

Video Article

Cell Co-culture Patterning Using Aqueous Two-phase Systems

John P. Frampton¹, Joshua B. White¹, Abin T. Abraham¹, Shuichi Takayama^{1,2}

¹Department of Biomedical Engineering, University of Michigan

²Department of Macromolecular Science and Engineering, University of Michigan

Correspondence to: John P. Frampton at frampton@umich.edu

URL: <https://www.jove.com/video/50304>

DOI: [doi:10.3791/50304](https://doi.org/10.3791/50304)

Keywords: Bioengineering, Issue 73, Biomedical Engineering, Microbiology, Molecular Biology, Cellular Biology, Biochemistry, Biotechnology, Cell Migration Assays, Culture Techniques, bioengineering (general), Patterning, Aqueous Two-Phase System, Co-Culture, cell, Dextran, Polyethylene glycol, media, PEG, DEX, colonies, cell culture

Date Published: 3/26/2013

Citation: Frampton, J.P., White, J.B., Abraham, A.T., Takayama, S. Cell Co-culture Patterning Using Aqueous Two-phase Systems. *J. Vis. Exp.* (73), e50304, doi:10.3791/50304 (2013).

Abstract

Cell patterning technologies that are fast, easy to use and affordable will be required for the future development of high throughput cell assays, platforms for studying cell-cell interactions and tissue engineered systems. This detailed protocol describes a method for generating co-cultures of cells using biocompatible solutions of dextran (DEX) and polyethylene glycol (PEG) that phase-separate when combined above threshold concentrations. Cells can be patterned in a variety of configurations using this method. Cell exclusion patterning can be performed by printing droplets of DEX on a substrate and covering them with a solution of PEG containing cells. The interfacial tension formed between the two polymer solutions causes cells to fall around the outside of the DEX droplet and form a circular clearing that can be used for migration assays. Cell islands can be patterned by dispensing a cell-rich DEX phase into a PEG solution or by covering the DEX droplet with a solution of PEG. Co-cultures can be formed directly by combining cell exclusion with DEX island patterning. These methods are compatible with a variety of liquid handling approaches, including manual micropipetting, and can be used with virtually any adherent cell type.

Video Link

The video component of this article can be found at <https://www.jove.com/video/50304/>

Introduction

Aqueous two-phase systems (ATPSs) form when solutions of two incompatible polymers are mixed together at high enough concentrations. Phase separation is influenced by a variety of factors that include the molecular weight and polarity of the polymers, temperature of the solutions, pH and ionic content of the aqueous solvent^{1,2}. The point at which the two polymer solutions separate is determined by the physiochemical properties of the chosen phase system, but generally occurs at low polymer concentrations (less than 20% wt/wt) under non-denaturing conditions, allowing ATPSs to be used for biotechnology applications³⁻⁹.

By far the most extensively studied ATPS is the polyethylene glycol (PEG)/dextran (DEX) system. The ATPS formed by these inexpensive and biocompatible polymers was originally described for the purification of biomolecules by way of molecular partitioning^{2,10}. Partitioning occurs when additional molecules or particles that do not contribute to the phase system are mixed with PEG and DEX. Based on their relative affinities for either DEX or PEG, the molecules or particles will preferentially reside within one of the two phases or at the interface. Another property of the PEG/DEX ATPS is the existence of interfacial tension between the two polymer phases. ATPSs formed by PEG and DEX generally display interfacial tensions that are much lower than other liquid-liquid two-phase systems such as oil and water; however, the interfacial tension forces still exert effects on small particles such as viruses, cells and protein aggregates^{2,11-13}. Finally, since higher molecular weight PEG and DEX separate at low concentrations (less than 5% wt/wt for high molecular weight polymers varieties) in the presence of physiological concentrations of salts, there are few if any deleterious effects on mammalian cells incorporated within these systems¹⁴⁻¹⁶.

Recently, the interfacial properties and partitioning effects of ATPSs have been applied by our lab for cell patterning^{14,16-20}. This was accomplished by micropatterning a denser DEX solution on cell culture substrates in the presence of PEG. When cells are incorporated into the PEG phase, they are excluded from entering the DEX droplets due to PEG/DEX interfacial tension²⁰. When cells are patterned in the DEX phase, they are retained at the surface of the cell culture substrate by interfacial tension and partitioning^{16,17,19}.

In contrast to other methods for cell patterning, ATPS cell patterning is easy to learn and only requires rudimentary knowledge about the polymers themselves, and the ability to perform cell culture and use a micropipettor. Other methods for cell patterning often involve specialized equipment and training that are not easily translated to the life sciences. For example, some methods (microcontact printing or inkjet printing) pattern cells indirectly by applying patterns of cell adhesive biomolecules to a culture substrate that subsequently serve as sites for cell attachment^{21,22}. Although indirect approaches are useful for some cell types, they require a high degree of user skill and specialized equipment to fabricate the patterning tool, and can lack specificity depending on the particular cell type/biomolecule pattern. Alternatively, cells can be deposited with high pattern specificity by way of direct patterning approaches that include laminar flow patterning, stenciling and inkjet printing²³⁻²⁶. However, these techniques also require user expertise and specialized equipment, and may damage cells during the printing process.

