Spherical microwell arrays for studying single cells and microtissues in 3D confinement

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Keywords: spheroids, microwell, cell-cycle arrest, substrate curvature, 3D cell culture, cell-material interactions

Abstract

Microwell arrays have emerged as three-dimensional substrates for cell culture due to their simplicity of fabrication and promise for high-throughput applications such as 3D cell-based assays for drug screening. To date, most microwells have had cylindrical geometries. Motivated by our previous findings that cells display 3D physiological characteristics when grown in the spherical micropores of monodisperse foam scaffolds (Lee et al 2013 Integr. Biol. 5 1447–55 and Lin et al 2011 Soft Matter 7 10010–6), here we engineered novel microwells shaped as spherical caps with obtuse polar angles, yielding narrow apertures. When used as bare substrates, these microwells were suitable for culturing cell spheroids; the narrow apertures sterically hindered unattached cultured cells from rolling out of the aperture, and cell polarity was oriented based on the distribution of extracellular matrix proteins in the microwells. Surprisingly, single fibroblast cells in spherical wells of various diameters (40–100 μm) underwent cell-cycle arrest, while cells in circular cylindrical microwells continued to proliferate. Spatial confinement was not sufficient to cause cell-cycle arrest; however, confinement in a constant negative-curvature microenvironment led to cell-cycle arrest. Overall, these investigations demonstrate that this spherical microwell substrate constitutes a novel basic research tool for elucidating how cells respond to dimensionality and microenvironment with radii of curvature at the cellular length scale.

1. Introduction

The reductionist approach of culturing mammalian cells on simple two-dimensional (2D) substrates, far removed from the complex interior of their animal hosts, has yielded considerable knowledge about the development, function, and molecular composition of living cells. However, 2D cell cultures often fail to capture the physiological functions or responses of tissues in vivo [1]; three-dimensional (3D) cell cultures may close this gap. Current in vitro 3D cell-culture methods (such as gel-embedded culture [2], prefabricated scaffolds [3], and scaffold-free methods such as hanging-drop culture [4]) exploit diversities in architectures and materials in order to change parameters such as porosity, pore size, permeability, and mechanical characteristics that capture in vivo tissue characteristics [5]. The behaviors of cells in 3D culture often differ from their counterparts in 2D cultures. Frequently reported differences include cell morphogenesis and migration [6–9], stem-cell differentiation [10, 11], drug toxicity/efficacy in pharmaceutical screening [12–14], organoid formation [15–17], gene expression [9, 18–20], and cell proliferation [11, 21]. Although 3D culture methods offer advantages over 2D systems, the complexity of 3D matrices complicates systematic fundamental studies on the microenvironmental variables which result in differences in cellular behaviors responding to the dimensionality.
Previously, our lab reported a monodisperse foam that served as a novel 3D matrix composed of interconnected uniform spherical pores [22, 23]. The negatively curved substrates of this system retained the simplicity of 2D cell culture but extended cell adhesions into 3D. Importantly, in the spherical pores of monodisperse foam scaffolds, fibroblasts lose the ventral-dorsal asymmetric distribution of stress fibers and focal adhesions that is typical of planar cultures; rather, they exhibit 3D distributions of thin stress fibers and focal adhesions that are reduced in both size and frequency [22]. However, the open foam character of this matrix precluded the isolation of individual pores, hindering long-term tracking and high-throughput screening.

Here we report a new 3D culture substrate that retains the feature of negative-curvature, but each pore is isolated as a microwell. The spherical microwell substrates (figure 1(a)) were made through a serial molding-based microfabrication pipeline; here polyacrylamide was used as the substrate hydrogel. We created bioactivated variants of the polyacrylamide microwells by conjugating extracellular matrix (ECM) proteins to various parts of the microwells; ECM protein distribution affected epithelial tissue polarity. When only the interior walls of a microwell were coated with ECM proteins, cultured cells were confined to the microwells. To our surprise, single fibroblasts did not undergo mitosis in isolated spherical microwells. This new finding showcases the utility of our novel spherical microwells for 3D cell-culture studies.

