Video Article

Fabrication of Micropatterned Hydrogels for Neural Culture Systems using Dynamic Mask Projection Photolithography

J. Lowry Curley, Scott R. Jennings, Michael J. Moore
Biomedical Engineering, Tulane University

Correspondence to: Michael J. Moore at mooremj@tulane.edu

URL: http://www.jove.com/details.php?id=2636
DOI: 10.3791/2636
Citation: Curley J.L., Jennings S.R., Moore M.J. (2011). Fabrication of Micropatterned Hydrogels for Neural Culture Systems using Dynamic Mask Projection Photolithography. JoVE. 48. http://www.jove.com/details.php?id=2636, doi: 10.3791/2636

Abstract

Increasingly, patterned cell culture environments are becoming a relevant technique to study cellular characteristics, and many researchers believe in the need for 3D environments to represent *in vitro* experiments which better mimic *in vivo* qualities [1-3]. Studies in fields such as cancer research [4], neural engineering [5], cardiac physiology [6], and cell-matrix interaction[7,8]have shown cell behavior differs substantially between traditional monolayer cultures and 3D constructs.

Hydrogels are used as 3D environments because of their variety, versatility and ability to tailor molecular composition through functionalization [9-12]. Numerous techniques exist for creation of constructs as cell-supportive matrices, including electrospinning[13], elastomer stamps[14], inkjet printing[15], additive photopatterning[16], static photomask projection-lithography[17], and dynamic mask microstereolithography[18]. Unfortunately, these methods involve multiple production steps and/or equipment not readily adaptable to conventional cell and tissue culture methods. The technique employed in this protocol adapts the latter two methods, using a digital micromirror device (DMD) to create dynamic photomasks for crosslinking geometrically specific poly-(ethylene glycol) (PEG) hydrogels, induced through UV initiated free radical polymerization. The resulting "2.5D" structures provide a constrained 3D environment for neural growth. We employ a dual-hydrogel approach, where PEG serves as a cell-restrictive region supplying structure to an otherwise shapeless but cell-permissive self-assembling gel made from either Puramatrix or agarose. The process is a quick simple one step fabrication which is highly reproducible and easily adapted for use with conventional cell culture methods and substrates.

Whole tissue explants, such as embryonic dorsal root ganglia (DRG), can be incorporated into the dual hydrogel constructs for experimental assays such as neurite outgrowth. Additionally, dissociated cells can be encapsulated in the photocrosslinkable or self polymerizing hydrogel, or selectively adhered to the permeable support membrane using cell-restrictive photopatterning. Using the DMD, we created hydrogel constructs up to ~1mm thick, but thin film (<200 μm) PEG structures were limited by oxygen quenching of the free radical polymerization reaction. We subsequently developed a technique utilizing a layer of oil above the polymerization liquid which allowed thin PEG structure polymerization.

In this protocol, we describe the expeditious creation of 3D hydrogel systems for production of microfabricated neural cell and tissue cultures. The dual hydrogel constructs demonstrated herein represent versatile *in vitro* models that may prove useful for studies in neuroscience involving cell survival, migration, and/or neurite growth and guidance. Moreover, as the protocol can work for many types of hydrogels and cells, the potential applications are both varied and vast.

Protocol

1. DMD Setup

1. The DMD board, UV light guide (with collimator) and 4x objective lens are all mounted vertically on a vibration isolation table.
2. The UV light guide should be adjusted so that the light hits the mirror array at an angle of 45° relative to the plane of the mirrors, and 24° below the plane of the mirrors (Figure 1).
3. The objective lens is mounted so that the distance from the DMD to the objective lens corresponds to the focal length associated with the lens.
4. An inverted microscope is placed below the objective lens, such that the focused light reflected from the DMD can be visualized through the microscope. The distance from the objective lens to the polymerization surface should approximately correspond to the working distance of the lens. The stage on the microscope can then be used to adjust this distance to finely focus the image. This distance may vary depending on the chosen polymerization surface.

