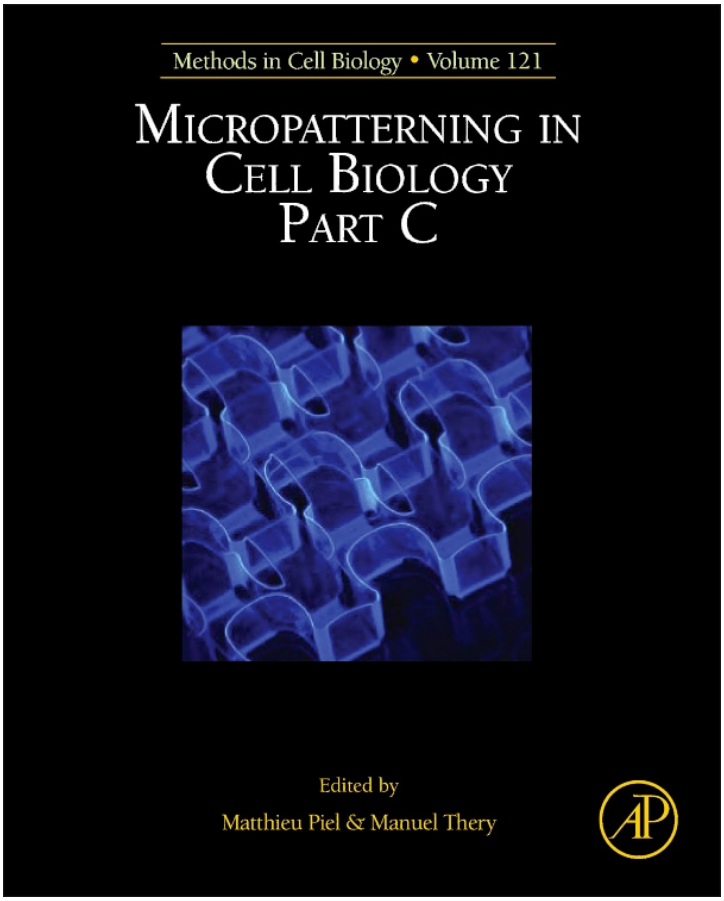

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CHAPTER

11 Micropatterned Porous Membranes for Combinatorial Cell-Based Assays

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Abstract

Here, we describe a protocol for producing micropatterned porous membranes which can be used for combinatorial cell-based assays. We use contact printing to pattern the surface of a porous filter membrane with a thin layer of polydimethylsiloxane (PDMS). This allows the porosity of the filter membrane to be altered at selected locations. Cells can be grown on one side of the filter membrane, while drugs and reagents can be deposited on the porous areas of the other side of the membrane. The reagents can diffuse through the pores of the membrane to the cells. The first part of the protocol describes how to design a stamp and use it to contact print PDMS. The second part describes how to create microprinted membranes for cell-based assays. The method is simple, highly customizable, can be performed at the bench, and can be used to perform combinatorial or time-dependent cell-based assays.

INTRODUCTION

The combination of microfluidics and fluorescence microscopy has been instrumental in recent developments in quantitative cell biology (Bennett & Hasty, 2009; Sia & Whitesides, 2003). In particular, several microfluidics systems which allow the chemical environment of eukaryotic cells to be altered while simultaneously measuring the expression of key reporter genes by fluorescence microscopy have been proposed (Jovic, Howell, & Takayama, 2009; Taylor et al., 2009). Yet, these systems are still complex to use. A number of commercial systems exist; however, they are often limited in terms of operability and, without solid expertize, cannot be used to perform combinatorial and/or time-dependent cell-based assays (Castel, Pitaval, Debily, & Gidrol, 2006; Evenou, Di Meglio, Ladoux, & Hersen, 2012; Wu et al., 2010). Here, we describe the setup of a simple device to perform semi high-throughput assays on a monolayer of adherent cells. Our method is inspired by the fabrication method of "paper-based microfluidics" (Carrilho, Martinez, & Whitesides, 2009; Nery & Kubota, 2013), which aims to create cheap and "ready to use" diagnostic systems with the use of paper. Paper-based microfluidics are made from a porous material (usually paper, but other porous materials can be processed) on which a pattern is printed using a hydrophobic wax (Carrilho et al., 2009). This pattern defines hydrophobic and hydrophilic areas. Liquids can permeate the hydrophilic porous areas but cannot infiltrate where the wax has been printed. This approach is simple and does not require expensive equipment or materials. However, the delivery of drugs or

