CHAPTER

Polyacrylamide Hydrogel Micropatterning

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CHAPTER OUTLINE

Methods in Cell Biology, Volume 120 **ISSN 0091-679X**

http://dx.doi.org/10.1016/B978-0-12-417136-7.00006-9

Abstract

This chapter describes the production of micropatterns of extracellular matrix proteins on a 2D flat polyacrylamide (PAA) gel. The technique is divided into two parts. First, micropatterns are produced on glass or directly on a photomask using deep UV. Then the micropatterns are transferred on acrylamide gel by polymerization of the gel directly on the template coverslip.

This procedure is easy to perform and does not require any expensive equipment. It can be performed in no more than 2 h once you get your hands on it. It combines the advantages of other existing techniques: good spatial resolution, suitable for very soft gel, no need for the use of chemical crosslinkers for attachment of the proteins to the acrylamide, no modification of the mechanical properties of the gel by the process, and suitable for multiple protein patterning.

We also discuss the storage issues of such substrates and provide a brief review of other existing techniques for micropatterning on PAA.

INTRODUCTION AND RATIONALE

Since the introduction of cell culture experiments in Petri dishes, several technical improvements have been developed to better reproduce *in vitro* the actual physiological metazoan cell microenvironment. Microenvironment geometry and architecture can be mimicked and modulated using surface micropatterning. It consists of the creation of extracellular matrix (ECM) protein islands of controlled size and shape, called micropatterns, surrounded by antifouling polymers preventing nonspecific protein and cell adhesion. Surface micropatterning has already revealed the implication of cell adhesive microenvironment geometry in the regulation of many critical physiological processes (cell shape, cell architecture, internal cell organization, cell migration, cell division, cell differentiation, tissue architecture, etc.) (Théry, 2010; Vignaud, Blanchoin, & Théry, 2012).

Microenvironment rigidity has also been shown to be a key parameter in the regulation of several key physiological processes, including pathological ones (cell polarity, cell growth, cell differentiation, tumoral transformation, etc.) (de Rooij, Kerstens, Danuser, Schwartz, & Waterman-Storer, 2005; Engler, Sen, Sweeney, & Discher, 2006; Klein et al., 2009; Pitaval, Tseng, Bornens, & Thery, 2010; Prager-Khoutorsky et al., 2011).

As both the spatial organization of the ECM protein and the substrate stiffness have implications for cell physiology it is relevant to combine both in order to faithfully reproduce and control cell microenvironment. Polyacrylamide (PAA) hydrogels have several interesting physico-chemical properties that are useful for protein and cell micropatterning. They are optically transparent, low cost, and chemically simple compounds that can be used in almost any lab. They have been used for decades in molecular biology, notably for the manufacturing of western blots, due to the possibility to modulate their mesh size by changing the ratio of monomers to crosslinkers before polymerization. Interestingly, this mesh size is also related to the stiffness of the gel and this technique has been successfully adapted for the production of cell culture substrates of defined mechanical properties (Pelham & Wang, 1997). Importantly for mechanical measurements, the stiffness of the gel does not depend on the applied strain (Storm, Pastore, MacKintosh, Lubensky, & Janmey, 2005). In addition, PAA has constitutive antifouling properties preventing nonspecific protein and cell adsorption. Alternatively, acrylamide groups can be used as a substrate to make covalent link with proteins of interest. Therefore, many recent efforts have been devoted to the development of experimental methods to micropattern proteins on PAA hydrogels (Damljanovic, Christoffer Lagerholm, & Jacobson, 2005; Grevesse, Versaevel, Circelli, Desprez, & Gabriele, 2013; Polio, Rothenberg, Stamenović, & Smith, 2012; Rape, Guo, & Wang, 2011; Tang, Yakut Ali, & Saif, 2012; Tseng et al., 2011; Versaevel, Grevesse, & Gabriele, 2012; Wang, Ostuni, Whitesides, & Ingber, 2002; Yu, Xiong, Tay, Leong, & Tan, 2012; Zhang, Guo, Rape, & Wang, 2013). However, these methods still have intrinsic limitations. Most of them involve a microcontact printing (μCP) step to physically pattern the proteins onto the PAA. This step is time consuming, poorly reproducible in terms of amount of transferred proteins, and has a limited spatial resolution (typically few micrometers). We circumvent these limitations by using a direct (one step) activation of the PAA with deep UV through a photomask in contact with the gel (Tseng et al., 2011) and improve the spatial resolution of the micropatterns. In addition, most methods require chemical crosslinkers such as sulfosuccinimidyl 6-[4′-azido-2′-nitro-phenylamino]hexanoate (sulfo-SANPAH) or *N*-Hydroxysuccinimide (NHS)–ethyl(dimethylaminopropyl) carbodiimide (EDC) to bind the proteins of interest to the PAA. However, these reagents are poorly stable in the presence of water and the efficiency of the crosslinking is variable. Recently, Wang and colleagues used PAA polymerization itself to directly bind the protein of interest and transfer prepatterned proteins onto the PAA (Rape et al., 2011). Here we propose two rapid, accurate, reliable, and easy-to-use methods that combine all these improvements. They are based on production of micropatterns on hard substrate using deep UV photopatterning followed by protein transfer on PAA hydrogel. Thereby, we associated the advantages of deep UV patterning (micrometer to submicrometer resolution, production of highly reproducible

micropatterns) to the efficacy and reproducibility of protein covalent linking with acrylamide polymerization. The "glass method" is based on the transfer from a micropatterned glass coverslip while the "mask method" produces micropatterns directly on the quartz photomask before transfer on PAA, thus bringing the resolution of the technique to submicrometer level (Fig. 6.1I and II).

6.1 SAFETY RECOMMENDATIONS

Many of the reagents used during the processes described below are potentially dangerous. Acrylamide, acetone, isopropanol, silane, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) are very volatile compounds that should be handled under a chemical hood with adapted personal security protection (lab coat, gloves, protective glasses) and should be discarded specifically as they usually have particular destruction circuits.

The UV lamp will produce some ozone gas by the reaction of UV with the dioxygen from the air. As a consequence, the UV lamp should also be placed in a chemical hood.

6.2 PREPARATION OF REAGENTS COMMON TO BOTH TECHNIQUES

6.2.1 Materials

- Glass coverslip no. 1 (Knittek glass, Germany)
- Ethanol
- Silane solution (3-(Trimethoxysilyl)propyl methacrylate, M6514, Sigma, USA)
- pLL–PEG as powder (PLL20K-G35-PEG2K, JenKem Technology, USA)
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (HN 77.5, Carl Roth, Germany)
- Parafilm
- Ice
- Acrylamide solution (01697, Fluka Analytical, USA)
- *N*,*N*′-Methylenebisacrylamide solution (66675, Fluka Analytical, USA)
- TEMED (T9281, Sigma, USA)
- Ammonium persulfate (APS) (A3678, Sigma, USA)
- Water milliQ
- Filter 0.222 μm pore size (SLGP033RS, Millex, IRL)
- Acetic acid

6.2.2 Equipment

- pH meter
- Plasma cleaner
- Beaker
- Oven
- Your favorite metallic tweezers to handle glass coverslip
- Upright fluorescence microscope

