Scaffold-free three-dimensional cell culture utilizing micromolded nonadhesive hydrogels

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Techniques that allow cells to self-assemble into three-dimensional (3-D) spheroid microtissues provide powerful in vitro models that are becoming increasingly popular—especially in fields such as stem cell research, tissue engineering, and cancer biology. Unfortunately, caveats involving scale, expense, geometry, and practicality have hindered the widespread adoption of these techniques. We present an easy-to-use, inexpensive, and scalable technology for production of complex-shaped, 3-D microtissues. Various primary cells and immortal cell lines were utilized to demonstrate that this technique is applicable to many cell types and highlight differences in their self-assembly phenomena. When seeded onto micromolded, nonadhesive agarose gels, cells settle into recesses, the architectures of which optimize the requisite cell-to-cell interactions for spontaneous self-assembly. With one pipetting step, we were able to create hundreds of uniform spheroids whose size was determined by seeding density. Multicellular tumor spheroids (MCTS) were assembled or grown from single cells, and their proliferation was quantified using a modified 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay. Complex-shaped (e.g., honeycomb) microtissues of homogeneous or mixed cell populations can be easily produced, opening new possibilities for 3-D tissue culture.

INTRODUCTION

Three-dimensional (3-D) in vitro cell culture, in which cells are grown in environments that more closely mimic native tissue architecture and function, have important applications in developmental/cell biology, drug screening, and regenerative medicine (1–9). Current methods that employ extracellular matrices, photolithography, cell printing, or laser tweezers are limited by expense and/or difficulty; therefore their use is not widespread (9–11). Numerous studies have shown that single-cell suspensions, in the absence of an extracellular matrix, will spontaneously self-assemble spherical microtissues, and mixed cell populations will self-segregate to form multilayered structures (4,12). Unfortunately, the methods to produce these microtissues, mainly spinner culture and hanging drops, have inconvenient design limitations such as spherical geometry, low throughput, or a high shear force environment (4,12–16). We present a straightforward platform that uses micromolded agarose to guide the spontaneous self-assembly of cells into 3-D microtissues. This platform represents significant improvements and additions to our previously published technique, including durable, autoclavable molds, a 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1)-based aggregate proliferation assay, as well as methods for viewing self-assembly in the vertical plane, clonal expansion of individual cancer cells into microtumors, and production of multicell-type microtissues with a prescribed shape (17). This micromolded agarose Petri dish is compatible with standard cell culture equipment, biochemical assays, microscopy techniques, and offers new opportunities in microtissue design.

MATERIALS AND METHODS

Cell Culture

MCF-7 human breast cancer cells (ATCC, Manassas, VA, USA) were expanded in RPMI-1640 medium (GIBCO®; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 1% solution, and 1% L-glutamine. Human umbilical vein endothelial cells (HUVEC; Cambrex, Walkersville, MD, USA) were expanded in endothelial growth medium 2 (EGM2) (Cambrex). Normal human fibroblasts (NHF) derived from neonatal foreskins, rat hepatoma cells (H35), and rat glioblastoma cells (RG2) were expanded in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. MCF-7, HUVEC, RG2, and H35 were maintained at 37°C in a 5% CO2-95% air atmosphere in a humidified incubator. NHFs were incubated at 37°C in a 10% CO2-90% air atmosphere. Growth media were exchanged every other day.