metabolites to a pool of cells in such systems is limited (Derda et al., 2011). In particular, paper-based systems do not enable the possibility of repeated toxicity testing or pulse/chase experiments in cells. Indeed, liquids move by capillarity inside the porous meshwork and cannot be washed off or removed from the paper. Hence, such systems are usually designed for single use. Here, we use contact printing to deposit a thin layer of a biocompatible hydrophobic polymer (PDMS, polydimethylsiloxane) on top of a commercial filter membrane which has the properties of limited retention of liquids and proteins. In doing so, we modify the local porosity of the filter membrane (Evenou et al., 2012). Adherent cells can be cultured on the surface of the filter membrane, and chemicals can be delivered manually using a pipette from the other side of the membrane at selected locations where the porosity has not been blocked by the PDMS polymer. The resulting device, which we call a micropatterned membrane (μPM) is easy to produce and allows the effects of several drugs to be simultaneously tested on a monolayer of cells. We first describe the preparation of the porous substrate and then illustrate how it can be used for semi-high-throughput cell-based assays.

11.1 PATTERN DESIGN

The first step is to print the surface of a porous filter membrane with a thin layer of PDMS. This is performed using contact printing and requires the production of a master stamp; we have successfully used both direct micromachining and soft lithography (Whitesides, Ostuni, Takayama, Jiang, & Ingber, 2001; Xia & Whitesides, 1998) to fabricate stamps.

11.1.1 Pattern sizes and printing resolution

The design of the patterns will largely depend on the aim of the study. We have successfully printed different shapes—resulting in several patterns of porosity—ranging from simple lines and arrays of disks to more complex patterns. A few simple rules can help when drawing the patterns. First, the stamp should be made using PDMS, so that the uncured, liquid PDMS mixture to be used for contact printing will homogeneously spread on the surface of the stamp (i.e., good wettability). Once printed on the filter membrane, the thin layer of liquid PDMS will spread a little and blur the features of the pattern. This prevents the successful printing of fine details; sharp angles and tiny structures will not be faithfully reproduced. The printing protocol described later is limited to a typical resolution of 100 μm. A finer resolution can be obtained, but will require increased precision during the manipulation and deposition of the PDMS thin film. The nature of the filter membrane (wettability) and its porosity will also influence the final resolution of the pattern. The typical device that we use for cell-based assays consists of an 8×8 array of porous disks (1 mm in diameter), each separated by 1 mm nonporous areas. This pattern can be viewed as a miniaturized well-less plate, on top of which 2 μl drops of various reagents can be

deposited to perform screening or cell-based assays on cells growing on the other side of the porous areas. Note that chemicals will diffuse within the media surrounding the cells, and therefore may act on cells further away than the cells immediately below the porous area on which the chemical has been deposited. This places constraints on the minimum distance between two porous areas and on the duration of the exposure time. In most cases, a distance of 1 mm between two porous areas is sufficient to avoid cross-talk between two consecutive porous areas; however, this distance may need to be adapted depending on the experimental protocol.

11.1.2 Materials

Several software programs can be used to draw a simple, geometrical pattern and export it in a format which can be used directly by a high resolution printer. Professional software programs (*AutoCAD* from Autodesk Inc., *L-Edit* from Tanner EDA) include advanced drawing features but are expensive and often not very user-friendly. A variety of open source software are also available and, in our opinion, these programs are ideal to start with. In particular, *Layout Editor software* is a powerful, free solution. A list of other layout editors can be found on their website (http://www.layouteditor.net/links/).

Printing companies now offer printing on transparent plastic sheets up to a resolution of 50,800 dpi, which allows the creation of patterns larger than 10 μm when using soft lithography. These companies usually work with the file format GDSII: a standard binary file format which is used to represent complex layouts. However, since the resolution of the final pattern on the membrane is limited by the contact printing—and not by the fabrication of the stamp—we do not need to achieve such high resolutions. It is actually possible to speed up the printing process and reduce costs by using a simple, standard inkjet printer. In this case, any bitmap or vector image editor can be used to draw the pattern and print it. We commonly use the commercial software Adobe Illustrator (Adobe Systems Incorporated) or Inkscape (open source GNU software) to draw patterns. We then use a Canon Pixma IP400 inkjet printer (Canon Ltd) at a resolution of 600 dpi and clear inkjet films (e.g., 100–075 from Mega Electronics) to produce the final photomask.