2. Dual Hydrogel Constructs for Tissue Explant Cultures

A. DRG explant adhesion

1. Coat the walls of 6-well collagen coated cell culture inserts (Corning) with Rain-X, taking care to avoid the membrane itself. (Alternatively, a hydrophobic barrier pen may be used.)
2. Hydrate inserts overnight with 1.5 mL of adhesion medium in an incubator at 37 °C and 5% CO_2. Adhesion medium is neurobasal medium with 10% fetal bovine serum, 1% Penicillin/streptomycin, 0.5mM L-glutamine, and 20 μg/mL NGF.
3. Harvest embryonic dorsal root ganglia (DRG) from E-15 rat pups. DRG should be plated onto collagen coated 6-well inserts, as many as four per insert.
4. Incubate inserts for 2 hours to allow for DRG adherence to the membrane.

B. Dynamic mask photopolymerization

A digital micromirror device (DMD) is a 1024 x 768 array of individual mirrors, similar to that in projection televisions, which selectively reflects light based on mirror position. For our purposes, the DMD is used to pattern ultraviolet (UV) light onto photocrosslinkable hydrogels, creating specifiable hydrogel geometries in a simple and rapid manner. Figure 1 depicts the setup of the DMD and UV light path. Though our DMD is a standalone unit, the device can also be integrated for use with many existing microscopes.

1. Remove all excess liquid from insert, and add 500 µL of polymerization medium inside the insert. Polymerization medium contains 10% PEG (MW 1000) and 0.5% Irgacure 2959 dissolved in neurobasal medium supplemented with 20 µg/mL NGF and 1% Pen/Strep.
2. Place the insert underneath the DMD device on a Rain-X treated glass slide.
3. Load the appropriate black and white image to be used as a "photomask" on the DMD, through the use of the included ALP-3 basic GUI program. For our purposes, a bifurcating shape was chosen to allow for implementation of neurite guidance systems.
4. Using an inverted microscope for visualization, align the tissue explants with the appropriate location on the photomask using a visible light source reflecting off the DMD.
5. Switch the visible light source for the UV light source, and illuminate the PEG solution until crosslinking is sufficient. (For the conditions given and 5.0 Watts/cm^2 incident on the DMD, crosslinking can be completed in as little as 55 seconds.) Repeat for all four DRG on the insert.
6. Wash each insert three times with sterile DPBS and 1% Pen-Strep.
7. Add 1.5mL growth medium underneath the cell culture inserts, and allow to equilibrate in an incubator for 30 minutes. Growth medium is neurobasal medium containing 2% B-27, 1% Pen/Strep, 0.5mM L-glutamine, and 20 µg/mL NGF.

C. Secondary hydrogel

Puramatrix

1. For neuronal applications, 1% Puramatrix is diluted according to manufacturer's instructions to 0.15% in sterile H_2O and supplemented with 1 µg/mL soluble laminin.
2. All excess media must be removed from the PEG voids, which contain the DRG explants, using a sterile cotton tip applicator, Kimwipe, or micropipette.
3. Puramatrix is added to the PEG voids with a micropipette in order to fill the empty space without overflowing. Depending on void volume, typically ~ 1 µL is used per construct.
4. Puramatrix begins the process of self-assembly immediately upon contact with a physiological salt solution, i.e. the swollen PEG, but 1.5 mL of growth medium is added underneath the insert and placed in the incubator for one hour to ensure total gelation.
5. Initially, Puramatrix is slightly acidic, so change the media after one hour to allow the pH to equilibrate.
6. Media changes are required approximately every 48 hours. Be careful that all media is added underneath the insert, to protect the integrity of mechanically weak Puramatrix.

