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TITLE:

Propagation of Dental and Respiratory Cells and Organs in Microgravity

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SUMMARY:

This protocol presents a method for the culture and 3D growth of ameloblast-like cells in microgravity to maintain their elongated and polarized shape as well as enamel-specific protein expression. Culture conditions for the culture of periodontal engineering constructs and lung organs in microgravity are also described.

ABSTRACT:

Gravity is one of the key determinants of human cell function, proliferation, cytoskeletal architecture and orientation. Rotary bioreactor systems (RCCSs) mimic the loss of gravity as it occurs in space and instead provide a microgravity environment through continuous rotation of cultured cells or tissues. These RCCSs ensure a continuous supply of nutrients, growth and transcription factors, and oxygen, and address some of the shortcomings of gravitational forces in motionless 2D (two dimensional) cell or organ culture dishes. In the present study we have used RCCSs to co-culture cervical loop cells and dental pulp cells to become ameloblasts, to characterize periodontal progenitor/scaffold interactions, and to determine the effect of inflammation on lung alveoli. The RCCS environments facilitated growth of ameloblast-like cells, promoted periodontal progenitor proliferation in response to scaffold coatings, and allowed for an assessment of the effects of inflammatory changes on cultured lung alveoli. This manuscript summarizes the environmental conditions, materials, and steps along the way and highlights critical aspects and experimental details. In conclusion, RCCSs are innovative tools to master the culture and 3D (three dimensional) growth of cells *in vitro* and to allow for the study of cellular systems or interactions not amenable to classic 2D culture environments.

INTRODUCTION:

Gravity affects all aspects of life on Earth, including the biology of individual cells and their function within organisms. Cells sense gravity through mechanoreceptors and respond to changes in gravity by reconfiguring cytoskeletal architectures and by altering cell division[1-3].

Other effects of microgravity include the hydrostatic pressure in fluid filled vesicles, sedimentation of organelles, and buoyancy-driven convection of flow and heat[4]. Studies on the effect of loss of gravity on human cells and organs were originally conducted to simulate the weightless environment of space on astronauts during space flight missions[5]. However, in recent years, these 3D bioreactor technologies originally developed by NASA to simulate microgravity are becoming increasingly relevant as novel approaches for the culture of cell populations that are otherwise not amenable to 2D culture systems.

3D Bioreactors simulate microgravity by growing cells in suspension and thus creating a constant "free-fall" effect. Other advantages of the rotating bioreactors include the lack of air exposure encountered in organ culture systems, a reduction in shear stress and turbulence, and a continuous exposure to a changing supply of nutrients. These dynamic conditions provided by a Rotary Cell Culture System (RCCS) bioreactor favor spatial co-localization and three-dimensional assembly of single cells into aggregates[6,7].

Previous studies have demonstrated the advantages of a rotary bioreactor for bone regeneration[8], tooth germ culture[9], and for the culture of human dental follicle cells[10]. There has also been a report suggesting that RCCS enhances EOE cell proliferation and differentiation into ameloblasts[11]. However, differentiated cells were considered ameloblasts based on ameloblastin immunofluorescence and/or amelogenin expression alone[11] without considering their elongated morphology or polarized cell shape.

In addition to the NASA-developed rotating wall vessels (RWV) bioreactor, other technologies to generate 3D aggregates from cells include magnetic levitation, the random positioning machine (RPM) and the clinostat[12]. To achieve magnetic levitation, cells labeled with magnetic nanoparticles are levitated using an external magnetic force, resulting in the formation of scaffold-free 3D structures that have been used for the biofabrication of adipocyte structures[13-15]. Another approach to simulate microgravity is the generation of multidirectional G forces by controlling simultaneous rotation about two axes resulting in a cancellation of the cumulative gravity vector at the center of a device called clinostat[16]. When bone marrow stem cells were cultured in a clinostat, new bone formation was inhibited through the suppression of osteoblast differentiation, illustrating one of the dedifferentiating effects of microgravity[16].

In vitro systems to facilitate the faithful culture of ameloblasts would provide a major step forward toward tooth enamel tissue engineering[17]. Unfortunately, to this date, the culture of ameloblasts has been a challenging undertaking[18,19]. So far, five different ameloblast-like cell lines have been reported, including the mouse ameloblast-lineage cell line (ALC), the rat dental epithelial cell line (HAT-7), the mouse LS8 cell line[20], the porcine PABSo-E cell line[21] and the rat SF2-24 cell line[22]. However, the majority of these cells have lost their distinctive polarized cell shape in 2D culture.