11.2 MASTER STAMP FABRICATION

The first step is to transfer the 2D printed pattern (the photomask) into a 3D pattern (the master stamp). Classically, this is performed using soft lithography. This is the limiting step in terms of resources and equipment, since you will need access to a clean room, or at the very least to a spin coater, a strong ultraviolet (UV) source, and heating plates. However, this equipment is only required to create the master stamp, which can be easily reused several times and replicated using epoxy resists or PDMS.

11.2.1 Materials required for fabrication

- Transparency photomask (see above, minimum size should cover the size of a porous filter membrane)
- SU-8-2100 photoresist and SU-8 developer (Microchem Corp)
- Isopropanol (for the developing steps)
- Silicon wafer (e.g., from SI-Mat)
- A metallic spatula for dispensing resist
- Two glass crystallizing dishes (typically 140–190 mm in diameter) for the rinsing steps.

11.2.2 Equipment for fabrication (usually done in a clean room)

- Spin coater (e.g., Delta +6RC from SUSS MicroTec, Germany) with a small vacuum pump to hold the sample (e.g., #FB65455 from ILMVAC, Germany)
- Two heating plates set at 65 and 95 °C
- UV lamp for resist exposure (e.g., Spectra Physics/Newport ref 66902 or MJB4 from SUSS MicroTec)
- A plasma cleaner (e.g., Diener Electronics)
- An air gun for drying the final wafer.

11.2.3 Method

Soft lithography has been described in several key articles (Whitesides et al., 2001; Xia & Whitesides, 1998). Soft lithography can be used to convert a 2D pattern into a 3D structure. When using SU-8 as a photoresist, the photomask should be opaque at the locations where PDMS will be contact printed onto the membrane (see Fig. 11.1). SU-8 is a negative photoresist, meaning that all regions of the photoresist layer that are illuminated by UV light will appear in relief after curing and development. We mainly use SU-8-2100 photoresist and follow the manufacturer's protocol (which takes ~3 h) which can be found on their website (http://microchem.com/pdf/SU-82000DataSheet2100and2150Ver5.pdf). This photoresist allows the creation of deep reliefs (>100 μm), which are suitable for contact printing of large areas. For our purposes, it is not advisable to make the patterns less than 100 μm thick, and we usually work with a 200–250 μm resist thickness. We quickly describe the manufacturer's protocol, focusing on several tips that can help when dealing with SU-8-2100, which is a very viscous resist.

- (Facultative) wafer preparation (step duration 10 min, total duration $t = 10$ min)
  - Clean the surface of the wafer using O_2 plasma at 6 mbar for 6 min.
  - Prior to deposition of the photoresist, the wafer can briefly be heated (95 °C) to encourage the spread of the photoresist.
- Resist spreading (step duration 10 min, $t = 20$ min)
  - Photoresists are usually stored at 4 °C. It is important to make sure that the resist is preheated to room temperature to achieve the nominal viscosity.