2. Materials and methods

2.1. Monolithic mold fabrication

To create a reusable template, a monolithic mold consisting of arrays of spherical microstructures attached to a flat surface was made through two replicating steps (figures 1(b)(i)–(v)). The first step was to create arrays of cylindrical microwells made of polydimethylsiloxane (PDMS) using standard soft lithography [24]. The diameters (d) of the cylindrical microwell arrays determined the aperture (a) of the final spherical microwell arrays. The PDMS microwell surface was coated with 1% bovine serum albumin (Bionovas, Canada) in phosphate-buffered saline (PBS; Gibco, USA) for 30 min at room temperature. This coating acted as a lift-off layer, facilitating separation in the next replication step. Next, we used a 20 μl micropipette tip to manually place one glass microsphere (ThermoFisher Scientific, USA) into each cylindrical microwell under a microscope (figures 1(b) (i)). Microspheres of diameters φ = 40, 60, 80, 100, and 200 μm were placed into d = 26, 40, 53, 66, or 132 μm microwells, respectively. We were typically able to place 100 glass microspheres within 2 h.

After the glass microspheres were placed, we poured fast-curing silicone (Elite Double 32, Zhermack SpA, Italy; green in figures 1(b)(ii)) onto the microsphere-PDMS master. Upon separation after curing, Elite Double 32 contains the negative of the microsphere-PDMS master—an array of spherical microwells. The residual microspheres lodged in the Elite Double 32 were easily removed with adhesive tape. Finally, we applied a two-part 5 min epoxy resin (PowrBon, Taiwan or Devcon, USA; red in figures 1(b)(iii)) onto the Elite Double 32 negative mold and covered the epoxy with a slide. The monolithic mold was made once the epoxy hardened and was separated from the Elite Double 32 mold (figure 1(c)).

2.2. Polyacrylamide spherical microwell arrays

We employed commonly used polyacrylamide as the substrate material for microwell fabrication, and followed published methods [25]. Briefly, clean coverglass was silanized with 2% 3-(trimethoxysilyl) propyl methacrylate (Sigma-Aldrich, USA) along with 2% acetic acid (Merck, Germany) and 96% ethanol (Sigma-Aldrich). We sandwiched 100 μl polyacrylamide solution containing 8% acrylamide (Bio-rad, USA) monomer, 0.4% bisacrylamide (Bio-rad, USA) crosslinker, 0.15% N,N,N′, N′-tetramethylethylenediamine (Sigma-Aldrich) catalyst, and 0.5% ammonium persulfate (Sigma-Aldrich) between the monolithic mold and silanized coverglass. (figures 1(b)(iv)). When gelation was complete (~15 min at room temperature), the coverglass-bound microwell substrate was carefully separated from the epoxy template (figures 1(b)(v) and (d)). Afterward, the substrate was sterilized via ultraviolet irradiation for 30 min in the laminar hood, followed by storage in 500 units ml⁻¹ penicillin and 500 μg ml⁻¹ streptomycin (Gibco) at 4 °C. We refer to polyacrylamide microwells with no conjugation as ‘bare substrate’.

2.3. Conjugating polyacrylamide with ECM proteins and selective removal of exterior ECM proteins

To culture cells with strong cell-matrix adhesions, we followed a published method [26] to conjugate ECM proteins such as collagen (BD Bioscience, USA) or fibronectin (Sigma-Aldrich) to the polyacrylamide substrate using the bi-functional crosslinker sulfo-SANPAH (ThermoFisher Scientific) [27]. We refer to microwells that are conjugated with ECM proteins everywhere as ‘all-conjugated substrate’.

To confine cells in microwells, it is necessary to remove ECM proteins exterior to the microwells. We first prepared amine-silanized glass with (3-amino-propyl) triethoxysilane (Sigma-Aldrich) and treated it with 2.5% glutaraldehyde (Sigma-Aldrich). Finally, we juxtaposed glutaraldehyde-functionalized coverglass with ECM-conjugated polyacrylamide microwells overnight at 37 °C. Upon separation, exterior ECM proteins on the top surfaces of microwell substrates are transferred to the glutaraldehyde-functionalized
coverglass (figures 1(e)–(g)). ECM stripping can be repeated with fresh glutaraldehyde-functionalized coverglass to reduce residual protein. The efficacy of ECM stripping depends on the evenness of the polyacrylamide surface. Substrates with only the microwell walls conjugated with ECM proteins are referred to as ‘isolated microwells’. Last, to create a completely closed microwell, we added lids of thin polyacrylamide gel conjugated with ECM proteins onto microwells; we refer to this geometry as ‘closed microwells’. All substrates were sterilized with ultraviolet light and antibiotics and stored at 4 °C.

