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Three-dimensional Tissue Culture Based on Magnetic Cell Levitation

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Abstract

Cell culture is an essential tool for drug discovery, tissue engineering, and stem cell research.

Conventional tissue culture produces two-dimensional (2D) cell growth with gene expression,

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G.R.S., J.R.M., R.M.R., D.J.S., C.S.L., J.M., T.C.K., W.A., R.P. conceived and designed the experiments. G.R.S., J.R.M., T.C.K., D.J.S., C.S.L., J.S.A., J.M. performed the experiments. G.R.S., M.G.O., D.J.S., C.S.L., L.F.B., J.S.A., J.A.B., T.C.K., W.A., R.P. analyzed the data. R.M.R., M.M.G., J.A.B., J.G.G., T.C.K., W.A., R.P. contributed materials/analysis tools. G.R.S., R.M.R., M.G.O., L.F.B., J.A.B., J.G.G., T.C.K., W.A., R.P. co-wrote the paper.

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Competing Interests Statement

The University of Texas M. D. Anderson Cancer Center (UTMDACC) and Rice University (RU), along with their researchers, have filled patents on the technology and other intellectual property reported here. If licensing or commercialization occurs, the researchers will be entitled to standard royalties. G.R.S., R.M.R., C.S.L., and T.C.K. have equity in Nano3D Biosciences, Inc. UTMDACC and RU manage the terms of these arrangements in accordance to their established institutional conflict-of-interest policies.

signaling, and morphology that can differ from those in vivo and thus compromise clinical relevancy^{1–5}. Here we report a three-dimensional (3D) culture of cells based on magnetic levitation in the presence of hydrogels containing gold and magnetic iron oxide (MIO) nanoparticles plus filamentous bacteriophage. This methodology allows for control of cell mass geometry and guided, multicellular clustering of different cell types in co-culture through spatial variance of the magnetic field. Moreover, magnetic levitation of human glioblastoma cells demonstrates similar protein expression profiles to those observed in human tumor xenografts. Taken together, these results suggest levitated 3D culture with magnetized phage-based hydrogels more closely recapitulates in vivo protein expression and allows for long-term multi-cellular studies.

While protein-based gel environments or rotational/agitation-based bioreactors have been developed in attempts to allow three-dimensional (3D) cell culture^{1–5}, broad practical application of such methods has not as yet been achieved^{2–4}. Thus, a straightforward technology enabling 3D cell culture is still an unmet need^{3–4}. To address this challenge, we introduce a “3D bio-assembler” that relies on magnetic forces and cell levitation^{6–8}. The methodology is based on the cellular uptake and subsequent magnetic levitation of a bioinorganic hydrogel composed of bacteriophage (phage) plus magnetic iron oxide (MIO; Fe₃O₄, magnetite) and gold nanoparticles that self-assemble into hydrogels (Fig. 1a). Incorporation of MIO nanoparticles creates a new material that retains the biocompatibility of gold-phage hydrogels^{9, 10} while adding capabilities for the culture and magnetic manipulation of cells.

The technology reported here provides an alternative to biodegradable porous scaffolds and protein matrices^{11–12}. In general, biodegradable scaffolds may suffer from slow or delayed propagation of cells and establishment of cell-cell interactions. Existing commercial products, such as Matrigel¹¹, are useful and not particularly expensive but their chemical composition is fixed (i.e., unchangeable). In contrast, our bio-assembler enables adaptable magnetic-based cell levitation and it may provide for improved 3D cell growth conditions in certain settings. The methodology is cost-effective because it does not require specific medium and it is compatible with standard 2D cell culture techniques.

Biological application of magnetic forces has long been studied^{13–18}. For instance, Magnetic Resonance Imaging is a mainstay of clinical diagnostic radiology, relying on superconducting magnets and large magnetic fields. Magnets have also been used to levitate biological samples through the natural diamagnetism of organic materials⁶. Incorporation of MIO nanoparticles has further enabled manipulation of surface patterns^{7, 13}, contrast-enhanced Magnetic Resonance Imaging¹⁴, cell sorting¹⁴, mechano-conditioning of cells^{14–16}, studies of mechano-sensitive membrane properties¹⁷, and cellular micromanipulation¹⁸. However, while MIO nanoparticles can be modified to target proteins or coupled to cationic liposomes for delivery and concentration¹³, a combination of ligand peptide-mediated targeting and magnetic levitation towards 3D cell culture has not yet been systematically applied.