Agarose

1. For neuronal applications, agarose is diluted to a 1% solution in growth medium and placed in a 60 °C water bath until the agarose fully dissolves (~1 hr). The solution is then supplemented with 1 µg/mL soluble laminin.
2. All excess media must be removed from the PEG voids.
3. Agarose is added to the PEG voids in order to fill the empty space without overflowing. Depending on volume of void, typically ~ 1 µL is used per construct.
4. 1.5 mL of growth medium is pre-chilled (8 °C) in a 6-well plate, and the agarose containing inserts are transferred to the chilled media and maintained in a refrigerator at 8 °C for at least three minutes to allow agarose to gel.
5. Finally, the inserts are transferred to 1.5 mL of pre-warmed growth medium (37 °C) and maintained in the incubator at 37 °C, with media changes required every 48 hours.

3. Dual Hydrogel 3D Cell Encapsulation

Dual hydrogel encapsulation is appropriate when using any self-assembling gel. The photocrosslinkable gel, in this case PEG, serves as a structural support for the geometric presentation of the self-assembling gel, for example Puramatrix or agarose. Some of the methods, specifically the type of gel and choice of photomask, will depend on the particular desired application.

1. Load an appropriate mask on the DMD. For our application, cell survival, we simply chose a cylindrical presentation of Puramatrix to aid in the imaging of cells. Researchers studying cell signaling might be interested in a compartmental geometry to allow for the diffusion of chemotactic molecules. Additionally, a rough approximation of an artery was shown to represent possible application to blood vessel research.
2. Treat the walls of a polyester cell culture insert with Rain-X, and place under DMD on Rain-X coated slide.
3. Add 500 µL of polymerization medium to the insert. Induce PEG crosslinking by exposure to UV light for 55 seconds.
4. Wash three times with sterile DPBS and 1% Pen-Strep.
5. Remove all excess media from the PEG voids.
6. Spin down cells at the desired density into a pellet. Care must be taken to remove all medium from the cell pellet prior to mixing, as Puramatrix begins self-assembly immediately upon contact with a salt solution. Suspend the cells inside 0.15% Puramatrix diluted in sterile H_2O supplemented with 10% sucrose.
7. Inject the Puramatrix/cell/sucrose mixture inside voids in PEG.
8. Add 1.5 mL of growth medium underneath the insert, and allow to gel inside incubator for one hour.
9. Change media after one hour, and ~every 48 hours thereafter.

4. Single Hydrogel 3D Cell Encapsulation

A single hydrogel encapsulation would be appropriate for any situation where the cells can be examined inside of a photocrosslinkable hydrogel.

1. Load an appropriate mask on the DMD. For cell survival studies, we again chose a basic circular mask to represent a cylinder. Masks similar to those shown in method 4 could again be applied for the appropriate research field.
2. Treat a polyester cell culture insert with Rain-X, and place under DMD on Rain-X coated slide.
3. Cells at any desired concentration can be added directly to the 10% PEG solution, mixing well to ensure homogenous distribution.
4. Add 500 µL of polymerization medium to the insert. Induce PEG crosslinking by exposure to UV light for 55 seconds.

5. Wash three times with sterile DPBS and 1% Pen-Strep.
6. Fill with growth medium both below and on top of the insert, changing every ~2 days.

5. Thin Film Hydrogel Polymerization

1. Load an appropriate mask on the DMD.
2. Treat a collagen-coated polyester cell culture insert with Rain-X, and place on Rain-X coated slide.
3. Add sufficient polymerization medium to just cover the bottom of the insert (~250-300 µL for 6-well plate inserts). Allow the media to permeate the insert membrane for 30-45 minutes at room temperature.
4. Remove excess polymerization medium from the insert with a micropipette or Kimwipe. Add a sufficient amount of UV-transparent oil to completely cover the bottom of the insert. Allow the insert to sit for 15-30 minutes at room temperature, long enough for the oil to form a distinct layer above the polymerization medium saturating the insert membrane.
5. Place the insert and slide under the DMD. Induce PEG crosslinking by exposure to UV light. Because of the thinness of the PEG layer, crosslinking can be completed in as little as 15 seconds at 5.0 Watts/cm^2 incident on the DMD.
6. Wash the insert three times with sterile DPBS and 1% Pen-Strep. (If the persistence of an oily residue is a concern, a mild detergent such as Tween 20 (1%) may be added to the wash buffer.)
7. Store the insert in a 6-well tissue culture plate. Add growth medium to suspended cells, in the desired concentration, and pipette a sufficient volume of the cell suspension inside the cell culture insert to obtain the desired cell density. Then, add enough growth medium below the insert to completely maintain cell viability (~1.5 mL).
8. After 48 hours have elapsed, wash the insert three times with sterile DPBS and 1% Pen-Strep to dislodge any unadhered cells. Change media approximately once every 48 hours.