Design and Fabrication of Micromolds

Molds designed using computer-assisted design (CAD; Solid Works, Concord, MA, USA) comprised a cell-seeding chamber, cell aggregation recesses, and medium exchange ports. The cell-seeding chamber is a relatively large rectangular recess that collects the cell suspension and distributes cells into the smaller aggregation recesses as cells settle under gravitational force. Cell aggregation recesses extend downward from the floor of the seeding chamber increasing cell-to-cell contact as cells collect on their concave bottoms. Recess bottoms are located 1.2 mm from the bottom of the agarose gel allowing imaging with inverted microscopes. Medium exchange ports allow room to place a pipet between the hydrogel and the tissue culture plate to change medium without disrupting the cells. Rectangular molds designed for horizontal view microscopy were 3.53 mm in height, 5.9 mm wide, and 17 mm long, and contained a single row of aggregation recesses. CAD files were used to produce wax molds with a ThermoJet® rapid prototyping machine (3D Systems, Valencia, CA, USA). Preliminary testing of the resolution capabilities of the ThermoJet rapid prototyping...
Figure 1. Micromolded nonadhesive hydrogels guide cellular self-assembly. (A) Poly-dimethylsiloxane (PDMS) micromolds (top row) are utilized to cast micromolded agarose hydrogels (bottom row) designed to fit 6-, 12-, and 24-well tissue culture plates. (B) The self-assembly protocol. Hydrogels are cast and equilibrated in culture medium (i) before adding cell suspension to the seeding chamber (ii). Cells settle into recesses under gravitational force (iii). Cells coalesce at the bottom of the concave recesses and self-assemble into microtissues (iv). A larger view of individual microtissue shown in the inset. (C) Photomicrographs of normal human fibroblasts (NHF) self-assembly demonstrate that cells settled into hydrogel recesses within minutes of seeding (top) and formed microtissues by 24 h (bottom). Side-on view of self-assembly of (D) NHF and (E) rat hepatoma cells (H35) in special polyacrylamide micromolds. Cells settle into the recesses immediately after seeding (top panels) and form spheroidal (NHF) or ellipsoidal (H35) microtissues by 1 day (bottom panels). Scale bars, 200 μm.

Casting of Micromolded Nonadhesive Hydrogels

Autoclave-sterilized powder Ultrapure™ Agarose (Invitrogen) was dissolved via heating in sterile water to 2% w/v and pipetted into PDMS micromolds. Air bubbles were removed via pipet suction or agitation with a sterile spatula. After setting, gels were separated from the mold using a spatula, transferred to multi-well tissue culture plates, and equilibrated overnight with tissue culture medium.

Side view hydrogels with a single row of recesses were developed to allow observation of self-assembly in the vertical plane. Because of its superior optical properties, polyacrylamide was used, and gels were cast directly from the original wax molds. Acrylamide-bis-acrylamide 29:1 40% solution (1.75 mL; Sigma-Aldrich, St. Louis, MO, USA) was mixed with 1.63 mL phenol red-free DMEM, 1.63 mL Tris buffer, pH 6.8, and 25 μL 10% ammonium persulfate solution. The prepolymer was degassed, and 50 μL N,N,N′,N′-tetramethylethylenediamine (TEMED) were added to initiate polymerization. Four hundred microliters of polymer solution were added to the wax molds, and a glass coverslip was placed over the top surface to ensure even spreading. After 20 min, polyacrylamide hydrogels were removed with a spatula, transferred to a 6-well plate, and equilibrated with 2 mL phenol red-free culture medium overnight at 37°C. After equilibration, gels were rinsed and stored in 2 mL fresh medium.

Microtissue Production

Cells were trypsinized, counted, and resuspended to the desired cell density in the appropriate medium. Medium was removed from the tissue culture plates containing the micromolded hydrogels, and 150 μL cell suspension were added dropwise via micropipet to the center of the rectangular recess (seeding chamber) of each gel. Cells were allowed to settle into the recesses for approximately 20 min before additional medium was added to the cell culture plate by pipeting into the media exchange ports. Initial settling and rolling of cells into the deep wells due to some culture media movement ensures that the vast majority of cells enters and stays within the wells. Cells self-assembled within 24 h at 37°C. Homotypic (single cell type) microtissues were maintained with the appropriate medium and incubation conditions for the cells which comprised them (see section entitled Casting of Micromolded Nonadhesive Hydrogel). Heterotypic (multiple cell type) microtissues that contained HUVEC were grown in EGM2 medium (Cambrex), while all others were grown in DMEM with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. All heterotypic microtissues were incubated at 37°C in a 5% CO₂ atmosphere. Media were exchanged every other day.
Side-On Viewing of Self-Assembly