Our hydrogel uses M13-derived phage particles, displaying the ligand peptide CDCRGDCFC (termed RGD-4C) to target α_v integrins^{19, 20}, gold nanoparticles and MIO.

Phage are being investigated for biotechnological and materials applications²¹ such as targeted gene delivery²⁰, optical imaging microscopy⁹, and protein targeting¹⁹. Both phage and gold nanoparticles are potentially biocompatible and have even been approved by the United States Food and Drug Administration for use in several applications²². Under the conditions used, MIO nanoparticles are well-tolerated by mammalian cells, a result consistent with previous reports^{7, 13, 23}. As such, magnetic-based levitation of cell-targeted hydrogels may become a useful biotechnology tool.

To first evaluate and next confirm the presence of MIO in the hydrogels, we employed several methodologies including elastic light scattering, Magnetic Resonance Imaging, and Inductively-coupled Plasma Atomic Emission Spectroscopy (Fig. 1b–d and Supplementary Figs. S1–S2). Having demonstrated the presence of MIO within the targeted hydrogel, we subsequently used murine C17.2 neural stem cells²⁴ to evaluate the ability of the system to induce magnetic field-based cell levitation (Fig. 2). The admixture of neural stem cells and MIO-containing hydrogel co-rose to the air-medium interface but could not leave the medium, presumably due to surface tension (Fig. 2a–c). The observed cell levitation in the liquid medium confirms that the field from a permanent magnet is sufficient to overcome the gravitational force into a steady state, consistent with our theoretical estimates (Supplementary Fig. S3). While the liquid-air interface could also be thought of as a physical barrier for levitated cell culture, it lacks a solid material surface for cell attachment and growth. We observed that the magnetic field concentrated clusters of levitated cells in solution, triggering cell-cell interactions in a manner consistent with tissue engineering scaffolds³ designed to bestow cell growth advantage. In general, 3D cell cultures were axially symmetric and on a large-scale reflected the symmetry of the magnets used. But under magnification, we observed small-scale multicellular assembly features (Fig. 2d) with characteristic and reproducible branching morphogenesis²⁵.

In order to assess cell growth within the bio-assembler, we visually and quantitatively monitored the formation rate, size, and viability of genetically-modified human glioblastoma cells over a period of 8 d by monitoring the fluorescence from stable protein expression of mCherry (Fig. 3a). Within 30 min of levitation, cells were brought together (Fig. 3a, 30 min); moreover, a cohesive multicellular assembly emerged by 24 h and a spheroid shape formed between 3–8 d, reaching a maximum diameter of ~1 mm. We obtained similar results--albeit with a lower initial yield--with shorter incubation of cells and MIO-containing hydrogels (Supplementary Fig. S4).

Furthermore, morphological analysis of the architectural integrity of 3D structures was performed with transmission and scanning electron microscopy. In “small” spheroids, we noted viable cells throughout the structure; in contrast, we observed central necrosis surrounded by a viable outer region in “large” spheroids (Supplementary Figs. S5–S7). While such features may actually be desirable in emulating conditions within the tumor microenvironment (such as ischemia, acidosis, or nutrient transport, among other parameters), the formation of a heterotypic vascular supply from exogenous angiogenic endothelial cells is an active area of research and development. Together, future use of serial co-cultures of endothelium-derived cells and different ligand peptides or varying magnetic fields can also be envisioned.

The intense red fluorescence from mCherry protein expression confirmed cell viability within the 3D assembly, and cultures could be maintained for at least 12 weeks at which timepoint the experiment was terminated (Supplementary Fig. S8). We compared the growth rate of magnetically levitated cells with that of cells in standard 2D cultures (Fig. 3b). In contrast to the indicated exponential trend for the growth of levitated cells, cell culture in 2D showed a linear growth pattern, a feature of surface attached cultures²⁶. Because of the volume accessible during 3D growth, a large assembly can be accomplished without the de-attachment/re-plating cycles (“passage”) required in standard tissue culture.