In the present study we have turned to a Rotary Cell Culture Bioreactor System (RCCS) to facilitate the growth of ameloblast-like cells from cervical loop epithelia co-cultured with mesenchymal progenitors and to overcome the challenges of 2D culture systems, including reduced flow of

nutrients and cytoskeletal changes due to gravity. In addition, the RCCS has provided novel avenues for the study of cell/scaffold interactions related to periodontal tissue engineering and to examine the effects of inflammatory mediators on lung alveolar tissues *in vitro*. Together, results from these studies highlight the benefits of microgravity-based rotatory culture systems for the propagation of differentiated epithelia and for the assessment of environmental effects on cells grown *in vitro*, including cell/scaffold interactions and the tissue response to inflammatory conditions.

PROTOCOL:

All necessary institutional approval was obtained to ensure that the study was in compliance with TAMU institutional animal care guidelines.

1. Bioreactor assembly and sterilization

1.1. Sterilize four high aspect ratio vessels (HARV) for the bioreactor in an autoclave on the plastic instrument cycle for 20 minutes at 121 °C as recommended by the manufacturer.

1.2. After sterilization, assemble the vessels in a cell culture hood by tightening the screws provided with the bioreactor after which the vessels are ready to use.

2. **Scaffolds used for the bioreactor co-culture experiment**

2.1. Incubate the scaffolds together with the cells to provide support for cell attachment necessary for further growth.

2.2. Choose among PGA based scaffolds, hydroxyapatite scaffolds, graphene sheets, gelatin discs, and collagen based scaffolds for co-culture studies.

3. Cervical Loop (CL) dissection and single cell preparation

3.1. Sacrifice mice by decapitation following respiratory arrest by CO_2 overdose.

NOTE: All animal studies are in compliance with TAMU institutional animal care guidelines.

3.2. Dissect cervical loops from 6 days postnatal (DPN) mice following a modified version of a previously published protocol[23].

NOTE: In our study, the distal portion of the cervical loop not shown in the previously published protocol is included (**Figure 3**).

3.2.1. Wash the dissected tissue with cold sterile PBS prior to digestion with collagenase dispase at 37 °C for 30 minutes.

3.3. Pass the solution through a 70 µm sterile cell strainer and further centrifuge at 800 x *g* for 10 minutes.

3.4. Discard the supernatant. Centrifuge the pellet and wash twice with cold PBS at 800 x *g*. Discard the supernatant.

3.5. Count the cells using a hemocytometer and add 1 X 10^5 cells per vessel.

4. Dental pulp cell culture and cell line expansion

4.1. Plate dental pulp cells in a 100 mm tissue culture plate at passage 4 at a concentration of 1 x 10^5.

4.2. Prepare media for the culture consisting of DMEM high glucose media, supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic/antimycotic (P/S).

4.3. When the cells reach 85-90% confluence, aspirate the medium and wash the plates twice with pre-warmed PBS.

4.4. After the PBS wash, add a pre-warmed 0.25% Trypsin-EDTA solution to the plate, and incubate the dental pulp cell-containing plates at 37 °C for 3 minutes.

4.5. Confirm cell detachment by a visual inspection using a microscope.

4.6. Collect all the media along with the cells from the culture plate and transfer via a 25 mL pipette to a 50 mL sterile conical tube.

4.7. Centrifuge the cells at 800 x *g* for 8 minutes and discard the supernatant. Resuspend the cells in fresh media and count using a hemocytometer.

4.8. For co-culture with cervical loop cells, seed the dental pulp cells in the bioreactor at a concentration of 1 x 10^4 per vessel.

5. Co-culture of cervical loop/dental pulp cells on a scaffold within the RCCS bioreactor

5.1. Add both cell types, the cervical loop, and the dental pulp to the culture medium containing keratinocyte SFM media with supplemented growth factors, extracellular matrix proteins and scaffolds.