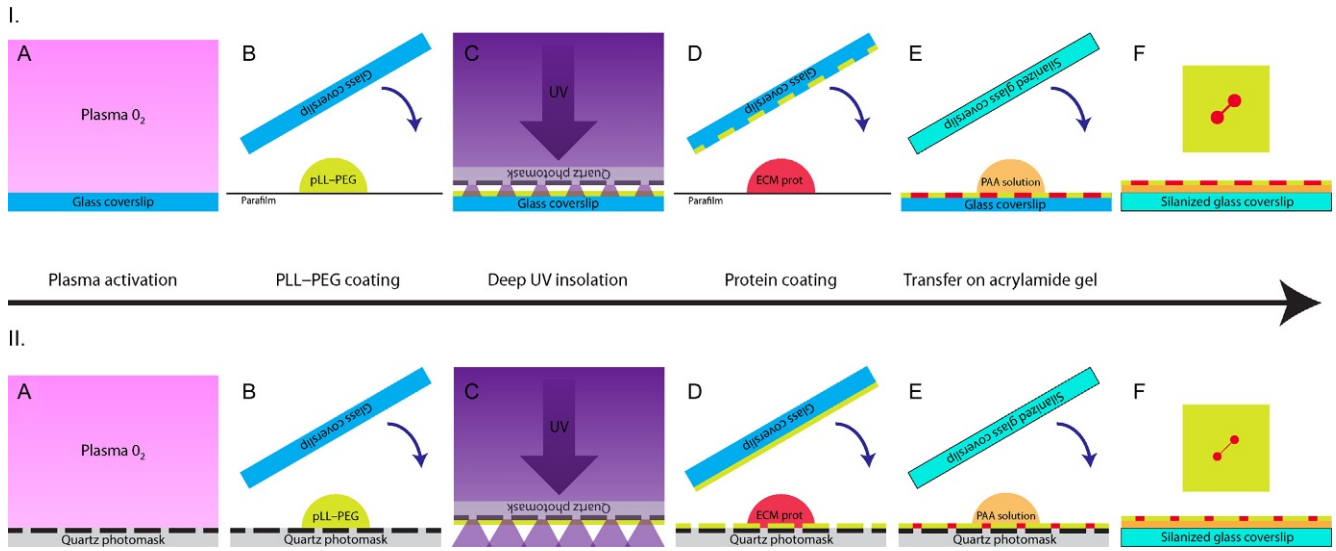

FIGURE 6.1

Description of the procedure. Two variants of the same technique are described. One is using the transfer from micropatterns produced on glass while the other from micropatterns directly produced on the quartz photomask. This latter procedure allows for production of micropattern with submicrometer spatial resolution. I. Transfer from a micropatterned glass coverslip (referred to as the "glass method"). This process consists in glass activation by plasma (a), coating with the repellent compound poly-L-lysine–PEG (pLL–PEG) (b), surface activation of the surface through a chrome photomask using deep UV (c), extracellular matrix (ECM) protein adsorption on the UV-activated sites (d) leading to the production of a glass micropatterned coverslip as previously described (Azioune et al., 2010). Then, a drop of PAA solution mix is sandwiched between the patterned coverslip and a silanized glass coverslip (e). After 30 min polymerization, the patterned coverslip is detached from the acrylamide gel while ECM protein remains on the gel (f). Note that due to the diffraction of the UV light at step (c), the shape of the final micropatterns is larger than the original on the photomask. II. Transfer from micropatterns produced directly on the quartz photomask (referred to as the "mask method"). This process is the same as previously except that the initial micropatterns are now produced directly on the quartz photomask. First, the mask and a glass coverslip are activated together with plasma (a), then a pLL–PEG drop is sandwiched between the chrome side of the mask and the glass coverslip (b). After 30 min incubation, the glass coverslip is removed and saved for step (d) as it is now a passivated surface. The photomask is exposed to deep UV from the quartz side (c), activating the pLL–PEG at defined loci with minimum loss of resolution due to diffraction. Then again, a drop of ECM protein is sandwiched between the mask and the passivated glass coverslip and incubated for 30 min (d). Transfer on acrylamide is then performed as in I (e and f).

6.2.3 Methods

6.2.3.1 Coverslip silanization

- This glass treatment is necessary to ensure a good attachment between the PAA gel and the underlying coverslip.
- As silane solutions are very toxic, as many steps as possible of this process should be performed under a chemical hood with appropriate user protection, at least the silane solution should not leave the hood outside of a hermetically closed container.
- Start the oven at 120 °C and let it warm up.
- Warm up the pump of the plasma cleaner for few minutes (according to manufacturer's instruction).
- In a 500 mL beaker, prepare a solution of ethanol containing 2% (v/v) 3-(trimethoxysilyl)propyl methacrylate and 1% (v/v) acetic acid (silanization solution).
- Put the glass coverslips in your plasma cleaner either horizontally or in a specific holder that will allow both sides of coverslips to be in contact with the ionized gas during the plasma treatment.
- Start pumping out the air in the reactor and wait for the pressure to stabilize (2 min).
- Open the oxygen inlet, set the gas flow to 5 mL/min (sscm) and wait 2 min for the pressure to stabilize (if you can control the pressure on your device, set it to 1 Torr = 133 Pa).
- Run the plasma for 3 min at 100 W.
- Close the gas inlet, stop pumping, and ventilate the reactor (a filter should be placed on the air inlet to avoid dust intake into the reactor).
- If the coverslips were horizontal in the reactor, flip them and repeat the plasma process (vacuum–oxygen–plasma).
- Soak the coverslips in the silanization solution one by one for 10 min altogether in the solution, shake occasionally.
- Discard solution.
- Rinse once with ethanol in the same beaker and then remove them from the ethanol one by one with tweezers (while others staying in the ethanol) and rinse once again in another beaker of ethanol (keep holding the coverslip with the tweezers) and finally blow off the ethanol carefully using pistol airflow and place them on the oven plate.
- Cure for 1 h at 120 °C.
- Blow off dust with pistol airflow and store at room temperature. This treatment is quite stable over few weeks so you can do many coverslips at the same time to avoid always repeating this fastidious time-consuming process.

6.2.3.2 pLL–PEG solution preparation

- This solution will be used for the passivation of coverslips before UV insolation and protein coating to avoid unspecific adsorption of protein outside of the insolated area.

- pLL–PEG is usually received as powder and should be stored under protective atmosphere (Argon) if possible, at −20 °C. The final concentration we want to achieve is 0.1 mg/mL. Since the powder is usually made of grains that weigh a few mg each, we first produce 1 mg/mL solution that is aliquoted and stored at −20 °C. The final solution will be diluted from stock.
- Prepare HEPES 10 mM from powder and milliQ water.
- Equilibrate the pH of the HEPES solution to 7.4 using NaOH.
- Weigh the pLL–PEG and add corresponding HEPES volume to reach a final concentration of 1 mg/mL. Then filter the solution using a syringe and a filter of 0.22 μm mesh size. Aliquot the solution and store at −20 °C.
- When needed, thaw an aliquot and dilute it 10 times in HEPES solution to achieve at 0.1 mg/mL pLL–PEG concentration. The pLL–PEG solution should be then stored at 4 °C and used within few days.

6.2.3.3 Preparation of acrylamide solution and polymerization reagent

- Again, as acrylamide is carcinogenic, handle it with care under chemical hood and using proper user protection.
- Gel stiffness from a given acrylamide/bis-acrylamide ratio was reproducible in our hands but the stiffness measured using atomic force microscopy (AFM) for a given acry/bis-acrylamide ratio was very different from those reported by others, so we highly recommend to verify the actual stiffness of the gel in your own experimental conditions. As a starting point, one can use the table from Tse and Engler (2001) which covers a wide range of stiffnesses.
- Mix acrylamide and bis-acrylamide solution in water to obtain the desired concentration.
- This solution can be stored for few weeks at 4 °C.
- TEMED solution was used as received without further preparation.
- APS solution was prepared from powder in water milliQ at a concentration of 10% w/w and immediately frozen in small 10 μL aliquots and stored at −20 °C.
- Since APS is not very stable, one aliquot was used for each experiment and the remaining solution was systematically discarded.