To explore the biological attributes afforded by magnetic levitation in our system, we cultured human glioblastoma cells and observed not only morphological but also molecular similarity to orthotopic human tumor xenografts from immunodeficient mice (Fig. 3c). We evaluated the marker N-cadherin, a transmembrane protein mediating cell-cell contact through homotypic cell adhesion interactions²⁷. N-cadherin expression in 3D cultures suggests that magnetic levitation may recapitulate at least some in vivo-like traits. Indeed, 2D cultures show N-cadherin scattered in the cytoplasm and nucleus but absent from the membrane, while 3D-levitated cells express N-cadherin in the membrane, cytoplasm, and cell junctions (akin to the protein expression pattern observed in tumor xenografts). Our results are qualitatively consistent with those recently reported by Ofek et al.²⁷ in which cartilage grown in vitro also yielded differential N-cadherin expression patterns in 3D-relative to 2D-culture. In control experiments, we observed no alterations in N-cadherin expression under conditions including: (i) MIO-containing hydrogels with no magnetic field, (ii) MIO-containing hydrogels with a magnetic field but cultured in 2D, and (iii) cells alone under a magnetic field (Supplementary Fig. S9). Thus, magnetically induced 3D tissue culture in vitro may become a complementary and cheaper surrogate for the labor- and cost-intensive generation and maintenance of human brain tumor xenografts in immunodeficient mice.

To evaluate the ability of magnetic fields to provide a means to engineer cell culture shape, we levitated tumor cells with different ring-shaped magnets, assessed the magnetic field strength, and serially recorded culture morphology (Fig. 4). The resulting structures reflected the properties of the estimated and Hall probe measured magnetic field. For the large radius magnet (Fig. 4a–f), the field at the height of the culture had a central minimum, which led to a ring-shaped cell pattern at the onset of levitation produced by the force pulling cells towards the field maximum (Fig. 4a, b); this shape persisted as cells assembled (Fig. 4c–f) and formed a cohesive structure with enough integrity to remain intact when the magnet was removed (Fig. 4e–f). While ischemia can cause central necrosis of spheroids cultured for long periods, the shape generated at the early time point was attributable to the magnetic field profile at the liquid-air interface. The small magnet produced a central maximum of the field at the height of the culture and a compact cellular assembly (Fig. 4g–l). In this initial work, the volume of medium and the magnetic fields were held constant and volumetric variation did not adversely affect 3D culture geometry.

Finally, magnetic field manipulation may provide precise temporal and/or spatial control of distinct cell populations in an environment conducive to visualization or molecular imaging in situ. We demonstrate the feasibility of confrontation assays with co-culture of human

glioblastoma cells (GFP-transfected; green cells) and normal human astrocytes (mCherry-transfected; red cells). Initially, a clear interface separating the cell structures was evident. By 12 h, the populations began to fuse and lose their individual spherical shapes. After 3 d, the populations coalesced into a single spheroid with the human glioblastoma cells invading the structure composed of normal human astrocytes. Glioblastoma is the most common, invasive, and lethal type of astrocytic brain tumor²⁸, and these results illustrate the potential of this methodology for analysis of brain tumor invasiveness of normal brain in confrontation culture assays, which have been correlated with clinical outcome²⁹.

In conclusion, we have described a bio-assembler based on magnetic levitation with gold-phage-MIO as an enabling technology for 3D cell culture. We demonstrated control of culture shape, and the ability to bring cultures together for controlled interaction in a confrontation assay with in situ monitoring. The magnetic levitation methodology reported here does not require specific medium, engineered scaffolds, matrices, or molded gels. This simple, flexible, and effective method may be suitable for a range of applications in biotechnology, drug discovery, stem cell research, or regenerative medicine. Indeed, a potential long-term goal is the possibility of accomplishing the “engineering” of normal tissues or complex organs¹². Side-by-side comparison to various other 3D culture methods (including a panel of porous and biodegradable scaffolds) will ultimately determine the value of this technology in different experimental settings or applications.