NOTE: Growth factors are added at a concentration of 0.03 µg/mL bone morphogenetic protein 2 (BMP 2), 0.03 µg/mL bone morphogenetic protein 4 (BMP-4), 10 ng/mL human recombinant fibroblast growth factor (hFGF) and 15 ng/mL epidermal growth factor (hEGF), while ECM components included 200 µg/mL Matrigel, 5 µg/mL laminin and 5 µg/mL fibronectin.

5.2. Coat scaffolds with a mixture of 500 µL of collagen gel enriched with 1 mg of LRAP (Leucine-rich amelogenin peptide) peptide, 1 mg of lyophilized early stage enamel matrix prepared from porcine teeth[24], 5 µg/mL laminin and 5 µg/mL of fibronectin.

NOTE: This worked best for our experiments.

5.3. Following scaffold coating, add both cells and scaffold to the bioreactor, and fill the bioreactor with 10 mL medium per vessel.

5.4. Close the fill port cap of the bioreactor vessel after addition of media, cells, and scaffolds.

5.5. Attach the sterile valves to the syringe ports at the beginning of the experiment and keep them in place with a cover until the end of the experiment.

5.6. Once the vessel is 90% full and the fill port caps are closed and tightened, open up the sterile valves.

5.7. Use two sterile syringes (3 mL) every time a media change is required. Fill one syringe with fresh medium and the other syringe is left empty.

5.8. Open up both syringe valves and gently maneuver the bubbles towards the empty syringe port.

5.9. Once the fresh medium from the full syringe is carefully injected in the vessel, remove the bubbles through the empty syringe port.

NOTE: This procedure is performed until all the microbubbles are removed from the vessels.

5.10. Close the syringe ports with caps and discard the syringes in a sharps waste container.

5.11. Attach each vessel to the rotator base and place the rotator base in an incubator with 5% CO_2 and 37 °C temperature.

5.12. Turn on the power and set the rotational speed to 10.1 rpm.

NOTE: Adjust the speed to ensure that the scaffolds are in suspension without touching the vessel wall.

5.13. For media change, place the vessels in an upright position to ensure that the cells settle at the bottom. Open the syringe ports and connect the sterile syringes to the ports.

5.14. Follow the same procedure by aspirating 75% of the nutrition-depleted medium and injecting fresh medium through the other port.

6. Lung organ preparation and bioreactor based culture

NOTE: E15 wild-type mouse pups were used for the preparation of lung organs.

6.1. Dissect the lung tissue after euthanizing a pregnant female mouse with CO_2 asphyxiation.

6.1.1. Once the death is confirmed following asphyxiation by cervical dislocation, use a scalpel to expose the thoracic cavity. Thereafter, remove the pups from the uterus and euthanize by decapitation.

6.2. After euthanization, prepare the thoracic cavity by opening it with a scalpel to locate the lungs. Wash tiny segments of lung tissue with sterile PBS and place it on a pre-sterilized membrane for 2 hours with organ culture medium in an incubator containing 5% CO_2 and 37 °C temperature.

6.3. Once the lung tissues attach to the membrane in a 2D organ culture plate, transfer the samples to the bioreactor vessel.

6.4. Prepare the media for culture.

NOTE: The control group culture media contains 10% fetal bovine serum (FBS), 1% antibiotic solution (P/S) (penicillin, 100 U/mL, streptomycin, 50 µg/mL) and 100 µg/mL ascorbic acid (AA) and for the induction of inflammatory conditions, control medium is supplemented with 5 ng/mL IL-6 protein.

6.5. Carry out the lung culture experiment for 10 days with a media change every 48 hours in the bioreactor as described above.

7. Coated scaffold with Human periodontal ligament (hPDL) cell culture

7.1. Culture human periodontal ligament cells.

NOTE: The cells are isolated and cultured in our lab as per the protocol previously published[25].

7.2. Coat the collagen scaffold discs with 30 µL of PBS for the control group and the neuropeptide galanin (GAL) at a concentration of 10^{-8} overnight.

7.3. Seed human PDL cells on the control and GAL coated scaffold for 2 hours at 37 °C in a culture plate, and transfer the cell seeded scaffolds to a bioreactor vessel as mentioned above.

7.4. Culture the cell seeded scaffolds for 14 days in the bioreactor prior to harvesting the scaffolds for analysis.

8. Bioreactor cleaning and maintenance

8.1. After the scaffolds/cells are harvested from the bioreactor, remove the syringe ports from the vessel.

NOTE: The screws around the vessels are taken off and the vessel is gently washed with soap water.