2.4. Cell culture

Rat embryonic fibroblast (REF52) YFP-paxillin cells (gift from Dr C-H Yu at the School of Biomedical Science, University of Hong Kong) were cultured in growth medium consisting of Dulbecco’s Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (HyClone, USA) and 100 units ml⁻¹ penicillin, and 500 μg ml⁻¹ streptomycin (Gibco). Before seeding in microwells, REF52 cells were synchronized through contact inhibition [28] by maintaining cells for two additional days after confluency. Madin-Darby canine kidney (MDCK) cells stably
expressing mCherry-H2B (gift from Dr C.-L Guo at IOP, Academia Sinica) and transfected with EGF-PZO1 (selected using G418, from Gibco) were cultured in the growth medium consisting of Minimum Essential Medium (Gibco) supplemented with 10% fetal bovine serum, 100 units ml⁻¹ penicillin, 500 µg ml⁻¹ streptomycin, and 1 mM sodium pyruvate (Gibco). Both cell lines were maintained at 37 °C and 5% CO₂, with growth medium exchanged every other day.

To seed cells in microwells, we added 50 µl of a cell suspension (2 × 10⁴ cells ml⁻¹) onto the surface of the substrate. Then, in a dedicated cell-culture room, cells were manually ushered into the microwells by tilting the culture dish. Loaded substrates were rinsed with culture medium to remove excess cells from the substrate surface. All substrates loaded with cells were immersed in the appropriate growth medium and maintained at 37 °C and 5% CO₂.

2.5. Staining, imaging, processing, and analysis
Microwells were stained with tetramethylrhodamine-5-isothiocyanate (ThermoFisher Scientific). ECM proteins were stained with Dylight 405 NHS-ester (ThermoFisher Scientific) in accordance with the manufacturer’s instructions. Cells were rinsed in PBS, fixed in 4% paraformaldehyde (Santa Cruz Biotechnology, USA) for 15 min, and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 3 min. Cells were rinsed in PBS and stained with phalloidin-iFluor 647 (1:1000, AAT Bioquest, USA) for F-actin and with 300 nM 4,6-diamidino-2-phenylindole (ThermoFisher) for the nucleus. Stained cells were immersed in PBS and mounted in a custom-made rose chamber.

Image stacks were taken with a 40×/NA1.1 water immersion objective (Leica, Germany) on an inverted microscope (DMI 6000, Leica) with a confocal scanhead (CSU22, Yokogawa Electric, Japan), a laser merge module containing 405, 491, 561, and 642 laser lines (ILEE-400, Andor, UK), and an DV885 EMCCD (Andor). The microscope apparatus was controlled with MetaMorph 7.7.2 (Molecular Devices, USA). z steps were 0.5 or 1 µm. All microwell images were processed and analyzed using Fiji 1.52p [29]. Briefly, the image stacks were processed with medium filter, background subtraction, and segmented by threshold. Well elongation was measured by fitting an ellipse onto a binary z x image with the ellipse splitting plugin [30] (figure S1 is available online at stacks.iop.org/BF/12/025016/mmedia). For each well size, more than 50 microwell image stacks were analyzed. Cell images were processed and analyzed using Imaris 9.5 (Oxford Instruments, UK).