Although these approaches generally produce precise patterns of cells, for cell patterning to be a useful tool in the life sciences, it must be cost effective and simple to implement.

Here we report a detailed protocol for generating patterned cell cultures using the ATPSs described in our previously-published applications. Using only micropipettors, users can generate cell exclusion zones or cell islands for migration assays. This is achieved by way of PEG/DEX interfacial tension that either retains cells in the DEX phase or excludes cells deposited in the PEG phase from DEX. By combining these two fundamental patterning techniques, it is possible to rapidly generate co-cultures of cells such as liver-fibroblast cell co-cultures. Patterning methods, ATPS parameters and expected results are described in detail.

Protocol

1. Phase System Characterization: Determining Thresholds for Phase Separation

1. Prepare solutions containing PEG and DEX in the desired buffer or cell culture medium as shown in **Figure 1** (purple dots) in 15 ml or 50 ml conical tubes. Hereafter, PEG and DEX will refer to 35 kDa PEG and 500 kDa DEX; however, critical concentrations will change depending on the two polymers used. Record the mass of PEG and DEX in each solution. High-concentration polymer solutions may take several hours to dissolve. Vortexing can be used for serum-free solutions. For media containing proteins or serum, place the tubes on a rocking stage until both polymers are fully dissolved. Record the weight of the media used to dissolve the polymers and take note of the initial concentrations.
2. Once the polymers are fully dissolved, the solutions should appear cloudy. This is the first indication that phase separation has occurred. To confirm this, allow the polymer solutions to rest in a vertical position at room temperature for 20 min. Centrifugation at 1,000 x g can be used to accelerate the phase separation process. The denser bottom phase will be DEX-rich and the top phase will be PEG-rich.
3. Slowly add additional buffer or media to the tubes. Small increments should be used so as not to overshoot the phase separation point.
4. When the solution becomes clear and no longer phase-separates after centrifugation, the threshold for phase separation has been reached. Record the final weight of the tube.
5. Using the previous recorded weights for the polymers, along with the final weight after adding media, determine the % wt/wt of each the two polymers at which phase separation no longer occurs.
6. Plot these values as % wt/wt PEG on the y-axis and % wt/wt DEX on the x-axis. This plot, known as the binodal curve, can be used to determine the threshold concentration for phase separation for different concentrations of PEG/DEX in a specific cell culture medium.

2. Configuration 1: Exclusion Patterning (96-well Plate Format)

1. Prepare separate solutions of 5.0% wt/wt PEG and 12.8% wt/wt DEX in cell culture medium. Use ATPS solutions of at least twice the critical point to ensure that an ATPS remains after the polymers equilibrate with respect to each other. There is a small amount of flux of DEX into the PEG-rich phase and vice versa, therefore, working too near the critical concentration can result in loss of the phase system as concentrations drop below critical point. Similarly, transferring the cell culture dish to a humidity- and temperature-controlled incubator can alter the two-phase properties and make the two solutions miscible. Note: Some cells may perform better with other ATPS formulations. Acceptable formulations may be selected based on the binodal curves determined from Part 1.
2. Harvest the cells to be used for exclusion patterning. Determine the total number/concentration of cells available. Optional: label the cells with CellTracker or other non-cytotoxic labels for fluorescence microscopy.
3. Pellet the cells and resuspend the pellet in an appropriate volume of 5.0% PEG to achieve the desired number of cells for exclusion. For example, one well of a 96-well plate requires 37,500 fibroblast cells to be resuspended in 200 μ l of PEG to produce confluence the following day. Scale these numbers as appropriate for other cell types and culture substrate sizes.
4. Using a micropipettor, dispense 0.5 μ l droplets of DEX onto a dry cell culture substrate. Larger volume droplets produce larger exclusion zones. Droplets ranging in size from 0.1 to 1 μ l are recommended for exclusion micropatterning. Optional: DEX droplets can be deposited 24 hr ahead of time and allowed to dehydrate at room temperature. This can produce cleaner patterns.
5. Dispense 200 μ l of PEG cell suspension into the well to cover the DEX droplets.
6. Place in a humidified incubator for 12 hr at 37 °C, 5% CO₂. Make sure that the dish is not tilted during handling and that it is placed on a level incubator shelf to avoid disrupting the patterns.
7. Remove the PEG solution and wash three times with 200 μ l of culture medium.
8. Add fresh culture medium and return to the incubator.
9. Monitor the cultures periodically to observe cell movement into the exclusion zone.