6. Representative Results

Examples of dual hydrogel constructs containing DRG explants are shown in Figure 2. Notice that cellular migration and neurite extension is limited to the cell permissive region of the dual hydrogel construct. Figure 3 depicts dissociated cells encapsulated similarly inside the dual hydrogel constructs. Due to the dynamic nature of the DMD photomask, the geometry available for encapsulation is limited only by the dimensions and resolution of the optics. Cell encapsulation was also possible inside a single photopolymerizable hydrogel, PEG, and a live/dead viability test was performed as evidenced in Figure 4. Encapsulation in PEG is meant only as an example, as PEG does not represent an ideal environment for neural cells. Therefore, the cell viability realized in our PEG constructs is understandably low. Finally, examples of utilizing thin PEG films as a patterned restrictive layer for cell adhesion on cell culture inserts are shown in Figure 5. Additionally, examples of possible "bad" results are offered in Figure 6.

The results represent only a small fraction of the possible uses of the methods developed in our lab. They are meant to demonstrate the ease, versatility and viability of our approach, and could be treated as "proof of principle" for researchers to develop their own possible adaptations.

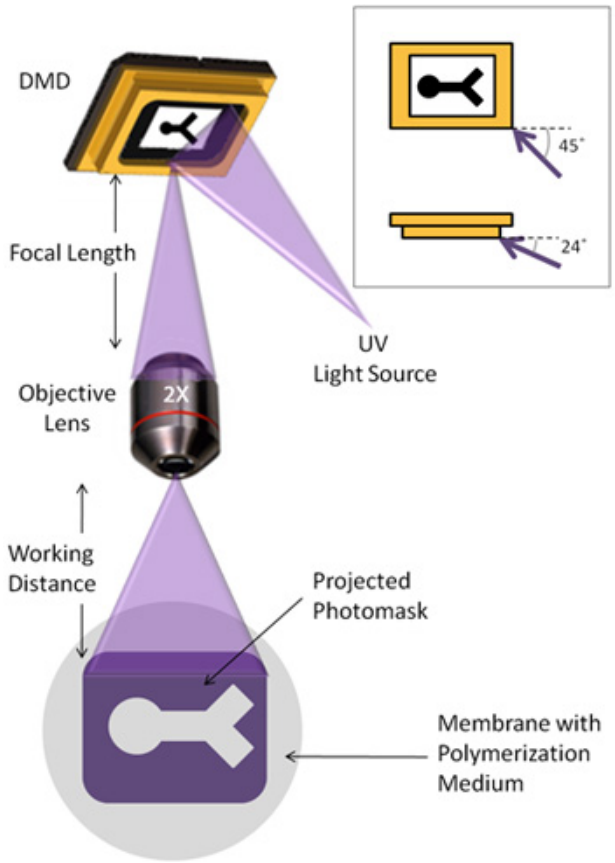

Figure 1. Schematic illustration of the light path used for photolithography. Inset: The UV light illuminates the DMD at an angle of 45°, and 24° below the plane of the mirror array.