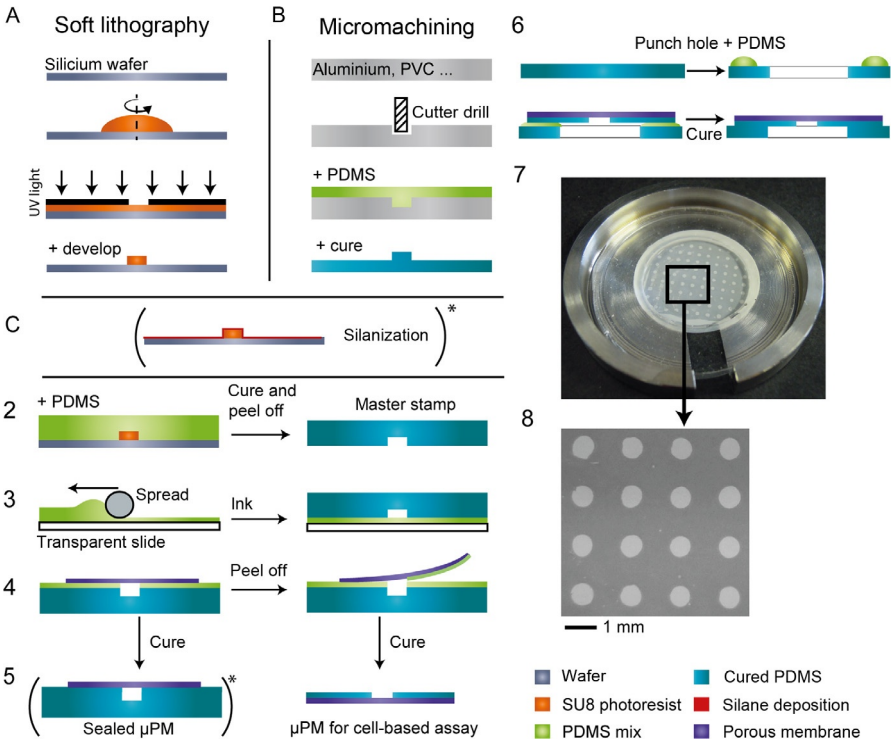

FIGURE 11.1

Patterned membrane fabrication. 1. Fabrication of the master pattern via soft lithography (A) or direct micromachining (B). (A) SU8 photoresist is evenly deposited on a flat silicon wafer by spin coating. The desired pattern is produced by UV exposure through a photomask followed by curing and development steps. (B) For simple patterns, one can also use micromachining and a hard material (such as metal or plastic). This can be performed using a microdriller or a microcutter drill. If required, the shape can be inverted by replica molding of PDMS. (C) A silanization step can be performed prior to master stamp molding; silanization is mandatory if the master pattern is created in PDMS (B), and advised if the substrate was produced by soft lithography to extend its lifetime. 2. The master stamp is fabricated by replica molding a 2–5 mm thick layer of PDMS on the master pattern. 3. A thin layer of uncured PDMS is spread on a flat surface such as printing transparency sheet. A tubular object such as a 10-ml pipette can be used to ensure homogeneous spreading. Ink the PDMS stamp by gently tapping it on the surface of the PDMS layer. 4. Turn the stamp upside down and place a porous filter membrane on top. Gently peel off the membrane and cure it to obtain a micropatterned membrane (µPM). 5. Direct curing can be used to obtain a microfluidic device with an integrated porous membrane, or the µPM can be cured alone on a hotplate at 100 °C. 6. To improve the ease of handling, a PDMS ring can be created by punching a hole in a 0.5–1 mm PDMS block. This ring is then attached to the µPM using uncured PDMS as a mortar followed by further curing. 7. A (metallic) holder can be used to facilitate cell culture. 8. View of the typical patterning of a µPM containing an array of millimeter scale porous discs.

  - Place the wafer on the spin coater and dispense around 5 ml of the photoresist on the wafer. If needed, use a spatula to spread the resist. SU-8-2100 is very viscous and it is important to initially help the spreading.
  - Wait for the resist to spread on its own for a few minutes (typically 5 min).
  - Spin the wafer in two steps (500 rpm for 120 s, 1500 rpm for 60 s). These steps need to be adjusted depending on the desired thickness of photoresist. The faster the rotation speed, the thinner the final photoresist layer. Note that the precise value of the thickness is not relevant here.
- Preexposure bake (step duration 46 min, $t = 1$ h 06 min)
  - Bake on hotplate for 6 min at 65 °C and then for 40 min at 95 °C. Do not allow the wafer to cool down between the two steps, as the gradual temperature increase is used to diminish mechanical tensions in the resist.
- Exposure (step duration 2 min, $t = 1$ h 08 min)
  - Place the transparency mask in contact with the photoresist layer after it has cooled down (this allows the photoresist to stiffen and become less sticky, thus preserving the mask). Expose to UV light (the exposure time depends on lamp power and focalization, and on the thickness of the pattern—we use 40 s at 10 mW/cm^2). Take into account that this time will also depend on the degree of UV absorption by the mask; a typical plastic mask will block 25% of the UV light. A good exposure time will result in a 90° edge on the photoresist/wafer interface, which is important for membrane patterning.
- Postexposure bake (step duration 18 min, $t = 1$ h 26 min)
  - Bake on hotplate for 5 min at 65 °C and then for 13 min at 95 °C. The resist should not wrinkle nor crack when heated. If this occurs, an intermediate 1 min temperature step (80 °C) can be added. In general, wrinkles should not be a problem for patterning, since such deformations will not affect the surface of the stamp that will be used for contact printing.
- Development and rising (18 min, $t = 1$ h 44 min)
  - Place the developer solution into a crystallizing dish and add the wafer. Agitate slowly for 16 min and then rinse the wafer with isopropanol in a new crystallizing dish. Isopropanol is best dispensed using a wash bottle. Carefully air-dry the wafer using compressed air (air gun). If white residue appears during the rinsing step, this means that either the development time was too short or that the developer bath is saturated with photoresist. Discard the entire contents of the developer bath in the proper manner and develop the wafer further in fresh developer solution.