3. Configuration 2: Island Patterning (96-well Plate Format)

1. Prepare solutions of 5.0% wt/wt PEG and 12.8% wt/wt DEX in cell culture medium, as above.
2. Harvest the cells to be used for island patterning. Determine the total number/ concentration of cells available.
3. Pellet and resuspend the cells in an appropriate volume of 12.8% DEX to achieve the desired concentration of cells for island patterning. Concentrations of 5,000 cells/ μ l or less are recommended for strongly adherent cell types. For cells that have difficulty attaching or cells that loosely adhere, concentrations of up to 10,000 cells/ μ l may be considered.
4. Working quickly to avoid drying, pipette 0.5 μ l droplets of DEX onto a dry cell culture substrate, as described above. Do not allow droplets to dry. Optional: 200 μ l of PEG solution can be added to the well ahead of time. DEX droplets can then be deposited into the PEG solution where they will sink to the bottom and contact the culture surface. This can produce cleaner island patterns.
5. Cover the DEX droplets with 200 μ l of PEG.
6. Place in a humidified incubator for 12 hr at 37 °C, 5% CO₂. Make sure that the dish is not tilted during handling and that it is placed on a level incubator shelf to avoid disrupting the patterns.
7. Remove the PEG solution and wash three times with 200 μ l of culture medium.
8. Add fresh culture medium and return to the incubator.

9. Monitor the cultures periodically to observe cell movement and proliferation outwards from the islands.

4. Configuration 3: Exclusion Co-cultures (96-well Plate Format)

1. Prepare solutions of 5.0% wt/wt PEG and 12.8% wt/wt DEX in cell culture medium, as above.
2. Harvest the cells to be used for exclusion and island patterning. Determine the total number/concentration of cells available for each cell type. Optional: Some cell pairings may display dramatically different proliferation indices. To prevent the excluded cells from overpopulating the island patterned cells (especially for long term cultures), treat the cells used for exclusion with mitomycin C for 2 hr or irradiate them before harvesting. This will prevent proliferation. Fluorescent CellTracker dyes can be used to distinguish the two cell populations if necessary.
3. Pellet the cells and resuspend the pellet for exclusion patterning in an appropriate volume of 5.0% PEG to achieve the desired number of cells, as above. Resuspend the pellet for island patterning in an appropriate volume of 12.8% DEX to achieve the desired concentration of cells, as above.
4. Using a micropipettor, dispense 0.5 μ l droplets of DEX cell suspension onto a dry cell culture substrate. Do not allow droplets to dry.
5. Cover the DEX droplets with 200 μ l of PEG cell suspension.
6. Place in a humidified incubator for 12 hr at 37 $^{\circ}$ C, 5% CO₂. Make sure that the dish is not tilted during handling and that it is placed on a level incubator shelf to avoid disrupting the patterns.
7. Remove the PEG solution and wash three times in 200 μ l of culture medium.
8. Add fresh culture medium and return to the incubator.
9. Monitor the co-cultures to observe interaction between cell populations. Optional: Controls can be prepared by performing exclusion or island patterning individually, by co-culture cells that do not interact or by blocking pathways of interest in one or both cell populations before or after patterning.

Representative Results

To select an appropriate combination of PEG and DEX for cell patterning it is important to determine the binodal curve. This curve delineates the points at which an ATPS can form and can vary for a given set of polymers based on temperature, pH and ionic content. For culturing cells that require customized medium formulations it may be necessary to experimentally determine the binodal curve. This is accomplished by generating a series of ATPSs that are far from the binodal and varying in their PEG and DEX contents (**Figure 1**, purple circles). When an ATPS is present, the polymer solutions will appear cloudy when mixed and will equilibrate into separate phases if left undisturbed. By adding additional solvent to the polymer mixture, the ATPS will approach 0% PEG/0% DEX. At some point, the mixture will no longer phase separate. The PEG/DEX concentration at which this occurs represents a point on the binodal curve; above that point an ATPS can form and below that point it cannot. The curves in **Figure 1** represent binodals for three common cell culture media with and without 10% fetal bovine serum (FBS). The concentrations at which an ATPS is formed are slightly higher in the presence of FBS.

In our previous reports, we used ATPSs based on a critical point (the point on the binodal at which equal volumes of PEG and DEX form after equilibration) of 2.5% PEG 35 kDa/3.2% DEX 500 kDa. The results from our binodal data are in close proximity to this critical point value. We tested nine phase system combinations for patterning, as shown in **Table 1**. Since pure solutions of PEG and DEX equilibrate with respect to their polymer concentrations after they are combined, some of these solutions did not form stable ATPSs, and were therefore not useful for patterning (**Table 1a**, x marks). Other polymer combinations produced recognizable patterns, but were not uniform enough for experimentation (**Table 1a**, x/# marks). Useful polymer formulations formed exclusion zones or islands that were nearly devoid of cells in the non-patterned regions (**Table 1a**, # marks).