Cells were trypsinized, counted, and resuspended in phenol red-free DMEM with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin to achieve a cell density of $3.28 \times 10^6$ cells/mL. Forty microliters of cell solution were added to each side view hydrogel. Seeded hydrogels were then incubated at 37°C with 10% (NHF) or 5% (H35) CO$_2$ for 45 min, after which 2 mL medium were added. Using a Mitutoyo FS-100 microscope modified to lie horizontally, images of the front face of the spheroids were acquired.

Spheroid Size Quantification

NHF spheroids were stained with calcein-AM. Excess medium was removed, and 300 μL phosphate-buffered saline (PBS) containing 2 μM calcein-AM (Invitrogen) were added to the seeding chamber. Plates were protected from light and incubated at room temperature for 45 min. Epifluorescent images were then acquired on an Olympus IX70 microscope (Olympus, Center Valley, PA, USA) using an AxioCam MRc camera (Carl Zeiss MicroImaging, Thornwood, NY, USA). Fluorescent images were manually thresholded so that spheroids were highlighted but background signal was eliminated, analyzed for spheroid area, and radii calculated from an assumed circular geometry using NIH Image J software.

RG2 spheroids were imaged using brightfield microscopy. A software-generated mask image containing an array of circular holes corresponding to the array of recesses on the micromolds was aligned to the image and used to subtract dark rings (artifact from viewing hydrogels in brightfield) around the edges of the recesses using Adobe® Photoshop®. These images were then imported into Image J, manually thresholded so that spheroids were highlighted but background signal was eliminated, and radii calculated from the areas.

WST-1 Cell Proliferation Assay

The WST-1 cell proliferation assay (Roche Applied Science, Mannheim, Germany) was adapted to measure proliferation of tumor spheroids. To establish a standard curve, MCF-7 cells were seeded at densities ranging from $0.5 \times 10^6$ to $8 \times 10^6$ cells/micromolded hydrogel. Cells were allowed to self-assemble for 1 day, excess medium was removed, and a 400-μL dilution of 10:1 medium and WST-1 reagent was added to the seeding chamber. Hydrogels were incubated at 37°C for 4 h, and 100 μL WST-1/medium solution were transferred to a 96-well plate. Optical density was measured at 440 (OD$_{440}$) minus 600 nm using a microplate spectrophotometer (SpectraMax® 384; Molecular Devices, Sunnyvale, CA, USA) and plotted with respect to the number of cells seeded on the previous day. Experimental hydrogels were seeded with $0.5 \times 10^6$, $1 \times 10^6$, or $1.5 \times 10^6$ cells/gel (six replicates each). Each day, proliferation was measured for one replicate of each seeding density as described previously. Cell proliferation was quantified by comparing OD$_{440}$ of unknown samples to the standard curve.

Live-Cell Fluorescent Staining

The relative locations of NHF, HUVEC, and H35 within heterotypic microtissues were visualized via staining with fluorescent dyes CellTracker Red CMTPX, CellTracker Green CMFDA, and CellTracker Blue CMAC (Invitrogen). Cells were loaded 1 day prior to seeding. Subconfluent flasks were incubated with DMEM containing 2.5 μM CellTracker for 45 min at 37°C. DMEM was then discarded and replaced with the appropriate dye-free medium. Stained cells were trypsinized and seeded into hydrogels with a 1:1 (heterotypic)
or 1:1:1 (tritropic) ratio of the appropriate cell types. CellTracker Red CMTPX, CellTracker Green CMFDA, and CellTracker Blue CMAC were imaged by confocal or epifluorescent microscopy with an excitation/detection of 577/602 nm, 492/517 nm, and 353/466 nm, respectively.

