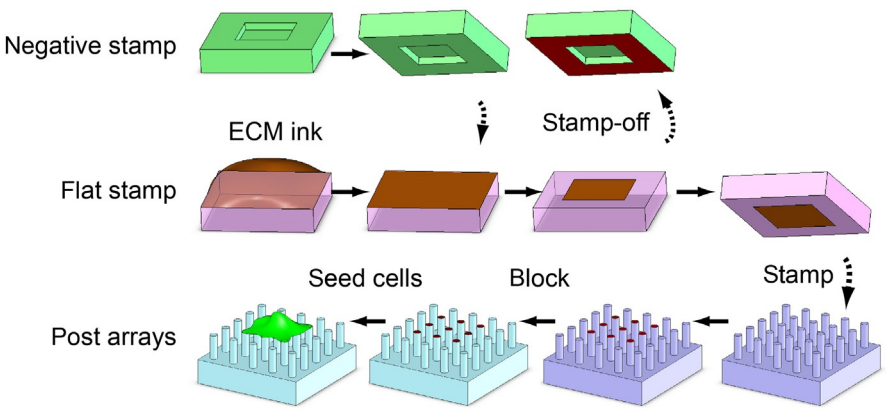

FIGURE 5.1

Stamping microposts for patterning cells.

Lemmon et al., 2005; Liang, Han, Reems, Gao, & Sniadecki, 2010; Liu, Sniadecki, & Chen, 2010a; Liu, Tan, et al., 2010b; Nelson et al., 2005; Pirone et al., 2006; Rodriguez, Han, Regnier, & Sniadecki, 2011; Ruiz & Chen, 2008; Saez, Buguin, Silberzan, & Ladoux, 2005; Saez, Ghibaudo, Buguin, Silberzan, & Ladoux, 2007; Sniadecki et al., 2007; Sniadecki, Lamb, Liu, Chen, & Reich, 2008; Tan et al., 2003; Tee, Fu, Chen, & Janmey, 2011; Ting et al., 2012; Yang, Sniadecki, & Chen, 2007).

In this chapter, we describe the approach used to make the stamps and micropost arrays. Portions of the steps to make microposts have been described elsewhere and are useful sources of information (Desai, Yang, Sniadecki, Legant, & Chen, 2007; Sniadecki & Chen, 2007; Yang, Fu, Wang, Desai, & Chen, 2011). However, the techniques described here are more specific to the approach used recently in our lab (Han et al., 2012; Ting et al., 2012).

5.1 MICROFABRICATION OF SU-8 MASTERS FOR MICROPOSTS OR STAMPS

The following steps describe the process to fabricate an SU8 master for the micropost arrays or stamps. Two layers of SU8 are used for the features of the SU master for the micropost arrays. The first layer is used to form a strong adhesion with the silicon wafer. The second layer is used to create the features of the microposts or the stamps.

5.1.1 Materials

- Bare silicon wafer
- SU-8 2000 series photoresist (Microchem, Newton, MA). SU-8 2005 and 2010 are used in this example
- Disposable wide-mouth pipettes to dispense SU8 onto the silicon wafers
- SU-8 developer (Microchem, Newton, MA)
- Chrome mask with features for microposts or stamps
- Acetone
- Isopropanol alcohol (IPA)
- Deionized water (DI)
- Clean room wipes
- Fluorosilane (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane) (T2492-KG, United Chemical Technologies).

5.1.2 Equipment

- Clean room environment (Class 1000 preferred)
- Photoresist spincoater with adjustable acceleration and velocity
- UV aligner
- Ozone cleaner (UVO cleaner; Jelight, Irvine, CA)

- Hotplates
- Clean Pyrex dishes
- Nitrogen gas
- Tweezers
- Digital timers.