Methods

Hydrogel self-assembly

Hydrogels were generated as described^{9, 10} except for the inclusion of MIO nanoparticles. MIO-containing hydrogels were prepared by mixing the gold nanoparticle solution (Optical absorbance 530 nm = 1.2–1.5 units) with MIO nanopowder (magnetite[Fe₃O₄], polydisperse particle size < 50 nm; stabilized with a surfactant of polyvinyl pyrrolidone; Sigma-Aldrich) to a concentration of 0.3 mg/ml. Magnetite nanoparticles were not placed in an oxidizing environment (i.e., chemical or heat) to avoid oxidation into another state such as maghemite [γ-Fe₂O₃]. Phage dilutions was prepared with 10⁹ transducing units (TU)/μl in picopure water. Finally, the phage solution and the gold nanoparticle plus iron oxide solution were mixed with equal volumes and allowed to stand overnight at 4°C for hydrogel formation.

Magnets

Ring-shaped, neodymium rare earth magnets from Gauss Boys (Portland, OR; part numbers MR32 and MR16) have outer (inner) radii of 12 (2.8), and 6 (1.7) mm and thicknesses of 5 mm and 3 mm. Permanent magnetization is parallel to the symmetry axis.

Cell lines

Bosc, normal human astrocytes, and human glioblastoma (LN-229 or U-251MG) cells were cultured in Dulbecco modified Eagle's medium containing 10% fetal bovine serum (FBS). Transfections and retroviral infections were performed as described, and cells were selected and maintained with blasticidin (mCherry) or puromycin (eGFP) selection³⁰. C17.2 murine neural stem cells were cultured in high-glucose Dulbecco modified Eagle's medium

containing 10% FBS supplemented with sodium pyruvate 2 mM, glutamine, penicillin, and streptomycin²⁴.

Magnetic levitation of cell culture in MIO-containing hydrogels

For levitated cell culture, surface attached cells (grown to ~80% confluence) were treated with 1 μ l of hydrogel per 1 cm² of surface area available for cell culture and incubated overnight. Treated cells were de-attached by PBS containing trypsin and EDTA and placed into a tissue culture Petri dish^{9, 10}. A cover top with an attached neodymium magnet was immediately put in place. Trypan-blue excluding cells were de-attached and counted. Half of the sample (~3 $\times$ 10⁴ cells) was transferred to seed a 2D surface-attached culture and the other half was seeded for a 3D levitated culture.

Orthotopic glioma xenograft models

Human glioblastoma cells (2 $\times$ 10⁶ cells per 10 μ l of PBS; LN229 vector-GFP containing cells) were orthotopically implanted into the brains of immunodeficient (SCID) mice by direct intracranial injection at the right frontal lobe. Tumor-bearing mice were closely monitored and killed upon any neurological signs of intracranial tumor burden. Tumor-containing brains were surgically collected and paraformaldehyde (PFA)-fixed for pathological analysis.

Immunofluorescence detection of N-cadherin

Human glioblastoma cells used for 2D cell culture were prepared by first plating 10⁵ cells on poly-d-lysine coated glass slide and allowing cells to attach overnight. Cells were subsequently washed with PBS and fixed with PBS containing 4% PFA for 30 min, and washed again. Next, cells were permeabilized with PBS containing 0.1% triton X-100 for 5 min, washed with PBS, and treated with Image-iT FX signal enhancer (Invitrogen) for 30 min. Blocking was performed with PBS containing 0.2% gelatin and 10% donkey serum (PBS-gel) for 30 min. 3D cell structures from levitated cultures or brain xenografts were prepared with standard histopathology procedures. Briefly, 3D structures were collected, washed twice in PBS, and fixed in PBS containing 4% PFA for 30 min, then washed again. 3D structures were subsequently embedded in paraffin blocks and sectioned. Samples were treated in xylene, hydrated through a series of ethanols (100%, 95%, 70%) and water. Samples were steamed in antigen retrieval solution (Dako) for 20 min, rinsed in water, and transferred to PBS. Blocking was done with PBS containing 10% donkey serum for 30 min.

Microscope slides of the samples were incubated with primary antibody (anti-N-Cadherin, Zymed) overnight in blocking buffer at 4°C. Cells were washed with PBS then treated with secondary antibody Alexa 488, 555, or 568 (Molecular Probes/Invitrogen) in PBS-gel for 1 h at room temperature (RT). Cells were washed with PBS-gel then treated with DAPI in PBS-gel for 5 min, washed, and mounted onto slides. Images (Z-stacks) were taken on a Zeiss deconvolution microscope and de-convolved with the Axiovert software.