With 10% PEG, we noticed that cell morphology was abnormally round and spindle-like after 24 hr, with cells displaying a diminished ability to attach to the culture surface (**Table 1b**, x marks). Morphology and attachment were normal for 2.5% and 5% PEG (**Table 1b**, # marks). We observed that serum precipitated from the culture medium at high PEG concentrations (**Table 1c**, x marks), suggesting that abnormal cell morphology and attachment in 10% PEG may be related to problems with serum access. Additionally, PEG is known to disrupt plasma membranes²⁷. Although these effects are only observed at high concentrations of low molecular weight PEG, it is best to use the lowest PEG concentration that produces reliable patterning.

Consistent with our previous reports, we concluded that 5% PEG/6.4% DEX and 5% PEG/12.8% DEX were well suited for cell patterning, with 12.8% DEX producing more uniform patterns. Expected results for three patterning formats using Cell Tracker-labeled HeLa cells are shown in **Figure 2**. By following each patterning approach, it is possible to create uniform patterns of each type, with very few cells outside of the patterned areas.

Using exclusion and island patterning it is possible to assess the proliferation and migration of patterned cells (**Figure 3**). Over the course of three days, HeLa cells filled the exclusion zones (**Figures 3a-c**). Island-patterned cells expanded outward from the initial patterns (**Figures 3d-f**). These changes can be quantified using standard imageJ measurement tools (**Figures 3 c,f**). It is important to note that when multiple cell populations are co-cultured (**Figure 2**, Format 3), one cell population may out-proliferate and replace the other. Exclusion patterning and island patterning can be useful tools to assess if this will be a problem. In situations where dramatic differences in proliferation index occur, it is recommended that one cell population be treated by irradiation or chemical factors to limit its proliferation. This is particularly useful in situations where one cell type is used as a support cell for a slower growing more sensitive cell type.

We demonstrated this principle by culturing HepG2 cells, a hepatocellular carcinoma cell line that is commonly used to model hepatocyte biology, with NIH 3T3 fibroblast that were arrested using mitomycin C (**Figure 4**). Over time, the HepG2 cells maintain their localization and colony shape (**Figure 4a**). By placing many droplets in the same plate and surrounding them with fibroblasts it is possible to grow these cells in a format that is potentially useful for multiplexed studies (**Figure 4b**). Cell island monocultures can be used with this format as a control for the influence of paracrine factors (**Figure 4c**).

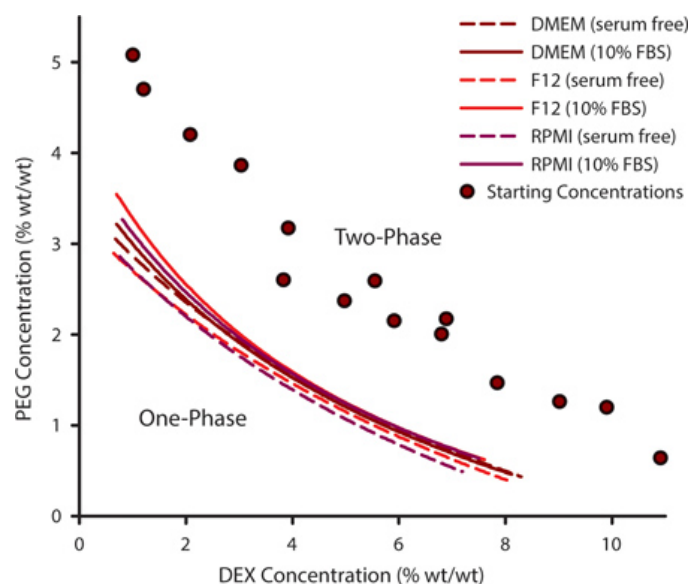


Figure 1. The polymer concentrations at which an ATPS can form can be extrapolated from experimentally determined binodal curves. This binodal curve was constructed using the cloud point method by adding additional solvent to measure the points at which two-phase mixtures of varying PEG/DEX concentrations (purple circles) no longer were capable of phase separating. Binodals were determined for DMEM, F12 and RPMI with and without serum. Data points were fitted with a three parameter rational function. N=3 for each data point.