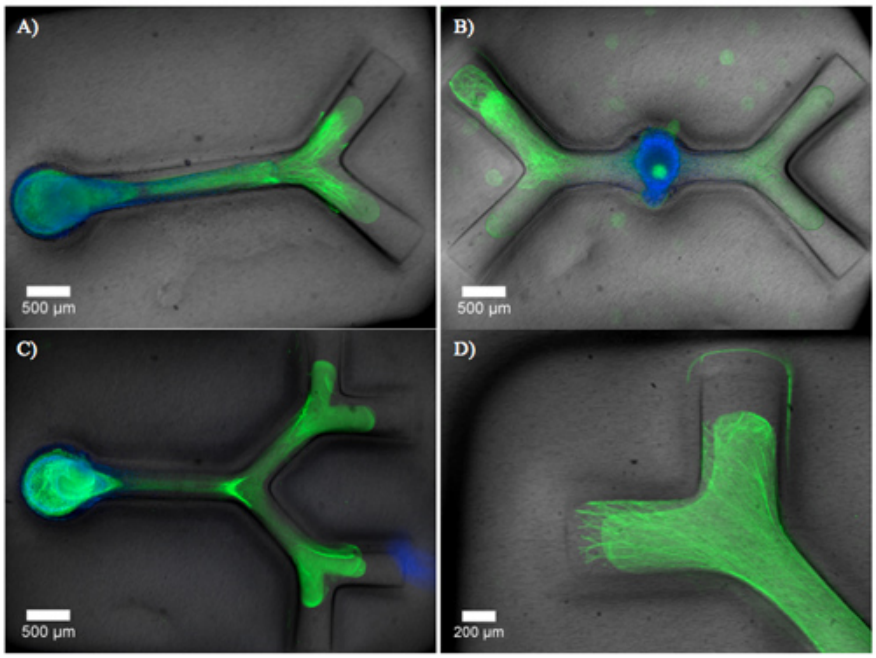

Figure 2. Labeled growth and proliferation in DRG containing dual hydrogel constructs. A-D) Images portraying polymerized PEG constructs (gray) with neurites labeled with Beta III tubulin (green), DAPI stained cell nuclei (blue). The DRG explants are contained in Puramatrix and located in the circular regions of the pattern, with neurites growing towards the bifurcation(s).

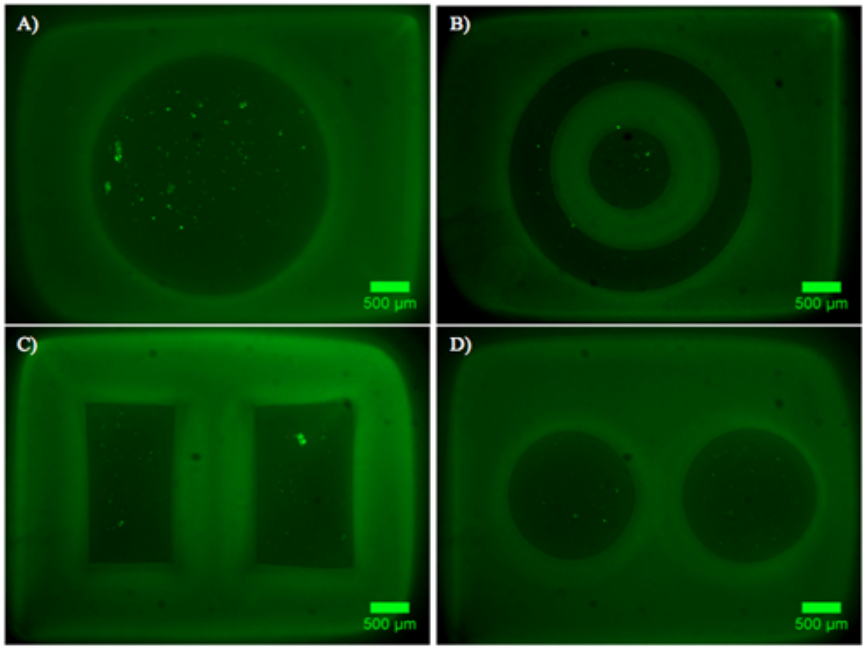

Figure 3. Dual hydrogel constructs containing cells labeled with calcein AM, a live cell marker, after 48 hours in growth medium. A-D) Various PEG shapes, filled with Puramatrix containing dissociated DRG neurons (~5x10^3 cells/mL).