RESULTS AND DISCUSSION

Micromolds for the directed self-assembly of microtissues were flexible, transparent, and could be autoclaved and re-used (Figure 1A). Agarose hydrogels cast from various micromolds (designed for 6-, 12-, or 24-well plates) formed precise negative replicates of their respective molds. Single-cell suspensions of NHF were added to the seeding chamber of the micromolded agarose where they sank into the recesses forming a sheet that conformed to the bottom of the well within 20 min (Figure 1, B and C). Since the hydrogel surface is nonadhesive for cells, cell-to-cell binding was promoted and, in <24 h, spheroids self-assembled. As with all spheroids and microtissues, limited access of the interior to nutrients may lead to necrosis, which could limit their applicability. This phenomenon is directly related to microtissue size and may vary with cell type and specific culture conditions. The ease with which microtissue size can be controlled using our method enabled us to self-assemble microtissues in which no cells were more than 150 μm from the microtissue surface, a typical diffusion limit for spheroids. We have shown that NHFs will survive in microtissues of this size for at least 2 weeks (17).

Horizontal microscopy revealed the vertical aspect of self-assembly. Single-cell suspensions of NHF and H35 were seeded into micromolds of polyacrylamide with a single row of recesses (Figure 1, D and E, respectively). Cells settled onto the bottom of the recesses and were funneled together due to the concave design of the recess bottoms maximizing cell-cell interactions. After 24 h, both cell types formed microtissues that appeared spherical via conventional microscopy, but horizontal microscopy revealed a significant difference. NHF spheroids were near perfect spheres, while H35 spheroids were shorter in the z dimension than the x, y dimension.

To evaluate the ability to control spheroid radius as a function of cell seeding density and to measure homogeneity of spheroid radius within relatively large batches, NHF and RG2 cells were seeded, (two gels per cell type, each containing 822 recesses/gel) at various seeding densities. After 1 (RG2) or 2 days (NHF) of self-assembly, image analysis was used to determine the radius distribution (Figure 2, A and B). Spheroid radius was proportional to seeding density, and spheroids were monodispersed about the mean radius. More cells settled into recesses along the periphery of the gel seeding chambers, resulting in larger spheroids. Radius distribution was measured across the entire gel. NHFs seeded at 0.25, 0.5, and 1 × 10^6 cells/gel resulted in spheroids with average radii of 53.3 μm with a coefficient of variation (CV) of 15.2%; 68.8 μm with CV of 8.7%; and 85.8 μm with CV of 10.6%, respectively. RG2s seeded at 0.18, 0.48, and 0.84 × 10^6 cells/gel resulted in spheroids with average radii of 63.0 μm with CV of 22.8%; 87.0 μm with CV of 23.8%; and 107.9 μm with CV of 17.2%, respectively. These CVs are comparable to published results of methods in which known numbers of cells are pipetted into individual hanging drops where CVs ranged from 10% to 15% for HepG2 cells and 5% for MCF-7 cells (18). The trend toward higher CVs using our method could be due to differences of when the CVs were measured (5–16 days after seeding versus 1–2 days in the present study), which could reflect differences in the short-term processes of self-assembly versus growth in 3-D. It could also be due to differences in the behavior of different cell types (e.g., HepG2 vs MCF-7) or to partitioning of cells into individual wells by a settling process as occurs in our method. More experimentation is needed to distinguish these possibilities.

To measure the growth of microtumor spheroids, MCF-7 cells were seeded at various densities (0.5, 1.0, and 1.5 × 10^6 cells/gel), and growth was measured by the WST-1 assay (Figure 2, C and D). MCF-7s seeded over a range of cell densities and self-assembled for 24 h yielded a standard curve with strong linear correlation (r² = 0.953) between cell number and optical density within the range of interest (0.5 to 8 × 10⁶). Spheroids displayed linear growth for the first 3 days, followed by slower growth from days 3–5. The slope of the growth curve for the initial 3 days was independent of initial spheroid size. We also assessed the smallest number of MCF-7s that could form a spheroid in this mold design. Small spheroids of MCF-7 cells could be self-assembled reliably (>95% of the recesses containing a single spheroid) by seeding as few as 3.1 × 10⁴ cells/gel (approximately 38 cells/recess).