5.1.3 Method

1. Clean the silicon wafer using a rinse of acetone for 10 s, a rinse of IPA for 10 s, and immersion in DI three times to remove organic residue that may be present on the wafer surface.
2. Place the wafer on a hotplate set to 200 °C for 10 min to dehydrate the wafer. This step is critical to ensure that the SU-8 adheres to the wafer surface. Cool the wafer to room temperature before the next step.
3. Spin a flat base layer of SU-8 2005 onto the wafer:
   a. Place the wafer on the spincoater and follow the manufacturer specifications for the chosen viscosity of SU-8 2000 series to set acceleration, rate, and time for spincoater. Some trial and error will be necessary to optimize the spinning process. Typically a base layer is thin, in the <5 μm thickness range so choose appropriately.
   b. Dispense the photoresist onto the wafer center in a smooth motion, using about 1 ml/in. in diameter of wafer to ensure even coverage. Allow the photoresist to spread momentarily before starting the spin cycle.
4. After the spin is completed remove the edge bead by either running an automatic edge bead removal cycle (available on most modern spincoaters) or by running a cleanroom wipe lightly wetted with SU-8 developer along the edge. This step is frequently necessary to ensure good quality photomask alignment in subsequent steps.
5. Place the wafer on a hotplate set to 95 °C for the manufacturer recommended preexposure bake time. A typical cycle is 3–5 min. This dries the SU-8 in place and prevents it from running.
6. Place the wafer onto a UV aligner. This step will not require a photomask because an unpatterned base layer is desired. Expose the wafer to UV light for the manufacturer recommended power and time. A typical time for the base photoresist is 3–10 s. An unpatterned base layer enables a strong adhesion for the second layer of SU-8 photoresist.
7. Place the wafer on a 95 °C hotplate for the manufacturer recommended postexposure bake time. A typical cycle is 3–5 min. Cool the wafer on a nonwoven wipe so that it does not cool too quickly and introduce thermal stress into the layer. If cracks in the SU8 film appear, they can be removed by annealing the wafer cyclically on a hotplate at 95 °C and cooling it several times.

8. Spin a second layer of SU-8 onto the wafer to create the micropost of the stamp pattern:
   a. Place the wafer on the spincoater and set the acceleration, rate, and time for the photoresist. A micropost layer will typically be 5–15 μm thick, while a stamp pattern will be from 5 to 50 μm thick.
   b. Repeat step 3b to dispense photoresist. A thicker photoresist will require more volume and more time will be required to allow it to spread properly.
9. Repeat step 4 to remove the edge bead.
10. Repeat step 5 for preexposure bake.
11. Move the wafer onto a UV aligner. Place the chrome mask into contact with the wafer ensuring that full contact is made between the mask and SU8 photoresist. Incomplete removal of edge beads can create a gap between the photoresist and the mask, which will cause diffraction of the UV light and poor pattern transfer. Expose the wafer to UV for the manufacturer recommended power and time. The micropost layer will typically require between 5 and 15 s, while a thicker stamp layer will require between 5 and 60 s.
12. Repeat step 7. Annealing may again be necessary to remove thermal stresses.
13. Develop the features by submerging the wafer in at least 1 cm deep dish of SU-8 developer. Agitate for the recommended time in developer. This will be between 5 and 15 min depending on SU-8 thickness, feature density, and feature size. Larger features with high aspect ratios or close packing will require more time for the developer to penetrate between the structures.
14. Remove the wafer, and while still wet, rinse with fresh SU-8 developer for 10 s on the top surface to remove the residual SU-8. Follow this rinse with an IPA rinse for 10 s. Use filtered nitrogen gas to dry the wafer.
15. A final annealing step may be used if cracks are seen in the SU8 structures. Place the wafer onto a 150 °C hotplate for 5 min and then cool slowly on a nonwoven wipe. This step has the potential to distort the features on the wafer due to reflow of the SU8 photoresist.
16. A final hard-bake step is recommended to fully cross-link the S-8 features on the master. A hard-baked master can be used for repeated polydimethylsiloxane (PDMS) casting without damage to the SU8 structures. Place the wafer onto a hot plate at 150 °C for 30 min, followed by a 200 °C hotplate for 30 min. Cool on a nonwoven wipe.
17. The wafer can now be cleaved and glued onto a glass slide to allow for easier handling and prevent breaking the master.
18. The master should be passivated using fluorosilane to prevent PDMS from permanently bonding its surface.
   a. Treat the master in an oxygen or air plasma for 60–120 s.
   b. Place the master into a silane desiccator with 50 μl of fluorosilane and place under vacuum. This vaporizes and binds the silane to the activated SU-8 surface; at least 1 h is recommended to achieve thorough coverage.

5.2 SOFT LITHOGRAPHY OF PDMS STAMPS AND MICROPOSTS

The following steps describe the process to fabricate PDMS stamps and micropost arrays from a SU8 master. PDMS stamps require a single-casting process from the SU8 master. Micropost arrays require a double-casting process from the SU8 master.