2.6. Live-cell tracking
Single synchronized REF52 cells in G1 phase were cultured in microwells (40, 60, 80, 100, and 200 µm diameter) and on planar polyacrylamide substrates for a total of 3 d; medium was refreshed once after 48 h. The whole field, including all microwells, was captured sequentially in bright-field using a 10×/NA 0.25 air objective (Leica) and a digital camera (RETIGA-EXi, QImaging, Canada) on a cell culture microscope (DM IL, Leica). Four timepoints from the time of seeding (t = 0) were taken for the time-lapse: initial (<1 h), 24 h, 48 h, and 72 h. For each condition at each timepoint, cell division was scored based on whether a second cell, which was not previously observed, had appeared. Microwells seeded with multiple cells at t = 0 were excluded to avoid cell–cell effects. Similarly, subsequent divisions of daughter cells were not considered. To track cell divisions, the spatial sequence at every timepoint was stitched together using the Grid/Collection stitching plugin [31] in Fiji 1.52p [29], thus reconstructing the entire field of microwells (figure S1). In this way, false negatives were identified and discounted: situations in which a cell division had occurred but one of the daughter cells migrated out of the microwell and out of the immediate field of view (figure S2).

3. Results

3.1. Replicated molds and microwells
The polyacrylamide microwells can be readily replicated from the glass–bead mold, but the beads would have to be placed on the cylindrical microwell arrays every time. In addition, we introduced two more replication steps to create microwells. Although this workflow required more cost and time for fabrication, the extra molds have advantages. First, the monolithic structure makes the molds robust and reusable through many replication processes. Second, using highly stretchable materials such as Elite Double 32 for the intermediate mold yields prolate ellipsoidal structures (figure S3). The final epoxy mold retains the spherical structure very well in microscale, with no apparent deformation evident upon scanning electron microscopy (figure 1(c)).

We characterized the final microwell geometry (table 1) by the well diameter (D₀), aperture diameter (a) with respect to Φ₀, and the diameter of the cylindrical microwell which holds glass beads (d) (Methods). In addition, there is elongation ε along the z axis (figure S2). Microwell diameters deviated by <3% from the diameters of the original glass beads, and the

| Table 1. Summary of microwell and template geometry. All units except ε are µm. |
|---|
| φ | D₀ | d | a | ε |
| 40 | 41.0 ± 2.3 | 26 | 33.6 ± 4.0 | 1.13 ± 0.05 |
| 60 | 59.4 ± 3.8 | 40 | 46.1 ± 6.2 | 1.15 ± 0.04 |
| 80 | 80.3 ± 3.3 | 53 | 62.9 ± 6.6 | 1.14 ± 0.03 |
| 100 | 97.7 ± 3.6 | 66 | 78.0 ± 4.7 | 1.15 ± 0.08 |
| 200 | 204.7 ± 7.7 | 132 | 145.2 ± 8.1 | 1.16 ± 0.03 |
values of $a$ were always larger than $d$. While wells slightly elongated along the $z$ axis due to swelling of the polyacrylamide, the elongation values agreed with swelling ratios reported previously [22, 32].

3.2. Epithelial cell culture

Establishment of proper apical-basal polarity in epithelial cells is important for cell functions. Tight junctions, which are located at the most apical regions of the lateral surface of the cell, are commonly used as polarity markers [33]. The ECM orients in accordance with the polarity of epithelial cells. When grown in reconstituted ECM gel such as collagen gel or Matrigel, some epithelial cells differentiate into a functional, multicellular structure of cells, or organoids [34]. The most widely-studied epithelial cell model is MDCK cells, which form cysts consisting of a spherical monolayer cell sheet enclosing a lumen [35]. The sizes of cysts grown from single cells are $50 \sim 60 \mu m$ after one week of culture [36]. The sizes fall into the range of the diameters of our spherical microwells. In order to determine whether our microwell substrates promoted the growth of epithelial tissues by providing external templates, we cultured epithelial cells on substrates conjugated with ECM proteins under four scenarios: bare substrate (no conjugation), all-conjugated substrate (ECM proteins everywhere), isolated microwells (only walls conjugated), and closed microwells (thin conjugated lids) (Methods and figure 2).

On bare microwells, MDCK cells did not adhere to the substrate and exhibited an inverted polarity, with tight junctions facing outward (figure 2), as previously reported [37] when MDCK cells were cultured in suspension. We performed day-to-day observation of inverted cysts grown in our microwells and quantified cell number by segmenting nuclei (figure S4). Even though there was no adhesion to the substrate, cysts were not lost when the medium was changed twice (figure S5), nor were they lost after sample fixation and washing (data not shown). We hypothesized that the narrow aperture of the microwell restricted the movement of inverted cysts under perturbation. To further test the effectiveness of the microwells in restricting spheroids, we subjected the 5 d spheroid samples in the microwells to strong agitation on an orbital shaker and we lost only one spheroid out of 32 spheroids (figure S5).