To determine if microtumor spheroids could be grown through clonal expansion of single cells, micromolds were seeded with a low cell density of H35 cells (approximately 800 cells/gel with 822 recesses) (Figure 2E). Over a 21-day period, spheroids (300-μm diameter) readily grew from a single cell. This method is especially useful for cells that are proliferation competent in aggregate culture, such as tumor cells or possibly stem cells, because it promotes aggregate growth and allows for quantification of proliferation.

To establish their ability to guide the self-assembly of cellular structures with complex geometries, micromolds with rod, toroid, loop-ended dog bone (pair of toroids connected by a rod), and honeycomb (a lattice of toroids) recesses were seeded with H35 or MCF-7 cells (Figure 3, A and B) or NHF (see Supplementary Movie S1 available online at www.BioTechniques.com). Time-lapse microscopy of NHF revealed the process of toroid self-assembly (see Supplementary Methods). Cells sank into the toroid recesses, conformed to the bottom of the well, and immediately began to aggregate. NHFs assembled into a torus-shaped structure that wrapped tightly around the central hydrogel peg and decreased in outer diameter with time. H35s self-assembled into rod and loop-ended dog bone structures. The loop-ended dog bones adhered tightly to the two hydrogel pegs on the outer edges. On
the inner edges, however, the dog bones pulled away from the pegs forming a cleavage, indicating that the structure is under tension. MCF-7 cells readily self-assembled toroid and honeycomb structures. In micromolds with toroid-shaped recesses, the microtissues were wrapped around the protruding hydrogel pegs. In micromolds with honeycomb-shaped recesses, the self-assembled honeycomb was held on the micromold by contact with the outer edges of the outermost hydrogel pegs. From these points of contact, the cellular honeycomb appeared to evenly distribute tension in the structure such that a hexagonal geometry was approximated and most of the inner pegs were not in contact with the cellular structure. After 5 days, the honeycomb microtissues were removed from the hydrogel and cultured on agarose coated plates for 3 additional days. The branches became slightly shorter and wider, as if removed from tension, but the honeycomb shape was maintained.

To demonstrate the ease with which micromolds can generate multilayered microtissues, various cell types were labeled with fluorescent tracking dyes, mixed, and seeded into hydrogels (Figure 3D). NHF/H35 and NHF/HUVEC mixtures were assembled into honeycombs and loop-ended dog bones, respectively. Epifluorescent and confocal microscopy revealed that cells also self-sorted in these structures, forming multilayered microtissues. Within each of the mixtures, the NHF (red) formed the core structure, while the H35 or HUVEC (green) formed an epithelial-like outer coating.

Tritypic spheroids were generated by seeding a 1:1:1 mixture of labeled NHF (green), HUVEC (red), and H35 (blue) cells (Figure 3D). Cell sorting occurred in the tritypic spheroids with an outer layer of H35s engulfing interspersed islands of NHFs and HUVECs. The sorting of only two different cell types within a spheroid is a well-known phenomenon thought to be influenced by the relative strength of homotypic and heterotypic cell-to-cell adhesion (12,19). Although more experimentation is needed, sorting within the tritypic spheroid appears to be more complex.

We have presented an easy-to-use, cost-effective, versatile, and scalable platform for controlled production of 3-D microtissues. Beyond spheroids, we have demonstrated the directed self-assembly of microtissues with prescribed shape and cell type composition. Because the method is scaffold-free and cells are not adhered to a surface, cells spontaneously self-assemble and reach a structural equilibrium that is governed by cell-to-cell interactions. The microtissues are in a hydrogel, so their surfaces can readily receive nutrients and exchange wastes in all three dimensions, whereas, these processes are not uniform for microtissues that are bound to a substrate such as polystyrene. This system has a wide range of applications in cell biology, cancer biology, high-throughput drug screening, and tissue engineering.

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The authors declare no competing interests.

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