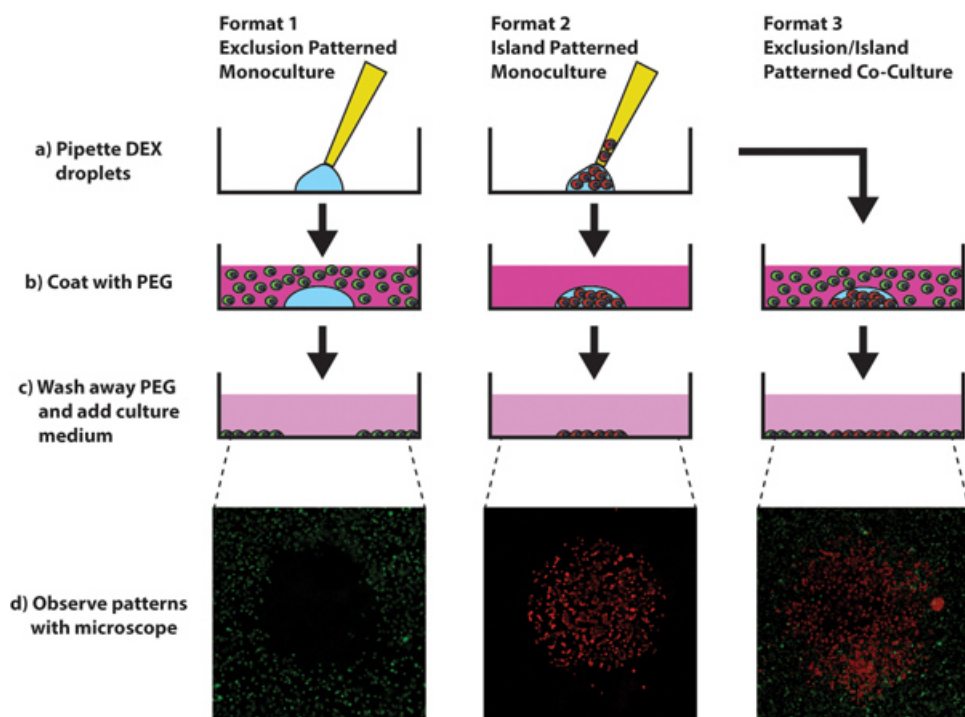


Figure 2. By dispensing ATPS solutions onto polystyrene plates, three formats for cell patterning can be produced. The procedure begins by pipetting DEX droplets **a)** that are then coated with PEG **b)**. Once the cells attach, the ATPS solutions can be washed away and replaced with culture medium (**c**, **d**). Fluorescence images for monocultures were stained with CellTracker dyes after patterning. For co-cultures, cells were stained separately with CellTracker dyes before patterning. HeLa cells were used to generate all three culture formats.

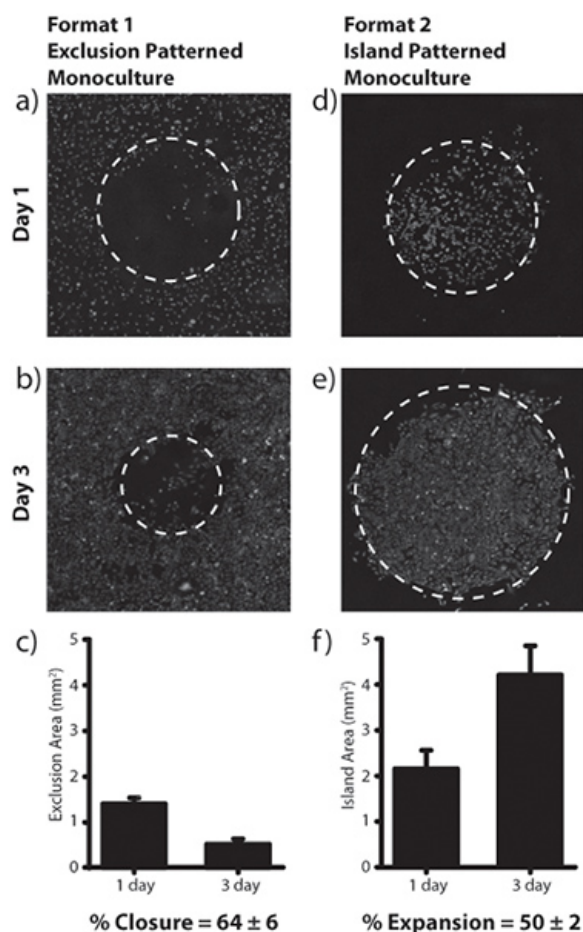


Figure 3. Exclusion patterning and island patterning can be used to assess cell migration and proliferation. **a)** Exclusion patterned HeLa cells 1 day after patterning. **b)** Exclusion patterned HeLa cells 3 days after patterning. **c)** Cells proliferate and migrate, significantly reducing the size of the exclusion zone. **d)** Island-patterned HeLa cells 1 day after patterning. **e)** Island-patterned HeLa cells 3 days after patterning. **f)** Cells proliferate and migrate outwards, significantly expanding the size of the island. Images were quantified using ImageJ software to measure the cell clearing and cell island areas before and after migration. Bars represent mean ± SEM of at least three independent observations.