6.3 ACRYLAMIDE PATTERNING FROM UV GLASS PATTERNING ("GLASS METHOD")

6.3.1 Materials

- ECM protein solution (i.e., Fibronectin solution (FF1141, Sigma, USA) and fibrinogen (FNG) Alexa Fluor 647 conjugate solution (F35200, Invitrogen, USA)
- Photomask (Toppan, France). Be careful to use a photomask compatible with deep UV exposure (see Azioune, Carpi, Tseng, Théry, & Piel, 2010 for more information)
- Silanized glass coverslip (see Section 6.2.3.1)

- Glass coverslips
- pLL–PEG solution (0.1 mg/mL in HEPES 10 mM, see Section 6.2.3.2)
- Sodium bicarbonate solution 100 mM pH 8.3 (0865 AMRESCO, USA)
- Trypsin/Ethylenediaminetetraacetic acid (EDTA)
- Cell culture medium
- Dulbecco's phosphate buffered saline (DPBS) (14190, Gibco, France)
- Cell culture dish

6.3.2 Equipment

- Deep UV lamp (UVO Cleaner Model NO.342A-220, Jelight Company, USA)
- Oxygen plasma oven
- Vacuum mask holder (custom, SMGOP, France) (see supplementary information for a picture of the mask holder) http://dx.doi.org/10.1016/B978-0-12-417136-7.00006-9
- Vacuum bell

6.3.3 Methods

6.3.3.1 pLL–PEG glass coating

- Remove dust from the glass coverslips.
- Warm up the pump of the plasma cleaner for few minutes (according to manufacturer's instruction).
- Put the glass coverslips in your plasma cleaner either horizontally or in a specific holder that will allow both sides of coverslips to be in contact with the ionized gas during the plasma treatment.
- Start pumping out the air in the reactor and wait for the pressure to stabilize (2 min).
- Open the oxygen inlet, set the gas flow to 5 mL/min (sscm), and wait for the pressure to stabilize (2 min).
- Run the plasma for 15 s at 30 W.
- Close the gas inlet, stop pumping, and ventilate the reactor (a filter should be placed on the air inlet to avoid dust intake into the reactor).
- Meanwhile put a drop of pLL–PEG solution (25 $\mu L/cm^2$) on parafilm.
- Take the coverslip with tweezers and flip it on the droplet in order to have the plasma-activated side of the coverslip facing the pLL–PEG solution and let incubate for 30 min.
- Afterwards, gently lift up the coverslip from the side using tweezers and put it vertically. Let the pLL–PEG run off by gravity. If needed, pipette the solution and put it back on the coverslip to help it run off the coverslip. Usually, one drop remains at the corner of the coverslip. You can remove it by gentle airflow from the other side of the coverslip in the direction of the corner.
- Store the coverslips at 4 °C with nothing in contact with the treated side of the coverslip and use within 2 days.

6.3.3.2 Deep UV insolation

- At this step, we will burn the passivized surface at specific positions by shining UV light through the chrome photomask. This will then allow us to adsorb protein at these specific positions.
- Heat up the UV lamp. This is very important. Power measurements of the lamp have shown that the steady state power is reached after 2–5 min (see supplementary information figure 1 for curve of the power in function of time) http://dx.doi.org/10.1016/B978-0-12-417136-7.00006-9 depending on the age of the lamp. We usually let it run for 5 min and then immediately put the sample inside the lamp and start the insolation process. The power measured at steady state was 6 mW/cm^2 at a distance of 1 cm from the lamp and a wavelength of 190 nm (you should take care to control the power frequently).
- Clean the photomask. First rinse it with milliQ water, then remove the liquid carefully using nitrogen gas flow, repeat the procedure with acetone and then isopropanol.
- Remove dust from the pLL–PEG coated glass coverslips, then put them on the vacuum mask holder, treated sides facing up. You should put at least three coverslips to have the mask horizontal. Then put carefully the mask on top, with the chrome side facing the coverslips. Plug the mask holder to house vacuum. The mask holder has a grid etched on it that corresponds to the grid we design on the photomask to easily find the shapes we want to produce. Then, put metallic pillars on two sides of the grip. Put the border of the mask in contact with these pillars in order to have a proper positioning of the coverslips in front of the desired matrix of shape on the mask.
- Put the entire setup in the warmed up UV lamp with the mask a few millimeters from the UV source. Expose for 5 min.
- Unplug the mask holder from the vacuum. Then carefully flip the photomask. If the contact between the mask and the coverslip was sufficient (no dust, no leaking of air from the side of the mask holder), the coverslips should stay stuck to the photomask.
- Detach the coverslips from the mask using a flexible tube connected to vacuum. Then store them activated side facing up at 4 °C and use within few days.

6.3.3.3 Protein coating and transfer on acrylamide gel

- The protein coating and the transfer on acrylamide gel should be performed in succession because otherwise the transfer is not as efficient. Here we will attach the ECM protein at the UV-activated sites on the glass coverslips and then transfer this protein on acrylamide gel by polymerization in contact.
- Prepare protein coating solution: we use a solution of 20 μg/mL of fibronectin diluted in sodium bicarbonate 100 mM. A small amount of fluorescently labeled protein could be added in order to see the micropatterns by fluorescence microscopy (typically 2 μg/mL of fibrinogen (FNG)—Alexa 647). Store the solution on ice.

- Rinse once the insolated side of the pLL–PEG coated coverslip with sodium bicarbonate and let the solution run of by putting the coverslip vertically. If some solution remains on the other side, it is not a problem.
- Put a droplet of protein solution on parafilm (25 μL/cm^2) and then put the pLL–PEG–UV-insolated side of the coverslip on the droplet. Protect from light and let it incubate for 30 min.
- In the meantime, aliquot the desired amount of acrylamide solution and allow it to degas in a vacuum bell.
- At the end of the incubation, put the glass vertically and let it dry. Then rinse three times with PBS.
- If you have used fluorescently labeled protein, check the quality of the procedure with fluorescence microscopy.

6.3.3.4 Transfer on acrylamide gel

- Here we will polymerize the acrylamide gel sandwiched between the patterned coverslip and the silanized coverslip. During detachment, the gel will stay attached to the silanized coverslip and the protein from the patterned coverslip will be transferred to the free surface of the acrylamide gel, resulting in a micropatterned acrylamide surface.
- If possible, one should use a silanized coverslip and patterned coverslips of two different sizes because it will then be much easier to detach them from each other.
- Put the larger of the two coverslips on a parafilm with the side of interest facing up. If both are of the same size, put a small drop of water on the parafilm and cover it with the patterned coverslip with the side of interest facing up. The small drop of water will prevent the acrylamide solution from sliding under the patterned coverslip.
- Collect the acrylamide solution from the vacuum bell and keep the container closed.
- *Optional*: If you want to add some fluorescent beads to your gel for force measurements, they should be added at this stage of the process in the acrylamide solution and the solution should be sonicated for 5 min to destroy any bead aggregates that could have formed during storage.
- Prepare TEMED, APS, and other coverslips. You will add TEMED and APS solution to the acrylamide with the following proportions: 1 μL of TEMED and 1 μL of APS 10% for 165 μL of acrylamide solution. You should proceed as fast as possible in the next steps.
- Add TEMED to the acrylamide solution, mix briefly but vigorously.
- Add APS solution to the acrylamide solution, mix briefly but vigorously.
- Put a drop of 7 μL/cm^2 of the acrylamide polymerization mix on each glass coverslip previously placed on parafilm.
- Slowly place the other coverslip of interest on top while taking care to avoid bubbles.

- Put a cap (to prevent evaporation) and let the gel polymerize for 30 min. Keep the rest of the acrylamide in a closed container as a control of gel polymerization.
- Once the polymerization is finished (you should check it by detaching the remaining acrylamide from the tube, it should have the shape of the container and be elastic if you try to pinch it with a pipette tip), immerse the sandwiched coverslips in PBS and let the gel hydrate for 5 min.
- Detach the patterned glass coverslip from the acrylamide gel using a scalpel, make sure that the gel is fully immersed during the entire detachment process, otherwise you will end up with collapsed micropatterns.
- Rinse the acrylamide gel attached to silanized coverslip (acrylamide coverslips) in PBS several times.
- Control quality with fluorescence microscopy if possible.
- Store at 4 °C and use as soon as possible.