5.2.1 Materials

- SU8 masters for the stamp and micropost array
- Sylgard 184 (PDMS) base and curing agent (Dow Midland)
- Plastic cup
- Plastic stirrer (e.g., plastic 5 ml pipette)
- Aluminum foil, dish, or boat. Its size should match that of the SU8 master
- Razorblade
- Fluorosilane (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane) (T2492-KG, United Chemical Technologies)
- Glass slide
- Glass pasture pipette.

5.2.2 Equipment

- Digital balance
- Vacuum desiccator chamber
- Convection oven
- Plasma chamber (Plasma Prep II, SPI, West Chester, PA)
- N_2 air.

5.2.3 Method for stamps

1. Prepare 20:1 mixture of base and curing agent by weight in a plastic cup using a digital scale.
2. Stir PDMS mixture for 5 min with a plastic stirrer.
3. Degas PDMS mixture in the desiccator for 1 h to remove air bubbles caused by stirring.
4. Place the SU8 master in an aluminum dish or boat and pour degassed PDMS over the master until it is 1 cm thick. Push down on the SU8 master at its corners using a disposable pipette to position it at the bottom of the aluminum dish.
5. Degas the PDMS for 30 min, or until almost all bubbles have dissipated. A gentle puff of N_2 gas can be used to pop air bubbles that are trapped at the surface.
6. Place the aluminum dish in a convection oven at 110 °C for 9–11 min or until the PDMS is firm.
7. Cut the aluminum dish away from the silicon master using a razorblade.

8. Cut away any access PDMS from the bottom of the SU8 master, and along the edges between the PDMS and the SU8 master.
9. Peel the PDMS away from the SU8 master very slowly.
10. Trim and cut PDMS to form 1 cm × 1 cm × 1 cm stamps. Cut a notch at one corner to mark the side opposite of the stamp features.

5.2.4 Method for negative molds for micropost

1. Prepare 10:1 mixture of base and curing agent by weight in a plastic cup using a digital scale.
2. Repeat steps 2–9 above for making PDMS stamps to create several negative copies of the SU8 master of the micropost arrays. These copies will be the negative molds used to make PDMS micropost arrays.
3. Plasma treat the negative molds inside the plasma chamber at 100 W for 90 s to activate the surface of the PDMS. Power and exposure times will differ if another plasma chamber is used.
4. Place negative molds into a glass desiccator with the features oriented face-up. Use tweezers to handle the negative molds.
5. Spread 25–50 μl of fluorosilane on a glass slide in the center of the desiccator using a glass pipette and place the pipette with fluorosilane in its tip on its side inside the desiccator.
6. Apply vacuum to the desiccator and expose the negative molds to fluorosilane vapor for 1 h.

5.2.5 Method for micropost arrays

1. Prepare 10:1 mixture of base and curing agent by weight in a plastic cup using a digital scale.
2. Stir PDMS mixture for 5 min with a plastic stirrer.
3. Degas PDMS mixture in the desiccator for 1 h to remove air bubbles caused by stirring.
4. Oxygen plasma treat the glass slides inside the plasma chamber at 100 W for 90 s.
5. Pipette the degassed PDMS into the negative molds so that the features are completely covered. Remove any air bubbles with a gentle puff of N_2 gas. If necessary, place structure in degasser to remove further bubbles.
6. Place the plasma-treated glass slides on top of the PDMS in the negative molds with the plasma-treated side of the glass facing the PDMS. Start with one edge of the glass on the negative mold and slowly lower it without trapping air bubbles between the PDMS and the slide.
7. Place the negative mold into a convention oven at 110 °C for 6 h in order for the PDMS to fully cure.
8. Remove the negative molds from the oven and allow them to cool to room temperature.

9. Slowly peel the glass slide away from the negative mold to release the micropost arrays on a glass slide. It is recommended to peel along the diagonal direction of the micropost array to prevent the microposts from sticking to each other.
10. Inspect the micropost array under a light microscope to determine whether the array has collapsed microposts, which is not useable.
11. Trim any excess PDMS that could interfere with stamping from the sides and edges.

5.2.6 Discussion of micropost arrays

If we wish to use the same negative mold again, we should treat the molds with plasma and fluorosilane every two castings. However, we will need to inspect the mold under the light microscope before each use to ensure that it has not developed any surface cracks for distortions in the array (Tooley, Feghhi, Han, Wang, & Sniadecki, 2011).