On all-conjugated substrate, MDCK cells covered the entire microwell and flat surfaces over time, with the tight junctions facing the culture medium (figure 2(b)). The cell height of cells in the wells is not uniform. In isolated microwells, cells grew and divided on the curved wall, with the population of daughter cells eventually growing out of the microwell (figure S5). Cells inside wells are columnar with the cell height more than $20 \mu m$. The cell aggregates outside the well (not in contact with ECM proteins) exhibited random or inverted polarity, while the tight junctions inside the microwell faced the center of the microwell and showed cusp structures at tri-cellular regions (figure 2(c)). Cells grown outside the well did not close into a cystic monolayer and did not maintain their polarity as a cell sheet on the microwell wall (figure 2(c)). We often observe nuclear debris in the lumen area and it is probably due to anoikis [38]. To obtain a spherical monolayer of MDCK cysts, we closed the microwell with a lid after seeding it with MDCK cells (Methods). MDCK cells had their tight junctions facing the lumen (figure 2(d)), the same polarity as cysts grown in 3D gel in the literature [39]. The cell height here is more uniform about $10 \mu m$.

3.3. Fibroblasts cultured in isolated microwells

In addition to epithelial cells, we cultured fibroblasts in spherical microwells. When cultured on 'all-conjugated substrates', fibroblasts tend to migrate to the flat substrate (data not shown). When cultured in isolated microwells, fibroblasts exhibited cell-cycle arrest (figure 3). We seeded G1-synchronized REF52 cells into microwells 40, 60, 80, 100, or $200 \mu m$ in diameter, as well as on the control planar substrate and cylindrical microwells of $90 \mu m$ in diameter and tracked cell division every day for 3 d (figure 3, figure S2). On day 3, it is clear to see the smaller the radii of substrate curvatures, the less division the cells reside in (figure 3(b)). The division rate of cells in cylindrical wells are between the division rate of cells grown on flat and in spherical microwells but closer to the cells on the flat substrate. The flat bottom of the cylindrical well may have affected cellular behaviors more than the curved wall. Some cells start to divide 1 d after seeding in 80, 100, and $200 \mu m$ microwells but most cells do not divide in the first day even on the flat control and cylindrical well. We believe the divisions on Day 1 are due to incomplete synchronization before seeding. To remove the effect of non-synchronized cells, we normalize the undivided cells against the cells grown on a 2D substrate curvatures, the less division the cells reside in (figure S7). We can clearly see that cells in microwells of less than and equal to $100 \mu m$ behave similarly in cell division.

In addition, we characterized the morphology of cultured cells by staining F-actin and focal adhesions (figure S8). Compared to cells cultured on the 2D substrate, F-actin structures in cells cultured in our microwells were typically thinner, more diffuse and show no strong vental-basal asymmetry (figure S8). Similarly, focal adhesions of fibroblasts in microwells were generally smaller and less prevalent than those in cells grown on a 2D substrate (figure S8). We examined $>50$ cells in microwells and did not identify any super mature focal adhesions ($>5 \mu m$) [40], in agreement with our previous observations of fibroblasts in monodisperse foam scaffolds [22].
4. Discussion

Culturing cells in microwells is useful for single-cell culture, embryoid-body formation, and spheroid culture, with promise for high-throughput analysis [41–43]. The geometries of most present-day microwells include a uniform cross-sectional area in the z axis. Only a handful of investigations have created negative-curvature substrates, with hemispherical geometries at best [44, 45]. The narrow aperture of the near-spherical geometry of the microwells reported here functions as a choke, greatly reducing the chance of losing spheroids during medium exchange; this substrate may be compatible with bioreactors. Although this constriction also makes it difficult to remove spheroids, removal can be achieved by using hydrogels that can be enzymatically digested or deformed by pH or temperature [46].