6.3.3.5 Cell seeding

- Warm up your cell culture reagents as usual.
- Using ethanol sterilized tweezers, transfer the acrylamide coverslip in sterile tissue culture petri dish filled with sterile PBS (gel facing up).
- Rinse once with PBS and cover with warmed medium.
- In the meantime resuspend your cell in warmed medium.
- Remove the medium from the petri dish and center the coverslip in the middle of the dish. This way, if convection movements of fluid tend to aggregate cell in the middle of the dish, it will be over the acrylamide gel.
- Gently cover the gel with the cell suspension (a deposition of 100,000 cells/cm^2 has shown optimal cell attachment for RPE1, this should be adapted to your favorite cell line).
- Put the petri dish in the incubator.
- Check the cell attachment regularly (every 30 min). When a substantial number of cells have started spreading on micropatterns, renew the medium to remove unattached cells and replace in the incubator for further spreading.
- Have a nice experiment!

6.4 ACRYLAMIDE PATTERNING FROM PATTERNING ON QUARTZ PHOTOMASK ("MASK METHOD")

Here the procedure relies on the same principles except that the first patterned surface is produced directly on the chrome photomask. This allows higher resolution and thus will produce much more defined structures due to the absence of diffraction issues during insolation. We will activate the chromium side of the mask, coat it with pLL–PEG to prevent unspecific protein adsorption, burn the coating by shining UV through the shapes of the photomask, coat the insolated zones with ECM protein, and finally transfer these motifs by acrylamide polymerization in contact.

6.4.1 Materials

- ECM protein solution (i.e., Fibronectin solution (FF1141, Sigma, USA) and FNG (Alexa Fluor 647 conjugate solution (F35200, Invitrogen, USA))
- Photomask (Toppan, France). Be careful to use a photomask compatible with deep UV exposure (see Azioune et al., 2010 for more information)
- Silanized glass coverslip (see Section 6.2.3.1)
- Glass coverslips
- pLL–PEG solution (0.1 mg/mL in HEPES 10 mM, see Section 6.2.3.2)
- Sodium bicarbonate solution 100 mM pH 8.3 (0865 AMRESCO, USA)
- Trypsin/EDTA
- Cell culture medium
- DPBS (14190, Gibco, France)
- Cell culture dish

6.4.2 Equipment

- Deep UV lamp (UVO Cleaner Model NO. 342A-220, Jelight Company, USA).
- Oxygen plasma oven big enough for the photomask to fit in, you can also consider cutting the mask into pieces that you can handle separately, since you do not need to use the vacuum mask holder in this case.
- Vacuum bell.

6.4.3 Method

6.4.3.1 pLL–PEG quartz mask coating

- Remove dust from the glass coverslips and clean the photomask. First wash it with soap, then rinse it with water milliQ, then remove the liquid carefully using nitrogen gas flow.
- Put the mask (chromium side facing the air) and the coverslips in the plasma.
- Start pumping out the air in the reactor and wait for the pressure to stabilize (2 min).
- Open the oxygen inlet, set the gas flow to 5 mL/min (sscm), and wait 2 min for the pressure to stabilize (if you can control the pressure on your device, set it to 1 Torr = 133 Pa).
- Run the plasma at 100 W for 3 min. This time is higher than for glass because the photomask is reused several times while glass coverslips are usually already quite clean. Exposition to successive coatings of protein makes it necessary to use a long plasma treatment to clean the mask properly.
- Close the gas inlet, stop pumping, and ventilate the reactor (a filter should be placed on the air inlet to avoid dust intake into the reactor).
- Put one drop of pLL–PEG solution (25 $\mu L/cm^2$) on the region of interest on the mask.
- Cover the drop by flipping the activated glass coverslip on it and let it incubate for 30 min.
- At the end of the incubation, lift the coverslips carefully without scratching the coating on the photomask. Put the photomask vertically and let it dry. The solution should run off by itself. If it is not the case, you can collect again the solution that has

fallen down and put it back on the region of interest. This should help the drying process. As the coverslips have been coated at the same time, we will keep them for the incubation with the ECM protein; they will provide a fully passivized surface that will be used to sandwich the ECM droplet on the activated mask after UV insolation. You should rinse them once with water and let them dry. Be careful to remember which size has been coated with pLL–PEG to prevent any damage on this side.

6.4.3.2 Deep UV insolation and protein coating

- At this step, we will burn the passivized surface at specific positions by shining UV light through the chrome photomask from the unpassivized side. The UV light will burn the passivized treatment directly on the mask and this will then allow us to adsorb protein at these specific positions.
- Heat up the UV lamp. This is very important. Power measurements of the lamp have shown that the steady state power is reached after 2–5 min depending on the age of the lamp. We usually let it run for 5 min and then immediately put the sample inside the lamp and start the insolation process. The power measured at steady state was 6 mW/cm^2 at a distance of 1 cm from the lamp and a wavelength of 190 nm (you should take care to control the power frequently).
- Flip the mask to have the coated side away from the UV source. You can use small holders on the squares of the mask to prevent scratching of the coating. Expose to UV for 5 min.
- Prepare protein coating solution: we use a solution of 20 μg/mL of fibronectin diluted in sodium bicarbonate 100 mM. A small amount of fluorescently labeled protein could be added in order to see the micropatterns by fluorescence microscopy. Store the solution on ice.
- Remove the mask from the UV lamp and set it on a horizontal surface, passivized side now facing up.
- Put a droplet of protein solution (25 μL/cm^2) on the region of interest and then put the pLL–PEG coated coverslips (saved at Section 6.4.3.1) on the top, passivized side facing the droplet. Protect from light and let it incubate for 30 min.
- In the meantime, aliquot the desired amount of acrylamide solution and put it to degas in a vacuum bell.
- At the end of the incubation, remove the glass coverslips and discard them. Put the mask vertically and pour some bicarbonate solution on it to rinse. Let the solution dry by itself.

6.4.3.3 Transfer on acrylamide gel

- Here we will polymerize the acrylamide gel sandwiched between the patterned photomask and the silanized coverslips. During detachment, the gel will stay attached to the silanized coverslip and the protein from the patterned mask will be transferred of the free surface of the acrylamide gel, resulting in a micropatterned acrylamide surface.
- Set the photomask horizontally with the pattern side facing up. Make sure that you have waited long enough for the solution to dry.