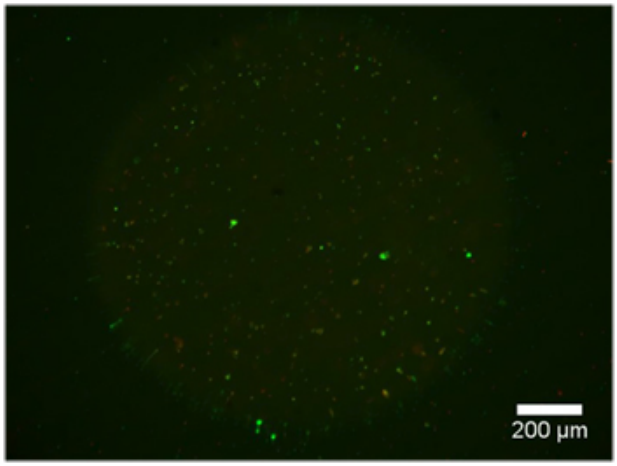

Figure 4. Single hydrogel construct containing live cells labeled with Calcein AM (green) and dead cells labeled with ethidium homodimer-1 (red) after 24 hours in growth medium ($5x10^3$ cells/mL).

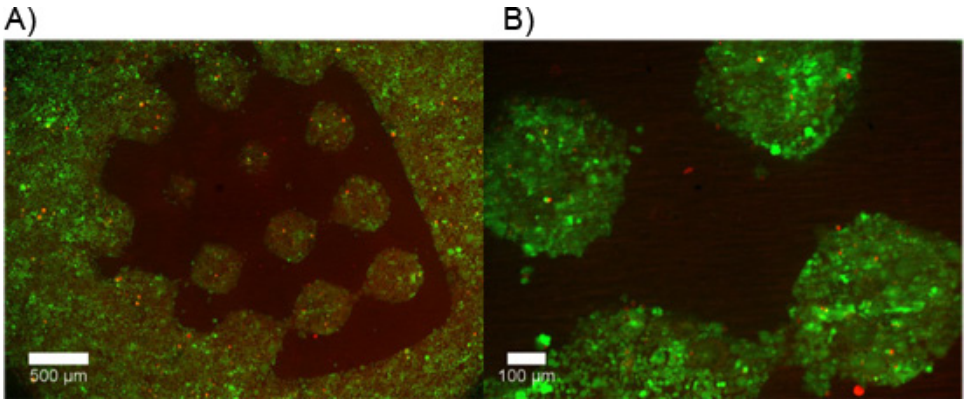

Figure 5. Cell restrictive PEG polymerized as a thin film using a "test pattern" to selectively adhere dissociated cells to the collagen coated membrane of permeable supports. A, B) Live cells are labeled after 48 hours with Calcein AM (green), while dead cells are labeled with ethidium homodimer-1 (red). Minimal cell adhesion occurs in the area containing the thin PEG film.