8.2. Rinse the vessels with clean water and tighten the screws once more.

8.3. Store the vessels after autoclaving until next use.

REPRESENTATIVE RESULTS:

The inside chamber of the bioreactor provides an environment for the cells to proliferate and differentiate, attach to a scaffold or congregate to form tissue like mass. Each HARV vessel holds up to 10 mL of medium and facilitates a constant circulation of nutrients so that each cell has an excellent chance to survive. **Figure 1A** illustrates the attachment of the syringe ports to the front plate of the vessel where sterile one-stop valves are attached. These valves act as doorkeepers to the culture chamber. Medium change requires the stop cock valve to be opened to facilitate attachment of a sterile syringe containing fresh medium. The microgravity environment within the bioreactor allows cells to attach to the scaffold within the vessel without requiring prior seeding onto the scaffolds. The scaffold (**Figure 1B and 1C**) placed in the bioreactor rotates constantly, allowing for cells to attach to scaffold surfaces and form cytoskeletal networks. The oxygenator membrane at the bottom of the vessel plate (**Figure 1B**) allows for a constant gas exchange to improve cell survival and longevity.

Various scaffolds were tested to identify the scaffold most compatible with the cells. The scaffold in **Figure 2A and 2B** is a graphene sheet composed of 75% graphene and 25% PLG (poly-lactic glycolide). This electrically conductive scaffold is frequently used for cells that may require electrical stimulation, such as skeletal muscle cells[26]. In our studies, the collagen scaffold surpassed all other studies tested in terms of biocompatibility, promotion of tissue growth and cellular differentiation. The specialized porous surface of this collagen-based scaffold (**Figure 2C and 2D**) allows the cells and nutrients to flow throughout the scaffold, increasing the area of cell attachment and cell proliferation.

The position of the cervical loop in relationship to the mouse mandible is illustrated in **Figure 3A and 3B**. To prepare mouse incisor cervical loop cells, a skeletonized mouse mandible was dissected and the distal-most portion of the lower mouse incisor was exposed. The precise position of the resected cervical loop is demarcated in the 6 days postnatal mouse incisor (**Figure 3B**), while a similar region in an adult skeletonized mouse mandible is provided as a reference in **Figure 3A** for orientation purposes. The area of the corresponding cervical loop in the adult skeletonized (**Figure 3A**) and the 6 dpn freshly prepared mouse incisor is framed by two broken lines (**Figure 3A and B**). The hemi mandibles is comprised of three mandibular molars and a constantly growing incisor, while the cervical loop cell niche is comprised of a varied cell population necessary for enamel formation such as the inner enamel epithelium, outer enamel

epithelium, stellate reticulum and stratum intermedium[19].

Figure 4 focuses on the successful differentiation of the cervical loop cells into elongated, polarized, enamel protein secreting ameloblast-like cells. The data revealed that successful differentiation of elongated, polarized, enamel protein secreting ameloblast-like cells requires the co-culture of the cervical loop cells with mesenchymal stem cells such as dental pulp stem cells. The cells were provided with a tailored microenvironment as described in step 3 of the protocol, resulting in the formation of polarized cells with the nucleus at one end and a long cellular processes at the other[27], a typical characteristic of ameloblasts (**Figure 4A**). Application of media alone and without growth factors and/or scaffold coating resulted in cervical loop cells that secreted key enamel proteins but did not elongate or polarize (**Figure 4B**).

Galanin-coated and non-coated collagen scaffolds were placed in a bioreactor with hPDL cells for fourteen days in a bioreactor. The cells survived a two-week culture period in the 3D culture system and the galanin-coated scaffold group demonstrated a significantly higher proliferation rate (**Figure 5B**) as compared to the control group containing uncoated scaffolds (**Figure 5A**). The cells in the experimental group also demonstrated a significantly higher level of extracellular matrix containing connective tissue fibers as compared to the control.