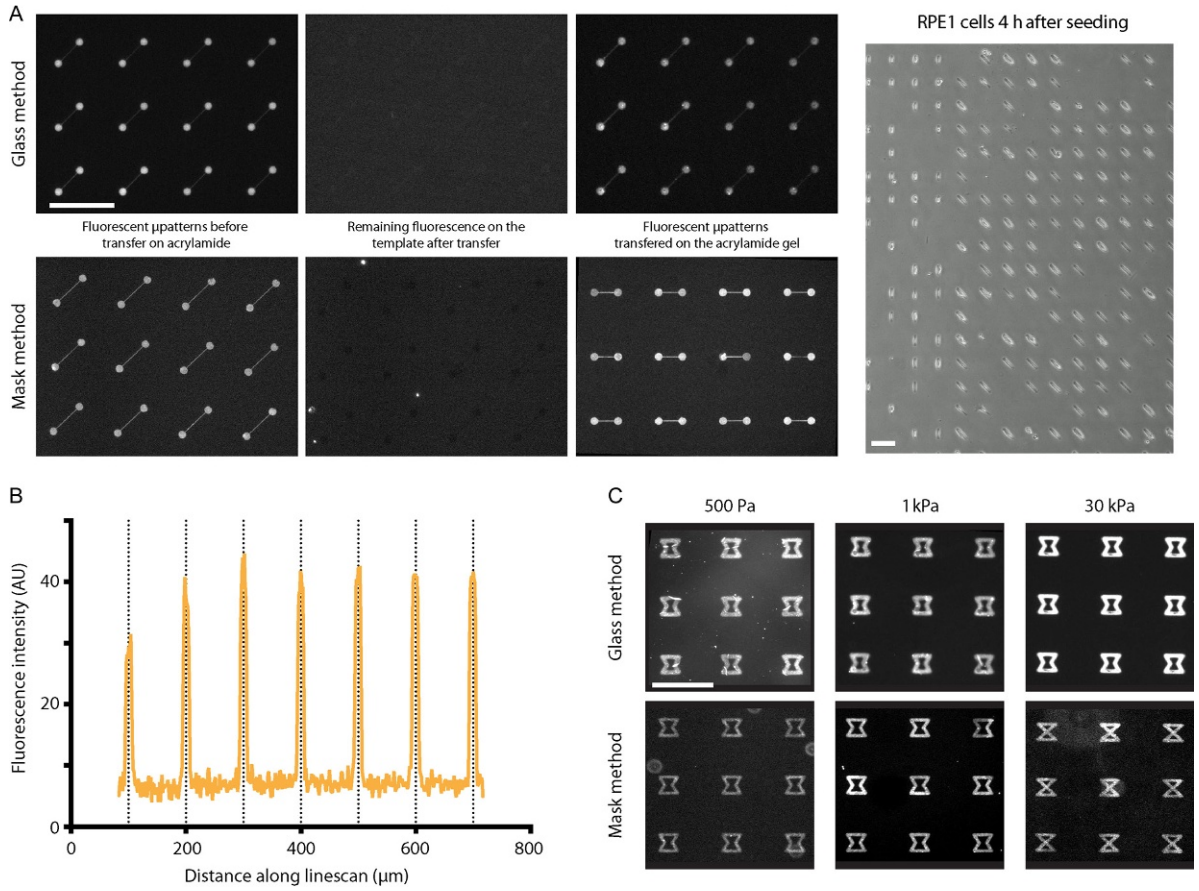

FIGURE 6.2

Both techniques provide robust protein transfer over a wide range of PAA stiffnesses. (A) Both techniques are presented in parallel. (Left) Micropatterns labeled with FN-Cy3 before transfer on acrylamide, (middle—left) remaining fluorescence on the initial micropatterned surface after transfer on acrylamide, (middle—right) micropatterns on acrylamide gel after detachment from the initially patterned surface. (Right) Phase contrast images of RPE1 cells 4 h after cell seeding. (B) Linescan of ECM protein fluorescence taken along several micropatterns, showing reproducible spacing between micropatterns and robust fluorescence intensity. (C) Fluorescence pictures of micropatterns labeled with FN-Cy3 produced using both techniques on PAA gel of 0.5 (left), 1 (middle), and 30 kPa (right). All scale bars correspond to 100 µm.

- Collect the acrylamide solution from the vacuum bell and keep the container closed.
- *Optional*: If you want to add some fluorescent beads in your gel for force measurements, they should be added at this stage of the process in the acrylamide solution and the solution should be sonicated for 5 min to destroy any bead aggregates that could have formed during the storage.
- Prepare TEMED and APS and silanized coverslips. You will add TEMED and APS solution to the acrylamide with the following proportions: 1 µL of TEMED and 1 µL of APS 10% for 165 µL of acrylamide solution. You should proceed as fast as possible in the next steps.
- Add TEMED to the acrylamide solution, briefly but vigorously mix.
- Add APS solution to the acrylamide solution, briefly but vigorously mix.
- Put a drop of 7 µL/cm^2 of the acrylamide polymerization mix on the mask in each patterned area of interest.
- Slowly place the silanized coverslip on top while taking care to avoid bubbles.
- Put a cap (to prevent evaporation) and let the gel polymerize for 30 min. Keep the rest of acrylamide in a closed container as a control of gel polymerization.
- Once the polymerization is finished (you should check it by detaching the remaining acrylamide from the tube, it should have the shape of the container and be elastic if you try to pinch it with the a pipette tip), cover the coverslips with PBS and let the gel hydrate for 5 min.
- Detach the acrylamide gel by carefully lifting the silanized coverslip using a razor blade. Due to the silanization process, the gel will stay attached to the coverslip. Make sure that the gel is fully immersed during the entire detachment process otherwise you will end up with collapsed micropatterns.
- Rinse the acrylamide gel attached to silanized coverslip (acrylamide coverslips) in PBS several times.
- Control quality with fluorescence microscope if possible.
- Store at 4 °C and use as soon as possible.

6.4.3.4 Cell seeding

- For the cell seeding, proceed as described in Section 6.3.3.5.

6.5 DISCUSSION

6.5.1 Storage

The best results were obtained when the gels were used immediately after production. If one wants to use a lot of gels on the same day, the "glass method" can be interrupted at the step of protein coating on the template. You can then produce many templates and store them in buffer for few days before transfer. The template should not be stored dry because it impairs the efficiency of the transfer process (Fig. 6.3A). If the storage of the gel is really necessary, one should store it wet in buffer as dry storage irreversibly deforms the micropatterns and detaches part of the protein coating (Fig. 6.3B).

6.5.2 Chemical modifications of protein for stronger protein adhesion to the PAA gel

Here we have described a protocol of protein transfer on acrylamide gel that does not require any specific chemistry for the crosslinking of the ECM protein to the gel. Observations of the fluorescence on the template micropatterned substrate (Fig. 6.2A and B) clearly show that the proteins were efficiently transferred on the acrylamide gel and cells were stably confined for a few days but not weeks. The technique was efficiently used on PAA gels of stiffnesses from 0.5 to 30 kPa (Fig. 6.2C) and used to perform traction forces experiments (Schiller et al., 2013).

Interestingly, if a polystyrene-coated coverslip was used for the template production, the transfer was then inefficient. The polystyrene coating has been shown to increase micropatterns stability for cell lines producing strong forces. In that case, the strength of the bond between acrylamide and the ECM protein is likely to be stronger than between ECM and glass, but weaker than between ECM and polystyrene (see Tang et al. (2012) for a detailed description of the detachment process and critical parameters). This strength is sufficient for the cell lines we have been using (RPE1, MCF10A, mouse fibroblasts) but it could be insufficient for other cell lines or for very long cell confinement. The passivation of the PAA could be improved by bovine serum albumin (BSA) incubation after micropattern production, and the stability of the micropatterns can be increased via chemical crosslinking strategies. Some groups have already put effort in this direction. Damljanovic et al. (2005) used a reducing agent, hydrazine hydrate, to modify nonreactive amide groups in PAA to highly reactive hydrazide groups that can form covalent bonds with aldehyde or ketone groups in oxidized proteins. ECM proteins were oxidized using sodium periodate (Polio et al., 2012) dissolved NHS ester in neutralized acrylamide solution before polymerization. NHS groups then react with amino groups on the proteins to form covalent bonds. The rest of the gel was then passivized by BSA incubation. Grevesse et al. (2013) replaced NHS esters by *N*-hydroxyethylacrylamide monomers resulting in the presence of hydroxyl groups in the acrylamide gel that could form hydrogen bonds with proteins. One should play around with these solutions if an improvement of the stability of the protein attachment to the acrylamide gel is required.

6.5.3 Resolution considerations

Even though both methods seem very similar, the maximal resolution that each can achieve is not the same. The "mask method" allows for a very faithful reproduction of the shape on the mask because the UV light is burning the surface directly in front of the shapes on the photomask (Figs. 6.1II and 6.3C) whereas there is a small distance between the passivized coverslip and the photomask at the step of UV insolation in the "glass method." Due to diffraction of the UV light, the burned area on the coverslip will be larger and smoother than the original shape on the mask. The linescans on fluorescently labeled micropatterns suggest that submicrometer resolution is achieved using patterning on the photomask.