11.2.4 Alternative method: micromachining

For simple patterns such as a single circular porous area or a straight line, the fabrication can be performed directly by micromachining of plastic or metallic materials. We routinely use polymethyl methacrylate (PMMA) or metallic substrates. Note that the features of the pattern must be sharply contoured in order to enable

good pattern resolution. When drilling, do not go too deep (<1.5 mm), as it could then be difficult to replica mold the pattern using PDMS and/or use the PDMS replica for stamping due to too high an aspect ratio. Flat-bottomed millers are also preferred to classical drill bits. Note that replica molding the PMMA/metal piece with PDMS (Fig. 11.1) is advised. Indeed, the PMMA/metal piece can be used directly for stamping the membrane; however, this is not advisable as it usually yields poor printing quality due to the suboptimal wettability of the liquid to be printed (uncured PDMS). Also, it is often easier to use a flexible stamp rather than a rigid one (see the succeeding text). Modern fabrication techniques such as 3D printing could also be used to achieve more complex patterns without having to deal with soft lithography.

11.3 CREATING THE FINAL PDMS STAMP

11.3.1 Materials

- Silane (e.g., trichloro(1H,1H,2H,2H-perfluorooctyl) silane, #448931-10G from Sigma-Aldrich)—note that silanes are usually a dangerous product and should be handled with care, while wearing protective gloves, eye protection, and a lab coat).
- Pressurized argon gas (to keep silane under a neutral atmosphere).
- Small beaker or plastic cup.
- PDMS kit (Silgard184 from Dow Corning).
- Transfer pipette for dispensing silane.
- A glass dish that can hold the silicon wafer and resist a temperature of 65 °C.
- Scalpel.

11.3.2 Equipment

- Chemical fume hood and safety equipment for handling silane.
- Vacuum chamber (e.g., #10528861 from Fisher Scientific, France) and pump (e.g., Alcatel Vacuum Pump Type 2002BB).
- Precision scale (mg).
- Vacuum chamber and pump for degassing PDMS (as above, but note that a smaller pump can be used, e.g., #10661633 from Fisher Scientific, France).
- 65 °C oven.

11.3.3 Method

11.3.3.1 Silanization of the wafer (optional, in a chemical hood, use protective equipment at all times)

Silanization is required to ensure that the PDMS can be easily peeled off the silicon wafer without damaging it. In the absence of silanization, the photoresist layout on the wafer may break after molding a few PDMS replicas. Silanization is also

required if using a master mold in PDMS (see Fig. 11.1B); however, it is not required when replica molding a PMMA/metal master directly.

- Place the wafer face up in a vacuum chamber. Beside the wafer, dispense three drops of silane in a small beaker (5 ml) or a plastic cup, and close the vacuum chamber.
- Apply a vacuum for 5 min, then stop the pump and let the wafer and silane sit under vacuum for 1 h to allow silane vaporization and deposition on the surface of the master pattern.
- Break the vacuum to recover the wafer. A good silanization process will leave little to no marks on the negative.

11.3.3.2 Casting PDMS on the wafer

- In a disposable plastic beaker (e.g., #11738549, Fisher Scientific, France) and using a precision scale, mix PDMS with its curing agent at a ratio of 10:1 by mass. Mix thoroughly using a spoon, a coffee stirrer stick, or a plastic pipette. The quantity of PDMS required will vary depending on the size of the stamp to be made. Easy-to-use thicknesses for the stamp are typically between 2 and 5 mm. A 3-in. wafer will fit into a 90-mm Petri dish and requires ~20–25 g of PDMS.
- Place the PDMS base and curing agent mixture in a vacuum chamber until all of the air bubbles are eliminated; the mixture will then be fully degassed. Note that the duration of this step may vary depending on the power of the vacuum pump. Also note that the PDMS liquid mixture will rise substantially due to bubble formation. To avoid overflowing, the volume of the beaker should be much larger than the volume of PDMS. We typically use a 150-ml beaker for 20–40 g of PDMS. Gently break the vacuum to recover the PDMS when no more bubbles are apparent.
- Carefully pour the PDMS over the stamp, face up. Incubate at 65 °C for at least 3 h; it is also possible to cure overnight.
- To peel off the stamp, carefully cut the PDMS around the zone of interest using a scalpel and gently pull the stamp away. Take care not to cut too close to the patterns, as the membrane surface must fit within the surface of the stamp, and be gentle so as not to break the silicon wafer.