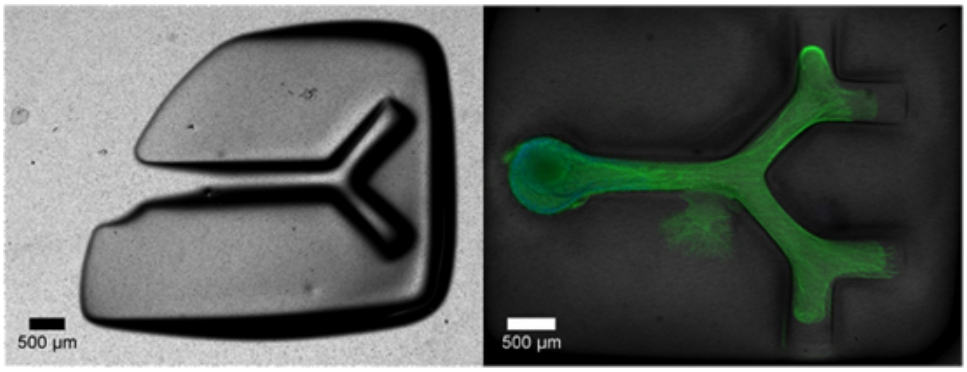

Figure 6. Representative images of undesirable results. A) Partial polymerization of PEG, leading to an unusable PEG construct. Improper polymerization can occur due to the presence of a meniscus in the pre-polymer medium, insufficient amounts of polymerization medium, insufficient UV exposure or improper focus of the optics. B) Image portraying polymerized PEG constructs (gray) with neurites labeled with Beta III tubulin (green), DAPI stained cell nuclei (blue). The neurites were able to grow outside of the patterned PEG channels. This often occurs in the condition that Puramatrix overflows on top of the PEG portion during injection.

Discussion

The method described herein could be used by any investigator seeking simple and reproducible cell culture systems. Theoretically, due to the wide variety of photopolymerizable hydrogels available, the environment could be tailored to allow for use with any cell type, including whole tissue explants. Additionally, the dual hydrogel system allows for improved spatial control in the presentation of self-polymerizing hydrogels, which tend to form amorphous shapes on their own. The resulting "2.5D" micropatterned hydrogel constructs provide a 3D matrix for neural growth presented in a 2D configuration that enables convenient microscopic evaluation. The substrate on which the gels are polymerized can also be varied, allowing for greater control in experimental design. Our methods are optimized for use with cell culture permeable supports, as we have seen improved viability (Figure 4) as compared to polymerization on glass slides (data not shown). However, other polymerization surfaces may be more applicable for different applications: fabrication on glass slides used in microfluidics experiments or cell aggregate formations, for example.

Our experience with these culture systems has led to the identification of potential areas of difficulty. First, careful techniques are required to maintain the sterility of the constructs. Due to the bulky nature of the DMD setup, it is difficult to operate polymerization steps under sterile conditions. To combat this issue, the rinse step described in the methods is helpful, and antibiotics should be used in all media. Additionally, the

final thickness and shape of the polymerized construct is highly dependent on the fluid behavior of the pre-polymer mixture, and the presence of a meniscus can result in gel constructs that are too thin or incompletely polymerized (Figure 6). Two steps can be taken to minimize the formation of a meniscus inside cell culture inserts. For thick hydrogels (>200 µm), a simple coating of Rain-X around the inside wall of the insert is sufficient. However, as briefly described above, for thin constructs (<200 µm), an oil layer is required to both minimize the meniscus and negate oxygen quenching of the free radical polymerization. Resolution was found to be dependent on thickness, with a decrease in feature size realized with increasingly thicker gels. The resolution also varied depending on whether the feature represented a positive or negative relief in the hydrogel. However, we achieved a sufficient resolution for constructs with minimum feature sizes on the scale of ~100 µm using only microscope objectives as focusing optics.

Our experiments have shown that the dual hydrogel constructs described here represent an excellent basis for the formation of basic *in-vitro* models of neurite growth and guidance. The micropatterning technique employed is an adaptation of existing methods[18,19], but our set-up emphasized a simple to implement design and was optimized for production of dual hydrogel constructs on cell culture inserts; cell culture inserts were vital for improving cell viability as well as crosslinking around previously adherent tissue explants. The scope of the results shown is limited by the interests of our lab; however, we believe that the methods described in this publication will prove useful to researchers searching for a relatively cheap, quick and easy to use method for the fabrication of 3D cell culture models.

Disclosures

No conflicts of interest declared.

Acknowledgements

The authors would like to thank the laboratory of Prof. Anthony Windebank for sharing their expertise on DRG dissection and culture, as well as Prof. Shaochen Chen for helpful discussions regarding the DMD setup. This research was funded in part by Tulane University and grants from the Louisiana Board of Regents (LEQSF[2009-10]-RD-A-18) and the NIH (NS065374).