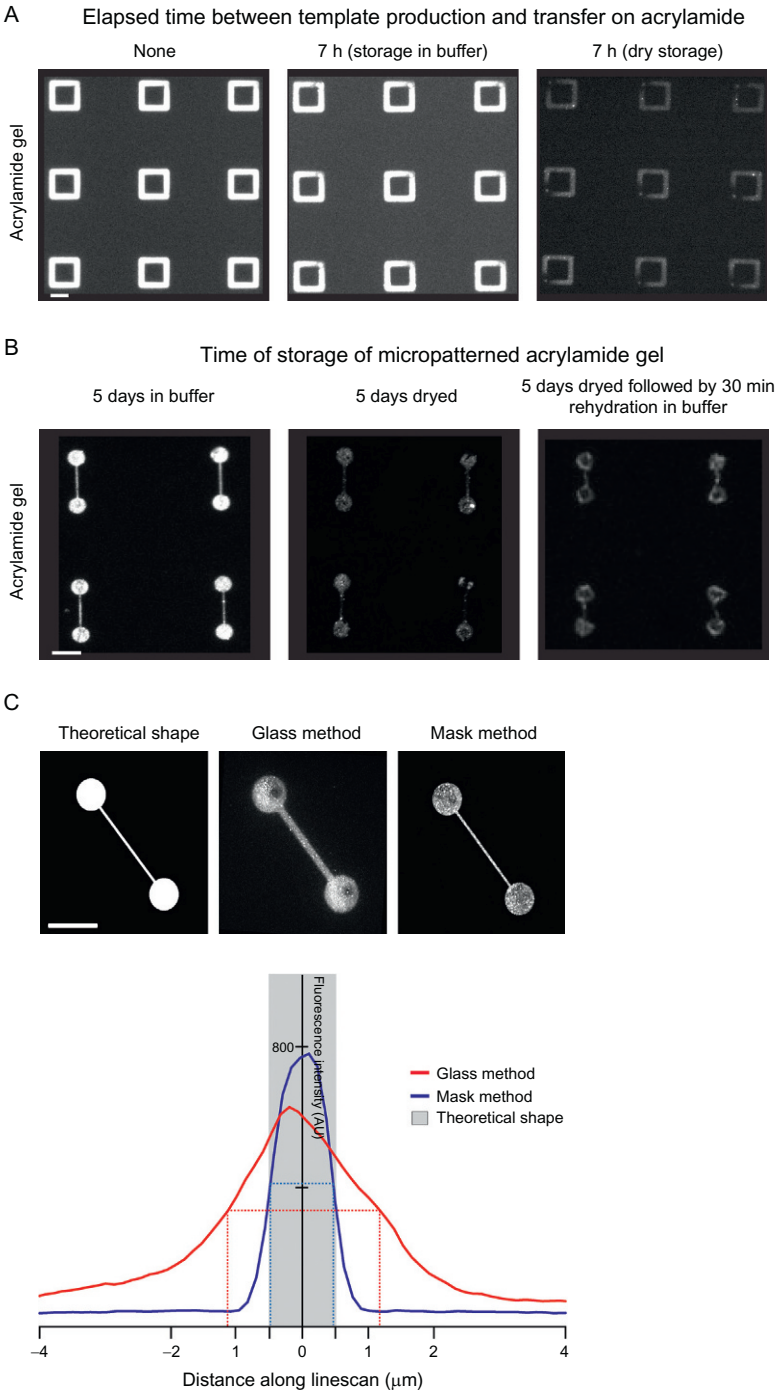

FIGURE 6.3

Conservation advice and micropattern resolution. (A) Fluorescent images of FN-Cy3 micropatterns on acrylamide gel. The template micropatterned glass coverslip was stored for 0 min (left), 7 h in PBS (middle), or 7 h dry (right) before acrylamide transfer. (B) Fluorescent

(*Continued*)

On the other hand, if your micropatterns can suffer a little enlargement then patterning on glass will allow you to produce many coverslips much more quickly. You can treat many coverslips with pLL–PEG at the same time, then insolate them sequentially and proceed through the other steps in parallel, while the full process has to be repeated sequentially when using the mask as the template surface.

The "glass method" might also be more suitable for sending coverslips to collaborators. Since the patterns on acrylamide should be used as soon as possible, one should consider sending UV-activated glass coverslips (produced at Section 6.3.3.2) that are more stable over time. The rest of the process (protein coating and transfer on acrylamide) does not require any expensive equipment and can be performed in any lab. You could even consider buying commercially available activated glass coverslips from micropatterning companies and just proceed through the protein coating and the transfer on acrylamide, with minimal equipment requirements.

6.5.4 Comparison to other techniques

The idea of producing micropatterns on acrylamide is not new. Several solutions have already been proposed to do this (Fig. 6.4 and Table 6.1), most of them are adaptations of existing techniques for micropatterning on hard substrates.

PDMS stencils (Fig. 6.4A) allow treatment at specific regions of the gel while keeping other regions unexposed. Once the gel is protected, the usual techniques of protein PAA functionalization can be used (Wang et al., 2002). Due to the elastic nature of the stencil, micropatterns may vary in their shapes due to deformation. Very small features are difficult to produce using this technique. If sulfo-SANPAH and UV insolation are performed through the stencil, a local modification of the stiffness of the acrylamide is likely to be created at the sites of micropatterns.

μCP (Fig. 6.4B) can also be performed on activated PAA (Damljanovic et al., 2005; Versaevel et al., 2012). A stamp covered with ECM protein is brought in contact with the activated PAA. This technique is difficult to perform on very soft gel and suffers from variability in protein transfer. Also deformation in the array of the micropatterns can occur if the PDMS is deformed at the step of stamping.

FIGURE 6.3—Cont'd images of FN-Cy3 micropatterns on PAA after storage of the gel for 5 days in PBS (left), 5 days dried (middle), 5 days dried, and after 30 min rehydration in PBS (right). (C) Assessment of the spatial resolution of both techniques. Theoretical shape of the micropatterns (up left), FN-Cy3 picture of a typical micropattern produced with the "glass method" (up middle) or the "mask method" (up right). Average of 20 linescans across the central line joining the head of the dumbbell shape of the micropatterns (down, red curve for the "glass method," blue curve for the "mask method," gray area for theoretical shape of the micropattern). The widths at half fluorescence maximum (doted lines) show that the "mask method" is very precise in reproducing the theoretical shape while the "glass method" suffers from enlargement of the micropatterns shape due to UV diffraction. All scale bars correspond to 20 μm.