To watch cells on microposts live under an inverted microscope, a glass coverslip should be used instead of a glass slide. The thickness from the top surface of microposts to the objective lens should be less than the working distance of an objective. For example, the working distance for a 40× oil immersion objective is approximately 200 μm. Thus, we use the microposts (on a cover glass) to the bottom surface of a Petri dish with a hole directly.

5.3 STAMPING MICROPOST ARRAYS FOR CELL CULTURE

A stamp-off method is used to print a pattern of extracellular matrix onto the tips of the microposts. These steps are minor modification of the steps described in Chapter 1. Two types of stamps are made: a "flat stamp" made by casting against a featureless master and a "negative stamp" with features used for removing protein from the flat stamps, that is, negative features (Fig. 5.1).

5.3.1 Materials

- PDMS flat stamp (1:20 PDMS)
- PDMS negative stamp (1:20 PDMS)
- PDMS micropost arrays (1:10 PDMS)
- Sterile DI
- 50 μg/ml human fibronectin (FN) in sterile DI water (BD Biosciences)
- Ethanol (100% and 70%)
- 5 μg/ml bovine serum albumin (BSA) conjugated with Alexa Fluor 594 (A13101, Invitrogen) or 1 μg/ml fluorescent lipophilic carbocyanine (DiI) solution prepared in DI (D3886, Invitrogen)
- 0.2% Pluronic F127 (BASF, Mount Olive, NJ)
- Phosphate buffer solution (PBS)
- Aluminum foil
- 100-mm Petri dish.

5.3.2 Equipment

- Sterile biosafety cabinet
- UV-ozone (UVO cleaner; Jelight, Irvine, CA)
- Nitrogen gas
- Sterile tweezers.

5.3.3 Method

1. All steps are to be completed while working in a sterile biosafety cabinet.
2. Place flat stamps in a Petri dish with working side up. Place droplets of FN solution onto the corners of the stamps, then on the edges, and lastly on the interior regions. As the protein in solution is adsorbed on the PDMS surface, the surface of the stamp becomes hydrophilic. This process of starting at the corners and edges requires a lower volume of FN solution to coat the stamps.
3. Incubate each stamp with FN solution for 1 h.
4. Wash stamps with DI water by pouring it into the dish at the edge and letting the water level rise over the stamps.
5. Transfer stamps into a second Petri dish filled with DI water. After washing, dry them with N_2.
6. Treat the negative stamps with UV-ozone for 7 min to make them more hydrophilic. This step is performed outside of the biosafety cabinet.
7. Using tweezers, place the patterned side of the negative stamps in contact with the protein-coated side of the flat stamps. Tap lightly on the stamps with tweezers to ensure full contact. Remove the stamps 5 s after contact. The flat stamps are now ready for patterning the microposts.
8. Treat the micropost arrays with UV-ozone for 7 min to activate the micropost surface for stamping. This step is performed outside of the biosafety cabinet.
9. Place the flat stamp in contact with the microposts. Tap lightly the top of the stamp with tweezers to ensure all areas have good contact, but be careful not to collapse the posts. It is helpful to practice this step under an inverted light microscope to figure out how much tapping pressure is needed. Carefully remove flat stamps with tweezers.
10. Submerge the micropost arrays in a Petri dish with 100% ethanol for 10 s. This step is used to wet the surface and then subsequent dilution washes the ethanol away.
11. Transfer micropost arrays to a Petri dish with 70% ethanol for 10 s. This step has a secondary benefit of sterilizing the micropost substrates and so from this point forward substrates should be handled using aseptic techniques.
12. Transfer micropost arrays to a Petri dish with sterile DI water for 10 s. The PDMS microposts are very hydrophobic and the surface tension of water can cause them to collapse together. Take caution in keeping the arrays from dewetting during transfer by moving carefully between dishes.
13. Repeat step 12 with two more dishes of sterile DI water.