The bioreactor environment proofed successful for the growth of lung segments in a 3D microgravity environment (**Figure 6**). For this study, lung tissue segments harvested from E15 mice (**Figure 6C**) were placed on a nitrocellulose membrane in an organ culture dish for two hours. Following initial tissue attachment to the membrane, the lung tissue/nitrocellular membrane composite was placed in the bioreactor vessel and was successfully cultured for 10 days (**Figure 6A**). To study the effects of inflammatory conditions on lung tissue growth, addition of IL-6 to the culture medium resulted in typical inflammation-associated changes in alveolar morphology similar to those seen *in vivo* (**Figure 6B**).

FIGURE AND TABLE LEGENDS:

Figure 1. Components of a bioreactor vessel. Figure 1 illustrates key components of a bioreactor vessel and their position at a pre-assembly stage. (**A**) Relative position of the reactors chamber, syringe ports, and the scaffold. (**B**) Half-open position of the front plate (clear cover) and the back plate (white plate covered by oxygenator membrane). The scaffold is positions in the center between front and back plate. The black-colored graphene scaffold (**C**) illustrates how a scaffold is placed within the vessel and used as a support for the cells to proliferate, differentiate and form a cellular network.

Figure 2. Representative illustrations of scaffolds tested in this study. Five scaffolds were tested for our bioreactor studies, of which two examples are presented here. (**A,B**) represent the graphene scaffold tested for the ameloblast-like cell growth while (C,D) represents collagen scaffold the scaffold most successful for our bioreactor based cell culture studies. Note the porous structure of collagen scaffold (**C,D**) versus the parallel array of surface embossments in the graphene scaffold (**A,B**). (**A,C**) are macrographs taken from a perpendicular perspective while (**B,D**) were imaged at a 45° angle.

Figure 3. Cervical loop preparation. The skeletonized adult mouse mandible in A demonstrates the cervical loop region used to prepare the cell niche ameloblast-like cells culture and differentiation. This macrograph also illustrates the position of anatomical markers, including the continuously growing incisor (inc) spanning almost the entire mandibular length, the first mandibular molar (m_1) along with the angle of the mandible, the coronoid process and the position of the mandibular condyle. This skeletal preparation serves as an anatomical orientation for the cervical loop region prepared in (**B**). Reference points include the mandibular bone (mand), dental papilla tissue (DP), the angle of the mandible (Angle) and the position of the cervical loop (CL) from which the cells for our ameloblast bioreactor studies were harvested. The broken line indicates the anterior and distal portion of the fenestrated mandibular window prepared to cervical loop dissection in the skeletonized adult mandible (**A**) and in the 6 dpn freshly dissected mandible (**B**).

Figure 4. Generation of ameloblast-like cells using a 3D co-culture approach. (**A**) Successful differentiation of ameloblast-like cells from cervical loop cells co-cultured with dental pulp stem cells in a suitable microenvironment. The resulting cell population was comprised of long, elongated, polarized cells with the ability to secrete enamel matrix proteins. These cells featured a nucleus at one end (nucl) and a cellular process (proc) resembling Tomes Process as observed in true ameloblasts at the other end (**A**). (**B**) illustrates a population of control group cells that were also subjected to co-culture conditions but with media free of growth factors or differentiation agents, resulting in rounded cells not representative of typical ameloblasts.

Figure 5. Long term 3D culture of a single cell periodontal progenitor (pdl) population with on coated and non-coated scaffold surfaces. Scaffold coating resulted in an increase in the hPDL cell proliferation rate in cells cultured on the galanin-coated scaffold (**B**) compared to the control group cells exposed to a non-coated scaffold (**A**). The cells from experimental group also secreted more extracellular matrix containing connective tissue fibers compared to the control group (B versus A).

Figure 6. Lung tissue culture in a bioreactor. (**A**) E15 lung tissue segments cultured on a nitrocellulose membrane in a bioreactor for ten days. (B) E15 lung tissue segments similar to (**A**) but subjected to inflammatory conditions by adding IL-6 to the media (**B**). (**C**) Paraffin section of a freshly dissected E15 lung tissue stained with H&E. Note similarities in alveolar type I and type II cell morphologies when comparing (**A**) and (**C**).