11.4 PATTERNED MEMBRANE FABRICATION

11.4.1 Membrane selection

Membrane patterning has been successfully applied to several commercially available membranes or filtration papers. Among those that we often use are polycarbonate track etched membranes (e.g., Isopore™ #GTTP02500—25 mm diameter; Millipore) which are the preferred choice in our lab; alumina-based filtration membranes (e.g., Anodisc™ #6809-6022; Whatman) which are very porous, but

brittle; or polyester-based membranes (Cyclopore™, #7060-2502; Whatman). These filtration membranes are available in different sizes (we usually use 25 mm in diameter) and with different pore sizes. Larger pores will facilitate increased diffusion of the reagents. Also, note that these membranes are opaque; therefore, the cells cultured on them can only be observed using epifluorescent microscopy. Transparent (less porous) membranes (e.g., Cyclopore™ from Whatman) also exist and may facilitate standard microscopy observations. There are three main criteria to consider when choosing a membrane:

- First, the membrane should be compatible with cell culture. Due to the size of pores, membrane ruggedness, or other factors, cells may not attach or may have difficulty growing on the membrane surface. To promote cell culture, the membrane surface should be coated with collagen, fibronectin, or other proteins that facilitate cell adhesion; alternatively one can use a thin layer of Matrigel™ (from BD Biosciences).
- Second, the membrane should be easy to pattern. Thin (<100–200 μm), chemically and mechanically resistant and highly porous membranes are the optimal choice, provided PDMS wettability is good. A high absorption capacity (e.g., Anodisc™) leads to a higher printing resolution, but may result in thickness printing heterogeneities in certain zones. Also, the membranes should be resistant to high temperatures (>100 °C) to enable rapid curing of the PDMS.
- Lastly, membrane porosity should be selected on the basis of the size and affinity of the diffusing drug molecules, and with respect to the possibility of cell protrusion through the membrane. We primarily use 0.2 and 1 μm pore sizes. A membrane with cross-linked pores or pores which are nonperpendicular to the membrane may be used, but will lead to a lower spatial precision in cell-based assays.

11.4.2 Materials for membrane patterning

- Filtration membranes (see Section 11.4.1 for references).
- Flat-tipped tweezers for membrane manipulation.
- Reusable spacers to avoid membrane/hot plate contact. We typically use 20 mm vulcanized fiber plumbing gaskets as a cheap source of spacer; these can be found in most hardware shops.
- Two to three grams of premixed/degassed PDMS (see Section 11.3.3.2). Sylgard 184 PDMS (Dow Corning) is a natural choice; however, it is often appropriate to use black PDMS (Sylgard 170, Dow Corning).
- A 10 cm × 20 cm piece of printing transparency sheet, or equivalently flat, smooth plastic for homogeneous spreading of the PDMS.
- A 10 cm smooth rod to spread PDMS (e.g., a 10 ml plastic pipette tip or a glass rod).
- Aluminum foil or an appropriate crystallizing dish to cover and protect the membranes during curing.
- A hotplate set at the appropriate temperature (e.g., 100 °C).