Importantly, the spherical microwell arrays reported here are well suited to long-term cell tracking and imaging; here we employed nuclear segmentation for these tasks. Although some of our 3D image stacks suffered spherical aberrations, lensing effects, and scattering from the lower part of the cells in the well (figure 2), complicating quantitative analysis, advances in adaptive optics microscopy [47] or light-sheet microscopy [48] may overcome this hurdle and make quantitative image analysis possible.

While the fabrication method reported here is low throughput, the second and third molds can be reused.

Figure 2. Organization of epithelial cell aggregates under four conjugation geometries. (a) A bare microwell 60 μm in diameter, after 4 d of culture. (b) An all-conjugated microwell 60 μm in diameter, after 6 d of culture. (c) An isolated microwell 60 μm in diameter, after 7 d of culture. The magenta in the lumen region are nuclear debris from dead cells. (d) A closed microwell 40 μm in diameter, after 7 d of culture. Left to right: schematics; confocal xy section; confocal xz section; rendering view of nuclei (magenta) and tight junctions (green). In the confocal images, collagen appears in cyan. Scale bars, 10 μm.
and proliferation rates increase as most fabricated wells are cylindrically shaped. 

Our consistently negatively curved substrates affect cell proliferation (figure 3), and further investigation is required to reveal the mechanism underlying this phenomenon. Concomitantly, we observed that both focal adhesions and stress fibers were smaller and appeared less frequently (figure S8). Cell proliferation may thus be inhibited through an integrin-regulated pathway [51]. When fibroblasts were previously cultured in 3D collagen gel, the cellular proliferation rate and size/frequency of stress fibers and focal adhesions declined as the collagen concentration decreased [52]. However, when we previously cultured fibroblasts in monodisperse foam scaffolds, we detected reductions in focal adhesions and stress fibers, but we did not observe cell cycle inhibition [22]. The difference between our earlier foam scaffolds and the current spherical microwells is the connectivity between pores, which is much higher in the foam scaffolds. Confinement achievable with our spherical microwells may also play an important role in inhibiting proliferation, as reported in another 3D cell culture in hydrogel [53]. Our results show that in 3D, cell proliferation can be regulated through physical constraints, a finding that may lead to applications in regenerative medicine, and cancer treatment.

Interestingly, here fibroblasts and epithelial cells responded to our negatively curved substrates very differently. Epithelial cells continued to proliferate and the cell bodies conformed to curved substrates very well (figure 2). In contrast, fibroblasts stopped proliferating and the cell bodies did not conform to the curved substrates (figure S8). It will be of fundamental interest to identify the internal differences between these two cell types that underlie these distinct behaviors, perhaps including cytoskeletal organization. Recent research has shown that cells respond to curvature at the cellular length scale, an effect known as curvotaxis [54]. More and more evidence shows that substrate curvature affects morphological and functional responses [55]. We found that epithelial cells in isolated open or close wells are taller than cells on the
flat substrate. This observation is in agreement with the previous observation in tubular confinement [56]. While fabricated substrates for curvature investigation are often cylindrically shaped, cells experience two different principle curvatures [56]. Our microwell substrate is ideal for investigating substrate curvature effect because within the microwell, the cells experience almost constant curvature in all directions.

5. Conclusion

Here we have demonstrated that a new geometry of spherical microwell arrays yields versatility in culturing spheroids, epithelial tissues, and single cells. Epithelial cells and fibroblasts displayed distinct proliferation patterns and morphology in confined spherical wells. While we do not know the mechanisms underlying these differences, the spherical microwells developed here constitute a useful tool for further investigation. These microwells can also potentially be used to culture functional spheroids and for image-based quantitative analysis. Improvements in the fabrication process will increase the throughput of analysis.

Acknowledgments

This work was supported by MOST 105-2112-M-001-015, 107-2622-M-001-001-C1 and the Career Development Award of Academia Sinica. C-K Huang thanks the Academia Sinica postdoctoral fellowship program for supporting this research. We thank Siram Muthu Irluapan of Academia Sinica for participating in part of this research and Meng-wei Hsueh for 3D schematic drawing. We also acknowledge technical support from the Flow Cytometry Core Facility at the Institute of Biomedical Sciences, Academia Sinica. K-l Lin is thankful for insightful discussions with Professor Yuli Wang from Carnegie Mellon University.

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