DISCUSSION:

Critical steps of the protocol for the growth of cells in microgravity include the bioreactor, the scaffold, the cells used for 3D culture, and the scaffold coating as a means to induce cell differentiation. The type of bioreactor used in our studies comprises the RCCS-4 bioreactor, a recent modification of the original Rotary Cell Culture System (RCCS) rotating cylindrical tissue culture device developed by NASA to grow cells in simulated microgravity. This RCCS-4 environment provides extremely low shear stresses, which enhances mass transfer and improves culture performance. This version of the bioreactor allowed for the simultaneous use of four

vessels for four different experiments at the same time. The RCCS-4 bioreactor was equipped with high aspect ratio (HARV) vessels, which ensured simple and straight forward culture conditions with a sufficient number of cells for our studies.

A second critical component of the approach is the scaffolds as they provide templates for floating cells to attach and form assemblies. While the use of scaffolds in 2D culture is limited due to the formation of necrotic cores, the enhanced diffusion, oxygen and nutrient flow in a rotary bioreactor improves upon the applicability of scaffolds as carriers for the propagation of cell assemblies[28,29]. In the present study we explored the efficacy of five types of scaffolds, the poly(lactic-co-glycolic acid (PLGA) scaffolds, a collagenous and porous scaffold, a graphene scaffold, as well as a gelatin disc and a hydrodroxyapatite disc. In addition, we transferred the membrane from the lung organ preculture environment into the bioreactor, with the cultured lung segment attached to it. Among these, the collagen scaffold emerged as the most favorable scaffold in our studies, possibly due to its collagenous nature and porous structure. The PLGA scaffold was also viable, while the other three scaffolds were less favorable in our hands. The nitrocellulose membrane of the original lung organ culture system proofed to be another effective scaffold as it successfully maintained the integrity of the cultured lung after transfer into the 3D bioreactor environment.

A third critical component of the strategy are the types of cells used to seed the vessel. For our amelogenesis studies we relied on cervical loop cells prepared from the continuously growing mouse incisors that were co-cultured with dental pulp cells. Cervical loop cells were chosen as the source of the original enamel organ progenitors that are continuously renewed in the rodent incisor. The mouse or rat incisor is a remarkable source of stem and progenitor cells that continues to exist throughout the life of the animal while the cells of the enamel organ are replenished for continuous eruption and amelogenesis[23,30]. Both periodontal progenitor cells and dental pulp cells were used as co-culture cell sources. The use of mesenchymal co-culture cell populations for the successful culture of epithelial cells is well established[31,32]. In our studies, dental pulp cells were more effective in inducing ameloblast differentiation than periodontal ligament progenitors even though based on their mesenchymal characteristics, both are suitable as odontogenic and mesenchymal co-culture candidates. When applied toward amelogenesis, dental pulp cells as the natural counterpart to odontogenic epithelium during tooth development might have triggered the appropriate epithelial-mesenchymal interactions suitable for the induction of terminal ameloblast differentiation[33,34]. However, for the study of scaffold interactions for periodontal tissue engineering, periodontal progenitors were ideally suited as they give rise to fully differentiated periodontal ligament fibroblasts[25,35]. Finally, for the culture of lung organs in a bioreactor environment, we relied on dissected embryonic murine lung segments. Procedures to culture embryonic lung organs in organ culture dishes have been described earlier[36] and a number of two-dimensional cell culture models combining lung epithelial cells with vascular or smooth muscle cells have been explored[37-39]. In the present study, the 3D bioreactor model maintained a robust level of surfactant secretion while preserving the integrity of the core of the cultured tissue block, rendering this model suitable for the study of environmental effects on lung tissue integrity.

The fourth significant aspect of our model is the application of cell-type specific coatings on the scaffold surfaces to trigger ameloblast-like cell differentiation. Specifically, components such as LRAP and enamel matrix emerged as a key contributing factors toward ameloblast-like cell differentiation as a lack of coating with LRAP and initial enamel matrix prohibited the formation of elongated and polarized cells. Together, the coating of scaffold surfaces provides a powerful tool to promote the tissue-specific differentiation of complex organs in bioreactors.

The most significant aspect of this study was the ability to restore the distinctive elongated and polarized cell shape of ameloblasts. This outcome is a unique benefit of the 3D bioreactor system over the limitations of 2D culture systems that highly rounded enamel-organ derivative cells. This outcome provides further evidence for the benefit of using bioreactor technologies when culturing cells incompatible with other culture technologies[40]. We attribute the success of the ameloblast co-culture studies to several unique attributes of the rotatory bioreactor system, including the continuous supply of nutrients, growth and transcription factors, and oxygen, as well as the ability of individual cells to aggregate and form social interactions between cells of various lineages and developmental stages. While the studies succeeded in growing elongated, polarized, and amelogenin secreting ameloblast-like cells, the ameloblast-like cells grown here remain in isolation, and the natural continuity of the ameloblast cell row was lost. In future applications, ameloblast-like cells grown with this technology might be used for enamel tissue engineering applications or as an experimental model to recapitulate aspects of tooth amelogenesis.