References

1. Abbott, A. Cell culture: biology's new dimension. Nature 424 (6951), 870-872 (2003).
2. Schindler, M. *et al.*, Living in three dimensions: 3D nanostructured environments for cell culture and regenerative medicine. Cell Biochem Biophys 45 (2), 215-227 (2006).
3. Maltman, D.J., & Przyborski, S.A. Developments in three-dimensional cell culture technology aimed at improving the accuracy of *in vitro* analyses. Biochemical Society Transactions 38, 1072-1075 (2010).
4. Smalley, K.S., Lioni, M., & Herlyn, M. Life isn't flat: taking cancer biology to the next dimension. In Vitro Cell Dev Biol Anim 42 (8-9), 242-247 (2006).
5. Irons, H.R. *et al.*, Three-dimensional neural constructs: a novel platform for neurophysiological investigation. Journal of Neural Engineering 5 (3), 333-341 (2008).
6. Bursac, N. *et al.*, Cultivation in rotating bioreactors promotes maintenance of cardiac myocyte electrophysiology and molecular properties. Tissue Eng 9 (6), 1243-1253 (2003).
7. Cukierman, E., Pankov, R., Stevens, D.R., & Yamada, K.M. Taking cell-matrix adhesions to the third dimension. Science 294 (5547), 1708-1712 (2001).
8. Cushing, M.C. & Anseth, K.S. Materials science. Hydrogel cell cultures. Science 316 (5828), 1133-1134 (2007).
9. Kloxin, A.M., Kasko, A.M., Salinas, C.N., & Anseth, K.S. Photodegradable hydrogels for dynamic tuning of physical and chemical properties. Science 324 (5923), 59-63 (2009).
10. Geckil, H., Xu, F., Zhang, X., Moon, S., & Demirci, U. Engineering hydrogels as extracellular matrix mimics. Nanomedicine (Lond) 5 (3), 469-484 (2010).
11. Luo, Y. & Shoichet, M.S. A photolabile hydrogel for guided three-dimensional cell growth and migration. Nature Materials 3 (4), 249-253 (2004).
12. Wylie, R.G. & Shoichet, M.S. Two-photon micropatterning of amines within an agarose hydrogel. Journal of Materials Chemistry 18 (23), 2716-2721 (2008).
13. Ji, Y. *et al.*, Electrospun three-dimensional hyaluronic acid nanofibrous scaffolds. Biomaterials 27 (20), 3782-3792 (2006).
14. Moeller, H.C., Mian, M.K., Shrivastava, S., Chung, B.G., & Khademhosseini, A. A microwell array system for stem cell culture. Biomaterials 29 (6), 752-763 (2008).
15. Xu, T. *et al.*, Viability and electrophysiology of neural cell structures generated by the inkjet printing method. Biomaterials 27 (19), 3580-3588 (2006).
16. Liu Tsang, V. *et al.*, Fabrication of 3D hepatic tissues by additive photopatterning of cellular hydrogels. FASEB J 21 (3), 790-801 (2007).
17. Beebe, D.J. *et al.*, Microfluidic tectonics: A comprehensive construction platform for microfluidic systems. Proceedings of the National Academy of Sciences of the United States of America 97 (25), 13488-13493 (2000).
18. Lu, Y., Mapili, G., Suhali, G., Chen, S.C., & Roy, K. A digital micro-mirror device-based system for the microfabrication of complex, spatially patterned tissue engineering scaffolds. Journal of Biomedical Materials Research Part A 77A (2), 396-405 (2006).
19. Naiser, T., Mai, T., Michel, W., & Ott, A. Versatile maskless microscope projection photolithography system and its application in light-directed fabrication of DNA microarrays. Review of Scientific Instruments 77 (6), 063711-063711-11 (2006).