14. Transfer the micropost arrays to a Petri dish with the fluorescent dye.
    a. For BSA, place droplets of BSA solution onto the bottom surface of a Petri dish and place the micropost arrays upside down onto each droplet.
    b. For DiI, submerge the micropost arrays in a Petri dish with DiI solution. DiI provides a stronger fluorescent signal than BSA.
15. Cover with aluminum foil and incubate with dye for 1 h.
16. Wash microposts with DI water for 10 s. For DiI, rinse the microposts thoroughly with sterile DI water after treatment to remove excess DiI, which can be taken up into the membranes of the cells during cell culture.
17. Place droplets of Pluronic solution in a Petri dish and place the micropost arrays upside down onto each droplet. Incubate for 30 min. This step is used to block the unstamped portions of the micropost, including the base and sidewalls, so that cells will not adhere there.
18. Repeat step 12 with two dishes of sterile DI water. Pluronic treatment makes the surface of PDMS hydrophilic so less caution is needed in transferring between dishes since they are not easily dewetted.
19. Transfer microposts into a dish with PBS. Microposts arrays in PBS can be stored at 4 °C for up to 1 week, but better results are obtained if used immediately for cell plating.

5.3.4 Discussion

Stamping is an essential step in the biofunctionalization of micropost arrays. During this step the matrix of interest is transferred to the tips of the posts for cells to adhere and spread on. We have been able to stamp fibronectin, fibrinogen, von Willebrand factor, collagen type I, and collagen type IV.

Stamping is challenging on softer microposts (low spring constant) for a large number of them collapse under the pressure of the stamp or during placement or removal of the stamp. In these cases occasionally stamping protocols are modified by stamping substrates under water. However, this modification adds to the limitations of the preparation of the substrate since once the posts are wet all the rest of protocol should also be done in a wet environment to avoid collapsing the micropost due to the force of water's surface tension.

An alternative method can be used to adsorb the protein of interest to the tips of the microposts which bypasses some of the limitations discussed. This method is called the *droplet adsorption method* and is based on the interaction of a water-based droplet with a hydrophobic surface. Micropost arrays are made of PDMS which presents hydrophobic characteristics and the topology of the microposts also adds to the overall hydrophobicity of the surface. In this method the protein of interest is dissolved in a water-based solution. The droplet is then placed on the micropost array. The overall hydrophobicity of the substrate prevents the droplet from penetrating between the microposts and wetting the side walls of the microposts. Therefore, the protein is only transferred to the tips of the posts, which is equivalent to regular stamping for stiffer substrates.

5.4 SEEDING CELLS

Follow these general procedures to culture cells onto the micropost arrays. Cell detachment procedures may differ depending on the cell type.

5.4.1 Materials

- Chosen cell line
- Trypsin–Ethylenediaminetetraacetic acid (EDTA)
- Growth medium
- Tissue culture dish
- PBS.

5.4.2 Equipment

- Sterile biosafety cabinet
- Sterile tweezers.

5.4.3 Method

1. Cells should be at or near confluence in the starting tissue culture dish or flask. This is necessary so that a sufficient number of cells are available to land on the microposts.
2. Micropost substrates should be placed onto a tissue culture dish containing prewarmed media and allowed to incubate for 1 h prior to seeding.
3. Aspirate away the growth media in the cell culture dish or flask.
4. Rinse the cells with PBS to remove residual media in the dish.
5. Detach the cells from the dish or flask using trypsin–EDTA solution. A typical volume is 1 ml of trypsin–EDTA per 50 cm^2 of surface area; however, this will depend on the cell type and cell age. Cells that have been cultured longer will typically require more time. Place the cells with the trypsin–EDTA solution in an incubator and check periodically to see if they have detached from the surface.
6. Once the cells have detached, resuspend the cells in prewarmed media with gentle agitation to separate groups into individual cells.
7. Pipette the cell solution into the dish containing the micropost substrates. Place this dish into the incubator and allow cells to settle onto the microposts. Take periodic observations to gauge the density of cells that have landed on the microposts. If a significant portion of them have settled, then a rinse with fresh prewarmed media can be done to remove unattached cells. This step may or may not be necessary depending on the type of cell being seeded.
8. Allow at least 24 h for the cells to spread and stabilize on the micropost tips before traction force measurements are taken.

GENERAL CONCLUSIONS

The steps described in this chapter cover the fabrication of stamps and microposts. These techniques are meant as a starting point for using microposts and not the *de facto* approach. We have found it necessary to adjust the steps or conditions in order to optimize the process to suit a particular set of experiments or cell type. Furthermore, it is highly recommend that a new user should watch a visual demonstration of some of the techniques used for micropost arrays in order to gain an understanding of how to work with them during their preparation (Desai et al., 2007).

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