Magnetic resonance measurements

MRI was acquired with a 4.7 T, 40 cm Bruker Biospec instrument. The MRI images were derived from a multi-gradient echo sequence with variance in T2*-weighting (500 ms

repetition time, 16 echo times ranging from 1.5 ms to 39 ms, 64×64 image matrix, 32 mm $\times$ 32 mm field-of-view). The characteristic time constant for exponential transverse signal decay (T_2^*) was calculated from the average signal level of a region-of-interest in the center of each phantom as a function of echo time. The image contrast between the MIO-containing hydrogel and the MIO-free control results from the reduction in T_2^* relaxation constant in the presence of iron oxide nanoparticles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a **b** **c** **d**

Figure 1. Magnetic iron oxide-containing hydrogels

a, Human glioblastoma cells (lower arrow) treated with magnetic iron oxide (MIO)-containing hydrogel held at the air-medium interface by a magnet. The image was captured at 48 h of culture and depicts a ~1 mm spheroid. Scale bar, 5mm. **b**, Vial of a MIO-containing hydrogel (arrow) in water. **c**, Scheme of electrostatic interaction of nanoparticles (spheres) with phage (elongated structures). Gold (yellow sphere) and MIO (brown sphere) nanoparticles are depicted (not drawn to scale). **d**, MRI image (T2*-weighted) of purified

hydrogel in solution: MIO-containing hydrogel (top panel), average $T2^* = 16$ ms and MIO-free hydrogel control (bottom panel), average $T2^* = 46.2$ ms. Scale bar, 2 mm.

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Figure 2. 3D cell culture with magnetic-based levitation

The top row shows the general cell levitation strategy and the bottom row shows the corresponding optical micrograph of neural stem cells at each stage. **a**, Hydrogel is dispersed over cells and the mixture is incubated. The dark blotches are fragments of hydrogel. **b**, Washing steps remove non-interacting hydrogel fragments. Fractions of phage, gold, and MIO nanoparticles enter cells or remain membrane-bound. **c**, Application of an external magnet causes cells to rise to the air-medium interface. Image shows culture 15 min after levitation. **d**, After 12 h of levitation, characteristic multicellular structures form (single structure is shown in the schematic). Scale bar, which applies to bottom row, 30 μm .

Figure 3. Comparison of 3D cell growth with standard 2D tissue culture

a, Phase contrast (top row) and fluorescence (mCherry; bottom row) images of levitated human glioblastoma cells monitored over 8 days. Cells coalesced within hours and formed spheroids by 24 h. Scale bar, 200 μm . **b**, Number of cells as a function of time for levitated cell culture (a; blue squares) and representative 2D culture (red triangles). Line fits indicate an exponential trend for levitated cells (blue line) and linear trend for surface attached (red line). **c**, Immunofluorescence detection of N-cadherin (red) and DAPI nuclear staining (blue)

in a mouse xenograft, 3D magnetic levitation for 48 h, and 2D standard culture with human glioblastoma cells. Scale bar, 10 μm .

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Figure 4. Shape control of magnetically levitated culture

3D culture derived from a magnet with a 12 mm (a–f) and 6 mm (g–l) outer radius. **a, g**, Magnetic field profiles were obtained by direct integration of the Biot-Savart law using Mathematica (Wolfram Research Inc.; Champaign, IL) and the surface current $\vec{K} = \vec{M} \times \hat{n}$ ($\vec{M}$, magnetization and $\hat{n}$, unit vector normal to magnet surface). **b, h**, Hall probe measurements along a diameter perpendicular to the symmetry axis at the air-medium interface. Lines are spline fits. **c–e, i–k**, Human glioblastoma cells at the onset of levitation

(c, i), 30 h (d, j), and following magnet removal at 30 h (e, k). **f, l**, Spheroid images after growth for one week. Scale bar, 400 μm .

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Figure 5. Confrontation assay of magnetically levitated multicellular spheroids

a, Brightfield and fluorescence images of human glioblastoma cells (green; GFP-expressing cells) and normal human astrocytes (red; mCherry-labeled) cultured separately and then magnetically guided together (time=0). **b**, Confrontation between human glioblastoma cells and normal astrocytes monitored for 10.5 d. Invasion of the spheroid composed of normal human astrocytes by human glioblastoma cells serves as a standard assay of glioma invasiveness²⁹. Scale bar, 200 μ m.