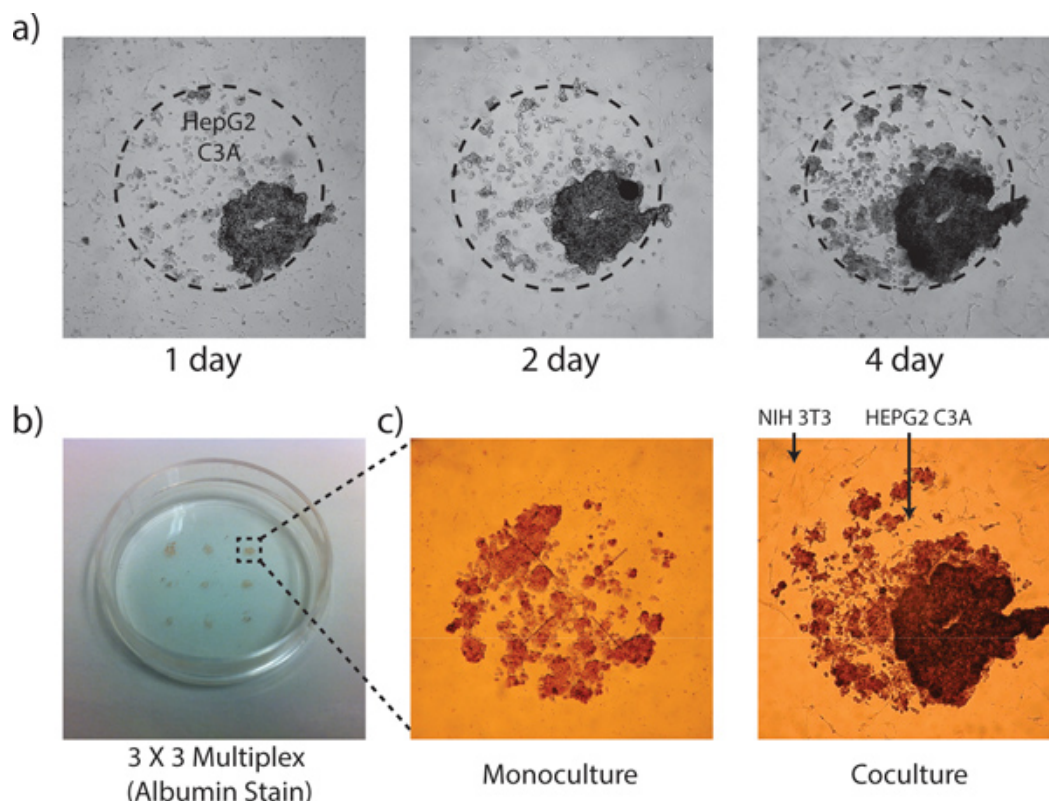


Figure 4. Liver cell/fibroblast cultures can be generated using ATPS exclusion co-culture patterning. a) These colonies maintain their organization for at least 4 days in culture. b) Multiple islands can be arrayed in a single dish with potential for multiplexed or high-throughput assays. c) Compared to non-co-cultured island patterns, the co-cultured cells display slightly higher levels of albumin production (brown staining) as evident from qualitative comparison of albumin stained co-cultures versus monocultures. Albumin is a protein produced by liver cells. Therefore, this result suggests that the function of liver cells is enhanced when co-cultured with fibroblast using ATPS.

a) Pattern Formed	DEX 3.2%	DEX 6.4%	DEX 12.8%
PEG 2.5%	x	x	x
PEG 5.0%	x/#	#	#
PEG 10.0%	x/#	#	#
b) Morphology	DEX 3.2%	DEX 6.4%	DEX 12.8%
PEG 2.5%	#	#	#
PEG 5.0%	#	#	#
PEG 10.0%	x	x	x
c) Serum Precipitation	DEX 3.2%	DEX 6.4%	DEX 12.8%
PEG 2.5%	#	#	#
PEG 5.0%	#	#	#
PEG 10.0%	x	x	x

Table 1. a) ATPS formulations that can be used for patterning are indicated by check marks, those that cannot are indicated by x marks. **b)** Formulations that retain normal cell morphology and attachment properties are indicated by check marks, those that cannot are indicated by x marks. **c)** Formulations that resulted in precipitation of serum proteins are indicated by x marks.

Discussion

The ATPS cell micropatterning method requires very little expertise beyond proficiency in cell culture techniques and can be quickly mastered. The advantages of this approach are that it is inexpensive, rapid and compatible with a variety of cell types and culture formats. For these reasons, our protocol should be easily adopted by life scientists, particularly those who study cell proliferation, migration and chemotaxis, and the influence of juxtacrine and paracrine interactions among cell populations. The assays presented here can be easily quantified at the cell population level using standard image analysis procedures available in software such as ImageJ.