11.4.3 Method

- Pour a few drops of PDMS on a sheet of printing transparency. Spread the PDMS using a rod to obtain an even layer approximately one-tenth of a millimeter thick. For this step, the use of black PDMS is ideal since it allows direct visualization of the homogeneity of spreading.
- Ink the stamp by placing the stamp upside down on the PDMS layer and gently tapping the stamp. This step is crucial to achieve good patterning. The stamp should be coated in an even, thin layer of PDMS, with an absence of edge beads or PDMS inside patterns. If the inking leads to an unequal layer of PDMS on the stamp, it is advised to wait a few seconds to allow the layer to equilibrate. In practice, it may take a few spreading/inking attempts to obtain an appropriate layer of PDMS. In this case, the stamp surface can simply be wiped clean using tissue paper, and if required cleaned with acetone and carefully dried before the process can be started again.
- Carefully place the membrane on top of the stamp. Wait a few seconds for total contact to occur and peel off the membrane using a pair of tweezers. Avoid sliding the membrane when peeling it off; this is highly likely to destroy the printed PDMS layer. When using brittle membranes such as Anodiscs™, the flexibility of the stamp is crucial to help membrane separation: in this case, the stamp is peeled off while the membrane remains untouched.
- Note that it is also possible to leave the membrane on top of the stamp and to cure it directly. We will not provide further details here; however, this is a simple method of including a porous filter in a microfluidics system.
- Quickly place the membrane inked side up on a spacer sitting on the hotplate (the inked side will hereafter be referred to as the recto side). The optimal temperature is around 100–120 °C, as it allows rapid curing of the PDMS and ensures a good pattern resolution. Most porous filtration membranes can resist such temperatures; however, some may be altered. In this case, the temperature can be lowered.
- Allow curing for 1 h. To avoid dust deposition, the membranes should be covered using aluminum foil or a crystallizing dish.
- The membranes can then be stored in a clean/dust free box.

11.4.4 Tip for membrane handling

To ease handling, the membranes can be attached to a 1-mm-thick PDMS washer. To do so, a 1 mm layer of PDMS mixture (see Fig. 11.1) can be poured in a Petri dish, cured at 65 °C overnight and then cut to the appropriate size using a scalpel. The PDMS washer can then be glued onto the inked side of the patterned membrane using drops of uncured PDMS as a mortar, followed by further curing at 65 °C.

11.5 MEMBRANE PREPARATION FOR CELL-BASED ASSAYS

11.5.1 Materials and equipment required for membrane preparation

- Round-tipped tweezers for membrane manipulation.
- UV lamp for sterilization treatment.
- Twenty milliliters of phosphate buffered saline (PBS).
- Five milliliters of coating solution to facilitate cell attachment to the porous filter, for example, 0.3 g/l type I collagen, fibronectin, or the relevant adhesion ligand for your chosen cell line.
- Five milliliters of sterile deionized water.

11.5.2 Method

- Membranes should be washed with PBS to remove any dust and should then be sterilized on both sides using UV light. This can be accomplished using a dedicated UV source. We usually expose the membranes for 30 min on each side to the UV light of a conventional cell culture hood.
- Incubate with the desired coating protein—for example, 0.3 g/l type I collagen—for 2 h at room temperature.
- Rinse once with water and twice with PBS.

11.5.3 Cell seeding

Prior to cell seeding, we routinely culture MadinDarby Canine Kidney (MDCK) cells in 25 cm^2 tissue culture flasks (Techno Plastic Products AG (TPP), Switzerland) in a 37 °C incubator with a 5% CO_2 atmosphere. The culture medium is Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin (Gibco), and 100 μg/ml kanamycin (Sigma-Aldrich). The MDCK cells are trypsinized, transferred for culture on the collagen-coated verso side of the μPMs, and allowed to grow to near confluence (2 or 3 days) in a 37 °C incubator with a 5% CO_2 atmosphere before the assay starts. Note that the best results are obtained when the cells are cultured on the verso side, possibly due to surface irregularities and the hydrophobicity of PDMS. It is also better to deposit the drops of the reagents to be tested on the printed PDMS side, as the hydrophobicity of PDMS ensures the drops of reagent are tightly confined which prevents cross-contamination. Most cell lines will be suitable for culture on μPMs, as long as they can adhere to the coated surface. We have tested MDCK, HeLa, and 3T3 cell lines. Again, note that μPMs are not transparent; therefore, the cells can only be observed by fluorescence microscopy. This requires the use of either cell lines transfected with a fluorescent reporter gene or staining with fluorescent dyes appropriate to the cellular process under study.