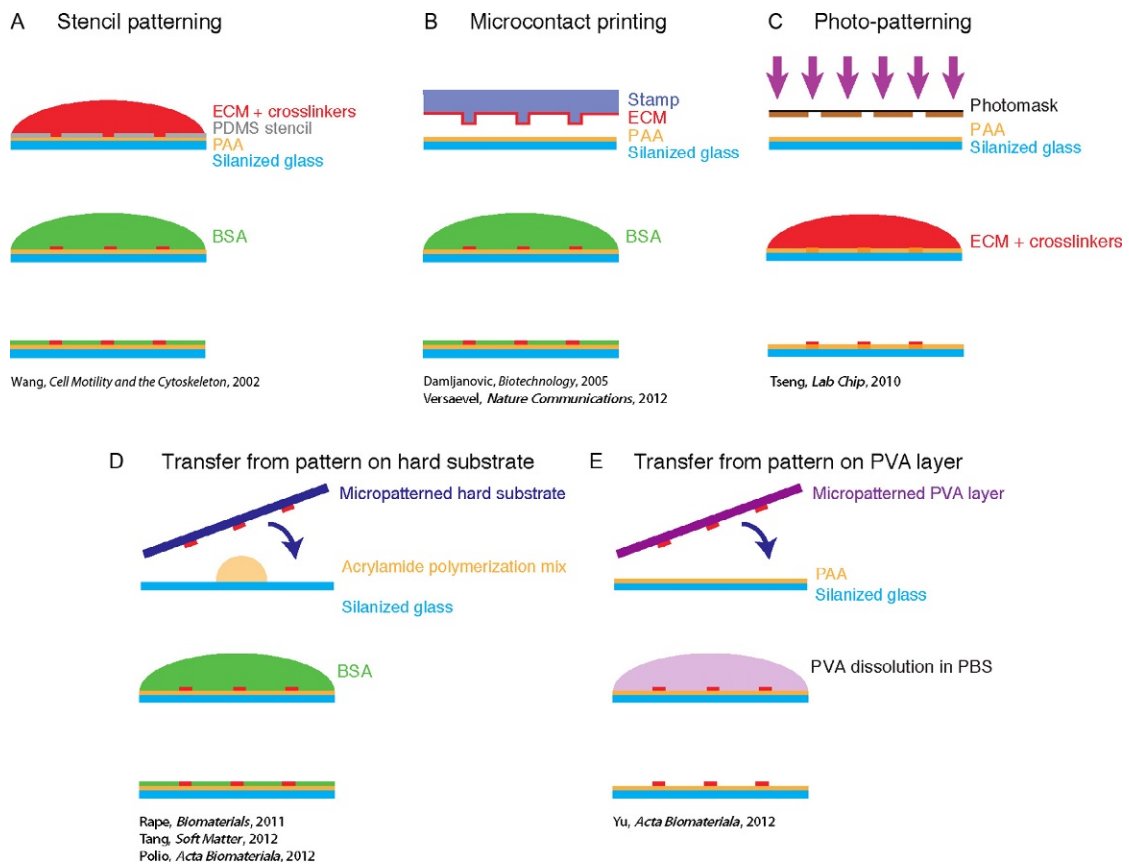

FIGURE 6.4

Existing techniques of micropatterning of polyacrylamide, see Section 6.5.4 for the description of each of them.

Table 6.1 Pros and cons of existing techniques for acrylamide patterning

	Spatial resolution (μm)	**Robust pattern shape (no elastic deformation)**	**Suitable for soft (<1 kPa) substrate**	**Crosslinker-free procedure**	**Unchanged micropattern stiffness**	**Sequential patterning**
Stencil patterning	1–10	−	+	−	+/−	+
Microcontact printing	1–10	−	−	−	+	+
Photo-patterning	>1	+	+	−	−	−
Transfer from pattern on hard substrate	1–10	−	+	+/−	+	+/−
Transfer from pattern on PVA layer	1–10	−	+	−	+	+
Mask method	>1	+	+	+	+	−
Glass method	1–2	+	+	+	+	+

Deep UV patterning (Fig. 6.4C) was successfully adapted to PAA patterning (Tseng et al., 2011). The gel is polymerized in contact with the photomask and then activated at specific loci using deep UV exposure. Incubation with chemical crosslinkers and ECM proteins leads to the production of micropatterned PAA gel. This technique creates patterns of very defined shape and organization due to the direct polymerization of the gel on the mask. This allows the development of force measurement techniques based only on the deformation of the micropattern with no need for traction force microscopy (TFM) expertise and cell detachment (Tseng et al., 2011). However, the use of sensitive chemical crosslinkers such as EDC introduces some variability in the protein attachment and deep UV exposure of the gel locally modifies the stiffness of the gel.

Transfer from micropatterned hard substrate (Fig. 6.4D) relies on polymerization of the acrylamide gel in contact with a previously patterned substrate (Polio et al., 2012; Rape et al., 2011; Tang et al., 2012). Crosslinkers in the solution allow for the transfer of ECM protein from the initial substrate to the surface of the acrylamide gel. This procedure is very attractive for laboratories that are already using micropatterns on hard substrate because only the transfer step has to be added to their standard protocol. However, the attachment between the initial hard substrate and the ECM protein has to be weak enough to allow for the transfer of the protein to the acrylamide gel. The resolution of the micropatterns is quite good in this case. However, it is less accurate than deep UV patterning due to the limitations of the technique used for the production of the template micropatterned substrate (μCP, stencil, etc.).

Transfer from patterned polyvinyl alcohol (PVA) film (Fig. 6.4E) (Yu et al., 2012) has been developed to solve the issues of μCP such as gel deformation due to mechanical contact and pattern deformation due to sticky interaction between the stamp and the PAA gel. Again, patterns are first produced on PVA film, then the PVA is put into contact with the activated soft substrate and dissolved in PBS. Thus, no deformation of the gel is induced by stamp detachment and the patterning is more accurate. However, the elastic properties of the layer could introduce some deformations in the micropatterns as for the stencil method. This technique is very promising for the patterning of curved surface such as implants or surgical tools since the initial PVA layer is flexible.

The method described here is combining many advantages that are found isolated in the other methods: no need for a chemical crosslinker, easy stamp production, no modification of acrylamide substrate due to crosslinkers or UV insolation, compatible with very soft gel (>1 kPa). Then one will have to choose between higher spatial resolution ("mask method") and higher throughput or multiple protein patterning ("glass method").

6.5.5 Future challenges and development

The process described here allows the robust and precise production of micropatterns on acrylamide gel of various stiffnesses. Multiple patterning can also be performed easily by the "glass method" technique with sequential insolation and protein coating on the glass template.

Submicrometer multiple patterning could be achieved using sequential laser patterning (Doyle, Wang, Matsumoto, & Yamada, 2009; Kim et al., 2010; Nakanishi et al., 2007) on glass or PVA and then transfer on acrylamide gel but the "mask method" is not suitable for this purpose. This will then provide heterogeneous cell environment for cell culture experiments that is more likely to reproduce complex *in vivo* cellular environment. It allows for the study of complex processes such as asymmetric stem cell division or tissue self-organization.

Traction force measurement based on dots micropatterns has been proposed as an alternative to fluorescent beads embedded in the acrylamide gel (Polio et al., 2012). This creates a platform for force measurement that is then quite similar to micropatterned PDMS microposts (Han, Bielawski, Ting, Rodriguez, & Sniadecki, 2014, Chapter 5 of Vol. 121) including the aspects of force computation from displacements. The technique described here is perfectly suited for this purpose.

Transfer on acrylamide from heterogeneous patterns produced on 3D substrate represents the next step in the improvement of these techniques, that is, for tissue engineering. It could provide the control of both topography and spatial localization of ECM protein. As the transfer is done using polymerization in contact with the template, the reproduction of topographical features is completely feasible (Charest, Califano, Carey, & Reinhart-King, 2011). If one is able to produce micropatterns on topographical features, using laser patterning on PDMS or polystyrene microstructures for instance, the transfer in acrylamide is then just one step ahead.

Real-time modification of the micropattern is something very challenging. This is already possible on hard substrates (Mandal, Balland, & Bureau, 2012; Nakanishi et al., 2007; Vignaud, Galland, et al., 2012) but as not yet been done on acrylamide. It could be another very useful tool for cell behavior studies and tissue engineering.

Finally, micropatterning has recently also been used for *in vitro* experiments (Reymann et al., 2014, Chapter 2 of Vol. 121). Patterning the nucleation of cytoskeleton proteins makes it possible to precisely study the role of boundary conditions in cytoskeleton organization with a minimal reconstituted system. Using patterning on PAA in the same way could allow for the study of the forces produced by these minimal mechanical architectures.

Acknowledgments

We would like to thank Mithila Burute for useful discussions on the micropatterning process and manuscript editing, Ben Fogelson and Aldo Leal-Egana for careful reading of the manuscript.

References

Azioune, A., Carpi, N., Tseng, Q., Théry, M., & Piel, M. (2010). Protein micropatterns: A direct printing protocol using deep UVs. *Methods in Cell Biology*, *97*, 133–146. http://dx.doi.org/10.1016/S0091-679X(10)97008-8.

Charest, J. M., Califano, J. P., Carey, S. P., & Reinhart-King, C. A. (2011). Fabrication of substrates with defined mechanical properties and topographical features for the study

of cell migration. *Macromolecular Bioscience*, *12*, 12–20. http://dx.doi.org/10.1002/mabi.201100264.