To generate consistent patterns we advise the following precautions. First, the pipette tip used to dispense the DEX solution should be changed after each DEX droplet is deposited to provide more consistent droplet volumes. Since the DEX solution is relatively viscous, it is also important to avoid depositing excess DEX that might be present on the outer surface of the pipette tip and to ensure that the entire volume of DEX exits the tip. Second, the droplets can move if PEG is added too vigorously or if the dish is tilted. DEX disruption can be minimized by keeping the dish on a level surface and allowing the PEG solution to gradually cover the droplets from above without allowing large forces from the PEG meniscus to dislodge part of the droplet. Droplet disruption occurs more frequently with large DEX droplets, so droplets of 0.5 μ l or less should be used if possible. Apart from these technical issues involved with dispensing the solutions, there are very few pitfalls associated with this technique, provided that appropriate polymer molecular weights and concentrations are used.

Although ATPS patterning can be easily performed using a micropipette (as presented here), there are a variety of more sophisticated approaches that can be used to rapidly generate patterns of more complex geometric arrays (e.g. liquid handling robots and acoustic droplet ejection), as presented in our previous studies^{14, 15, 20}. It is also possible to generate DEX droplets that are much smaller in volume by pneumatically ejecting DEX through capillary orifices or by actuating an orifice in a microchannel to produce flowing DEX droplets sheathed by PEG¹⁹. These approaches may be of interest for those seeking to produce high-throughput or multiplexed co-culture or migration assays. In addition, using microfluidic approaches, it is possible to perform experiments with a small numbers of cells, or with cells in a microchannel where the effects of fluid flow and shear can be examined. However, these advanced methods are not required for most applications.

Aqueous two-phase patterning of cells is simple and easily adapted to a typical cell culture setting. This method allows any researcher with access to a typical cell culture lab (access to a hood, CO₂ incubator, and micropipettes) and the aforementioned polymers to reproducibly pattern cells in monoculture and co-culture. Our lab has demonstrated this capability by printing arrays of cells to study cell migration in a wound healing assay and to examine the effects of juxtacrine and paracrine signaling in the differentiation of embryonic cells^{16, 17, 20}. Other methods, including patterning of extracellular matrix²⁸, inkjet printing²⁹, and patterning by laminar flow in microfluidic devices^{25, 26} have also been used to localize cells. These other methods are effective approaches to achieving well-defined patterns of cells and can often achieve single-cell precision. However, these methods also require highly-specialized equipment and/or access to cleanroom facilities to fabricate the stamps used to print extracellular matrix proteins or produce microfluidics devices. Their connection to power supplies, syringe pumps, and other external components also hinders their implementation due to the costs associated with the equipment and user skill required to operate it.

In terms of future applications we expect that our method will be useful for developing culture systems that enable high-throughput analysis of cell movement and proliferation, as well as investigating cell-cell interactions among multiple cell populations. To this point, our reports have focused on examining interaction of only a few types of patterned cells at once. However, it is conceivable that many subpopulations of cells could be cultured with a common feeder layer to investigate the impact of paracrine and juxtacrine signaling of many cell types grown together in a single cell culture setup. Finally, tissue engineering applications frequently required spatial localization of one or more cell types. It may be possible to adapt our technique for use in patterning cells in order to produce more physiologically relevant tissue engineered disease models or to pattern cells on implantable materials for clinical applications.

Disclosures

The authors have no competing financial interests.

Acknowledgements

This work was supported by the Coulter Foundation, Beyster Foundation, the Undergraduate Research Opportunity (UROP) summer program for ATA and a National Science Foundation Graduate Student Research Fellowship (Grant no. DGE 0718128; ID: 2010101926) for JBW.