11.5.4 Method

- Take the μPM and rinse the cells with fresh culture medium.
- To avoid direct contact between the cells and the Petri dish, it is advisable to use a holder to support the μPM (Fig. 11.1). It can easily be made in PDMS by

punching a hole into a 1–2 mm thick PDMS layer. If possible it is more convenient to make a holder in stainless steel that can be autoclaved and reused.
- Mount the μPM on the holder, with the recto side up so that the cells are immersed in the culture medium, with the top of the membrane at the surface; we use 4 ml of culture medium in 35-mm dishes. To facilitate observation of the cells, one can use 35-mm Petri dishes with glass bottom (e.g., #81158 from iBidi).
- Dispense 1–2 μl drops (for 1 mm porous disk) of the reagents to be tested at separate locations on the recto side of the μPM.
- Let the cells incubate in a 37 °C incubator with a 5% CO_2 atmosphere for the desired period of time.
- Using a pipette, replace the drops of reagents with a drop of culture medium to stop the assay.
- If required, repeat the deposition of the drops of reagents to be tested for a time-course experiment or a combinatorial assay. In the case of repeated assays at the same locations, the first drops can be aspirated using a micropipette, and the porous disks rinsed twice with drops of culture medium before depositing the next drops of reagents.
- Image the cells using fluorescence microscopy.

Note that the cells will remain upside down, and that imaging of the cells during this process will need to be done using an inverted microscope. Since the cells are not directly in contact with the bottom of the Petri dish, it is not possible to use a high magnification, and objectives with a long working distance are preferred. The drops of reagents can be manually added or removed at any time during image acquisition. Thus, it is possible to perform time-lapse microscopy. However, the user needs to be careful to not move the sample or the microscope stage when placing the drops. As a proof of principle, we were able to manually stain cells from the same monolayer at specific locations with different dyes using this method (see Fig. 11.2). It is also possible to stack different membranes in order to change the pattern of porosity seen by the cells as illustrated in Fig. 11.2. That way, complex drug delivery, with variations in space and time can be tested manually.

CONCLUSION

We have described a simple method to contact print a thin layer of PDMS onto a porous substrate. This method can be used to create arrays of millimeter scale porous areas which can be used as a substrate for cell-based assays. Compared to 96-well plates and other cell-based assay strategies, our method has several advantages: it is highly customizable, inexpensive, easy to fabricate and manipulate, and allows complex combinatorial and time-dependent assays to be performed on a monolayer of cells. The same technique can be applied to systems other than mammalian cells, for example, microorganisms for antibiotic or metabolic screening.

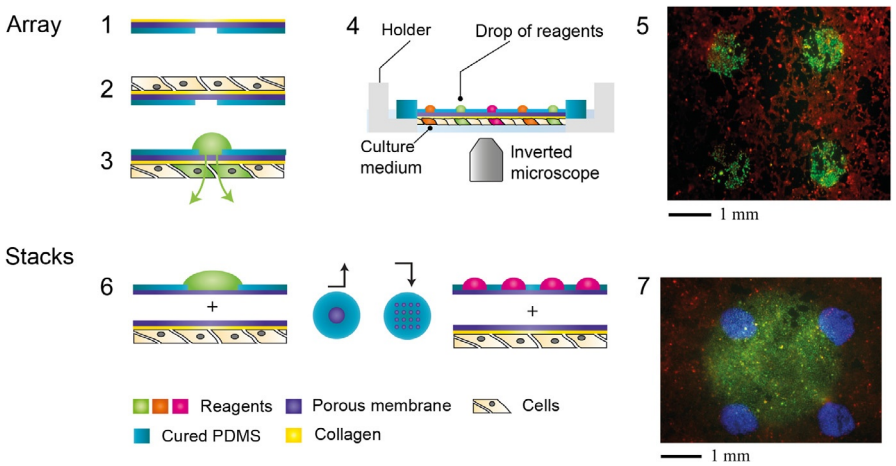

FIGURE 11.2

Cell culture assay. 1. Micropatterned membranes are coated with collagen prior to cell seeding. 2. Cells are grown to mid-confluence on the coated side of the membrane under normal cell culture conditions. 3. The μPM is then flipped upside down to allow the user to deposit drops of reagents on the porous areas. The drugs will selectively diffuse through the porous areas of the μPM to the cells growing immediately below. 4, 5. Multiple reagents can be tested at once, and their effects observed using an inverted fluorescent microscope. The hydrophobicity of PDMS allows precise deposition of the drops of reagents and selective cell stimulation. 6, 7. Stacking a nonpatterned membrane followed by a patterned membrane will allow the testing of multiple drugs or chemicals in different areas, in a combinatorial manner, by changing the μPM.

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