Damljanovic, V., Christoffer Lagerholm, B., & Jacobson, K. (2005). Bulk and micropatterned conjugation of extracellular matrix proteins to characterized polyacrylamide substrates for cell mechanotransduction assays. *Biotechnology*, *39*, 847–851. http://dx.doi.org/10.2144/000112026.

de Rooij, J., Kerstens, A., Danuser, G., Schwartz, M. A., & Waterman-Storer, C. M. (2005). Integrin-dependent actomyosin contraction regulates epithelial cell scattering. *Journal of Cell Biology*, *171*, 153–164. http://dx.doi.org/10.1083/jcb.200506152.

Doyle, A. D., Wang, F. W., Matsumoto, K., & Yamada, K. M. (2009). One-dimensional topography underlies three-dimensional fibrillar cell migration. *Journal of Cell Biology*, *184*, 481–490. http://dx.doi.org/10.1073/pnas.0604460103.

Engler, A. J., Sen, S., Sweeney, H. L., & Discher, D. E. (2006). Matrix elasticity directs stem cell lineage specification. *Cell*, *126*, 677–689. http://dx.doi.org/10.1016/j.cell.2006.06.044.

Grevesse, T., Versaevel, M., Circelli, G., Desprez, S., & Gabriele, S. (2013). A simple route to functionalize polyacrylamide hydrogels for the independent tuning of mechanotransduction cues. *Lab on a Chip*, *13*, 777–780. http://dx.doi.org/10.1039/c2lc41168g.

Han, S. J., Bielawski, K. S., Ting, L. H., Rodriguez, M. L., & Sniadecki, N. J. (2014). Decoupling substrate stiffness, spread area, and micropost density: A close spatial relationship between traction forces and focal adhesions. *Biophysical Journal*, *103*, 640–648. http://dx.doi.org/10.1016/j.bpj.2012.07.023.

Kim, M., Choi, J.-C., Jung, H.-R., Katz, J. S., Kim, M.-G., & Doh, J. (2010). Addressable micropatterning of multiple proteins and cells by microscope projection photolithography based on a protein friendly photoresist. *Langmuir*, *26*, 12112–12118. http://dx.doi.org/10.1021/la1014253.

Klein, E. A., Yin, L., Kothapalli, D., Castagnino, P., Byfield, F. J., Xu, T., et al. (2009). Cell-cycle control by physiological matrix elasticity and in vivo tissue stiffening. *Current Biology*, *19*, 1511–1518. http://dx.doi.org/10.1016/j.cub.2009.07.069.

Mandal, K., Balland, M., & Bureau, L. (2012). Thermoresponsive micropatterned substrates for single cell studies. *PLoS One*, *7*, e37548. http://dx.doi.org/10.1371/journal.pone.0037548.g001.

Nakanishi, J., Kikuchi, Y., Inoue, S., Yamaguchi, K., Takarada, T., & Maeda, M. (2007). Spatiotemporal control of migration of single cells on a photoactivatable cell microarray. *Journal of the American Chemical Society*, *129*, 6694–6695. http://dx.doi.org/10.1021/ja070294p.

Pelham, R. J., & Wang, Y. L. (1997). Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proceedings of the National Academy of Sciences of the United States of America*, *94*, 13661–13665.

Pitaval, A., Tseng, Q., Bornens, M., & Thery, M. (2010). Cell shape and contractility regulate ciliogenesis in cell cycle-arrested cells. *Journal of Cell Biology*, *191*, 303–312. http://dx.doi.org/10.1083/jcb.201004003.dv.

Polio, S. R., Rothenberg, K. E., Stamenović, D., & Smith, M. L. (2012). A micropatterning and image processing approach to simplify measurement of cellular traction forces. *Acta Biomaterialia*, *8*, 82–88. http://dx.doi.org/10.1016/j.actbio.2011.08.013.

Prager-Khoutorsky, M., Lichtenstein, A., Krishnan, R., Rajendran, K., Mayo, A., Kam, Z., et al. (2011). Fibroblast polarization is a matrix-rigidity-dependent process controlled by focal adhesion mechanosensing. *Nature Cell Biology*, *13*, 1–10. http://dx.doi.org/10.1038/ncb2370.

Rape, A. D., Guo, W.-H., & Wang, Y.-L. (2011). The regulation of traction force in relation to cell shape and focal adhesions. *Biomaterials*, *32*, 2043–2051. http://dx.doi.org/10.1016/j.biomaterials.2010.11.044.

Reymann, A.-C., Martiel, J.-L., Cambier, T., Blanchoin, L., Boujemaa-Paterski, R., & Théry, M. (2014). Nucleation geometry governs ordered actin networks structures. *Nature Materials*, *9*, 827–832. http://dx.doi.org/10.1038/nmat2855.

Schiller, H. B., Hermann, M.-R., Polleux, J., Vignaud, T., Zanivan, S., Friedel, C. C., et al. (2013). β1- and αv-class integrins cooperate to regulate myosin II during rigidity sensing of fibronectin-based microenvironments. *Nature Cell Biology*, *15*, 625–636. http://dx.doi.org/10.1038/ncb2747.

Storm, C., Pastore, J. J., MacKintosh, F. C., Lubensky, T. C., & Janmey, P. A. (2005). Nonlinear elasticity in biological gels. *Nature*, *435*, 191–194. http://dx.doi.org/10.1038/nature03521.

Tang, X., Yakut Ali, M., & Saif, M. T. A. (2012). A novel technique for micro-patterning proteins and cells on polyacrylamide gels. *Soft Matter*, *8*, 7197. http://dx.doi.org/10.1039/c2sm25533b.

Théry, M. (2010). Micropatterning as a tool to decipher cell morphogenesis and functions. *Journal of Cell Science*, *123*, 4201–4213. http://dx.doi.org/10.1242/jcs.075150.

Tse, J. R., & Engler, A. J. (2001). *Preparation of hydrogel substrates with tunable mechanical properties*. Hoboken, NJ: John Wiley & Sons, Inc.

Tseng, Q., Wang, I., Duchemin-Pelletier, E., Azioune, A., Carpi, N., Gao, J., et al. (2011). A new micropatterning method of soft substrates reveals that different tumorigenic signals can promote or reduce cell contraction levels. *Lab on a Chip*, *11*, 2231–2240. http://dx.doi.org/10.1039/c0lc00641f.

Versaevel, M., Grevesse, T., & Gabriele, S. (2012). Spatial coordination between cell and nuclear shape within micropatterned endothelial cells. *Nature Communications*, *3*, 671. http://dx.doi.org/10.1038/ncomms1668.

Vignaud, T., Blanchoin, L., & Théry, M. (2012). Directed cytoskeleton self-organization. *Trends in Cell Biology*, *22*, 671–682. http://dx.doi.org/10.1016/j.tcb.2012.08.012.

Vignaud, T., Galland, R., Tseng, Q., Blanchoin, L., Colombelli, J., & Théry, M. (2012). Reprogramming cell shape with laser nano-patterning. *Journal of Cell Science*, *125*, 2134–2140. http://dx.doi.org/10.1242/jcs.104901.

Wang, N., Ostuni, E., Whitesides, G. M., & Ingber, D. E. (2002). Micropatterning tractional forces in living cells. *Cell Motility and the Cytoskeleton*, *52*, 97–106. http://dx.doi.org/10.1002/cm.10037.

Yu, H., Xiong, S., Tay, C. Y., Leong, W. S., & Tan, L. P. (2012). A novel and simple microcontact printing technique for tacky, soft substrates and/or complex surfaces in soft tissue engineering. *Acta Biomaterialia*, *8*, 1267–1272. http://dx.doi.org/10.1016/j.actbio.2011.09.006.

Zhang, J., Guo, W.-H., Rape, A., & Wang, Y.-L. (2013). Micropatterning cell adhesion on polyacrylamide hydrogels. *Methods in Molecular Biology*, *1066*, 147–156. http://dx.doi.org/10.1007/978-1-62703-604-7_13.