References

- Hatti-Kaul, R. Aqueous two-phase systems : methods and protocols. In: *Methods in biotechnology.*, Humana Press, **xiii**, 440 (2000).
- Albertsson, P.A.k. In: *Partition of cell particles and macromolecules: separation and purification of biomolecules, cell organelles, membranes, and cells in aqueous polymer two-phase systems and their use in biochemical analysis and biotechnology.*, 3rd ed., Wiley, 346 (1986).
- Yamada, M., et al. Continuous cell partitioning using an aqueous two-phase flow system in microfluidic devices. *Biotechnol. Bioeng.* **88** (4), 489-94 (2004).
- Soohoo, J.R. & Walker, G.M. Microfluidic aqueous two phase system for leukocyte concentration from whole blood. *Biomed. Microdevices.* **11** (2), 323-9 (2009).
- Hahn, T. & Hardt, S. Concentration and size separation of DNA samples at liquid-liquid interfaces. *Anal. Chem.* **83** (14), 5476-9 (2011).
- Hatti-Kaul, R. Aqueous two-phase systems. A general overview. *Mol. Biotechnol.* **19** (3), 269-77 (2001).
- Hustedt, H., Kroner, K.H., Menge, U., & Kula, M-R. Protein recovery using two-phase systems. *Trends in Biotechnology.* **3** (6), 139-144 (1985).
- Keating, C.D. Aqueous Phase Separation as a Possible Route to Compartmentalization of Biological Molecules. *Acc Chem. Res.* **45** (12), 2114-24 doi:10.1021/ar200294y (2012).
- Helfrich, M.R., et al. Partitioning and assembly of metal particles and their bioconjugates in aqueous two-phase systems. *Langmuir.* **21** (18), 8478-86 (2005).
- Diamond, A.D., & Hsu, J.T. Prote. Partitioning in PEG/Dextran Aqueous Two-Phase Systems. *AIChE Journal.* **36** (7), 1017-1024 (1990).
- Wu, Y.-T. & Zhu, Z.-Q. Modeling of interfacial tension of aqueous two-phase systems. *Chemical Engineering Science.* **54** (4), 433-440 (1999).
- Liu, Y., Lipowsky, R., & Dimova, R. Concentration dependence of the interfacial tension for aqueous two-phase polymer solutions of dextran and polyethylene glycol. *Langmuir.* **28** (8), 3831-9 (2012).
- Rha, C.-W.K.a.C. Interfacial Tension of Polyethylene Glycol/Potassium Phosphate Aqueous Two-Phase Systems. *Physics and Chemistry of Liquids: An International Journal.* **38** (1), 25-34 (2000).

14. Fang, Y., *et al.* Rapid Generation of Multiplexed Cell Cocultures Using Acoustic Droplet Ejection Followed by Aqueous Two-Phase Exclusion Patterning. *Tissue Eng. Part C. Methods*. **18** (9), 647-657 doi:10.1089/ten.tec.2011.0709 (2012).
15. Tavana, H., *et al.* Nanolitre liquid patterning in aqueous environments for spatially defined reagent delivery to mammalian cells. *Nat. Mater.* **8** (9), 736-41 (2009).
16. Tavana, H., Mosadegh, B., & Takayama, S. Polymeric aqueous biphasic systems for non-contact cell printing on cells: engineering heterocellular embryonic stem cell niches. *Adv. Mater.* **22** (24), 2628-31 (2010).
17. Tavana, H., *et al.* Microprinted feeder cells guide embryonic stem cell fate. *Biotechnol. Bioeng.*, doi:10.1002/bit.23190 (2011).
18. Tavana, H. & Takayama, S. Aqueous biphasic microprinting approach to tissue engineering. *Biomicrofluidics*. **5** (1), 13404 (2011).
19. Frampton, J.P., *et al.* Precisely targeted delivery of cells and biomolecules within microchannels using aqueous two-phase systems. *Biomed. Microdevices*. **13** (6), 1043-51 (2011).
20. Hossein Tavana, K.K., Bersano-Begey, T., Luker, K.E., Luker, G.D., & Takayama, S. Rehydration of Polymeric, Aqueous, Biphasic System Facilitates High Throughput Cell Exclusion Patterning for Cell Migration Studies. *Advanced Functional Materials*. **21** (15), 2920-2926 (2011).
21. Falconnet, D., *et al.* Surface engineering approaches to micropattern surfaces for cell-based assays. *Biomaterials*. **27** (16), 3044-63 (2006).
22. Lim, J.Y. & Donahue, H.J. Cell sensing and response to micro- and nanostructured surfaces produced by chemical and topographic patterning. *Tissue Eng.* **13** (8), 1879-91 (2007).
23. Ringeisen, B.R., *et al.* Jet-based methods to print living cells. *Biotechnol. J.* **1** (9), 930-48 (2006).
24. Wright, D., *et al.* Generation of static and dynamic patterned co-cultures using microfabricated parylene-C stencils. *Lab Chip*. **7** (10), 1272-9 (2007).
25. Takayama, S., *et al.* Patterning cells and their environments using multiple laminar fluid flows in capillary networks. *Proc. Natl. Acad. Sci. U.S.A.* **96** (10), 5545-8 (1999).
26. Berthier, E., *et al.* Pipette-friendly laminar flow patterning for cell-based assays. *Lab Chip*. **11** (12), 2060-5 (2011).
27. Davidson, R.L., O'Malley, K.A., & Wheeler, T.B. Polyethylene glycol-induced mammalian cell hybridization: effect of polyethylene glycol molecular weight and concentration. *Somatic Cell Genet.* **2** (3), 271-80 (1976).
28. Johnson, D.M., LaFranzo, N.A., & Maurer, J.A. Creating Two-Dimensional Patterned Substrates for Protein and Cell Confinement. *J. Vis. Exp.* (55), e3164, doi:10.3791/3164 (2011).
29. Moon, S., Lin, P., Keles, H.O., Yoo, S., & Demirci, U. Title Cell Encapsulation by Droplets. *J. Vis. Exp.* (8), e316, doi:10.3791/316 (2007).