In conclusion, the 3D bioreactor emerged as a successful environment for the propagation of cervical loop/dental pulp co-cultures into ameloblast, for the growth of periodontal ligament progenitors in coated scaffolds and for the culture of entire lung organs. Based on these data, bioreactor-based technologies are likely to emerge as important vehicles for advanced tissue engineering or testing strategies in areas such as tooth enamel, periodontal, and lung research.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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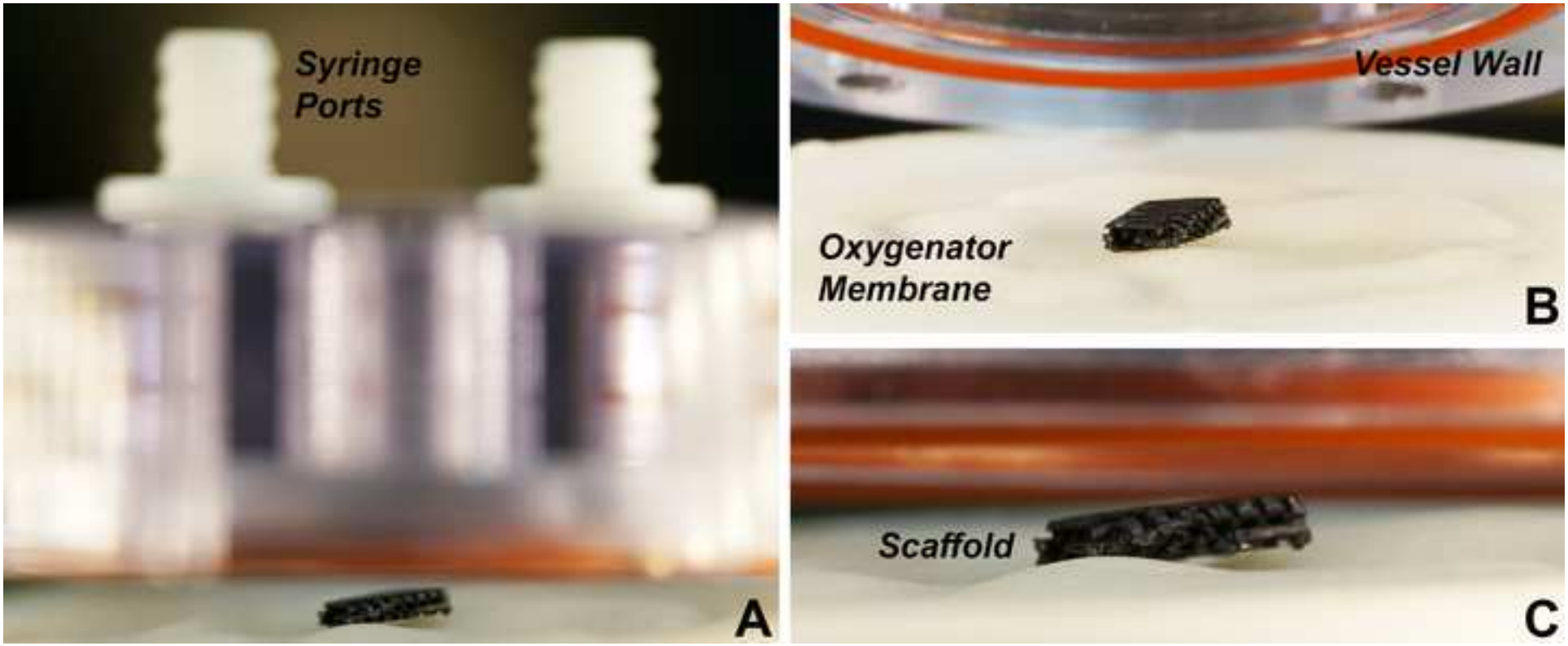
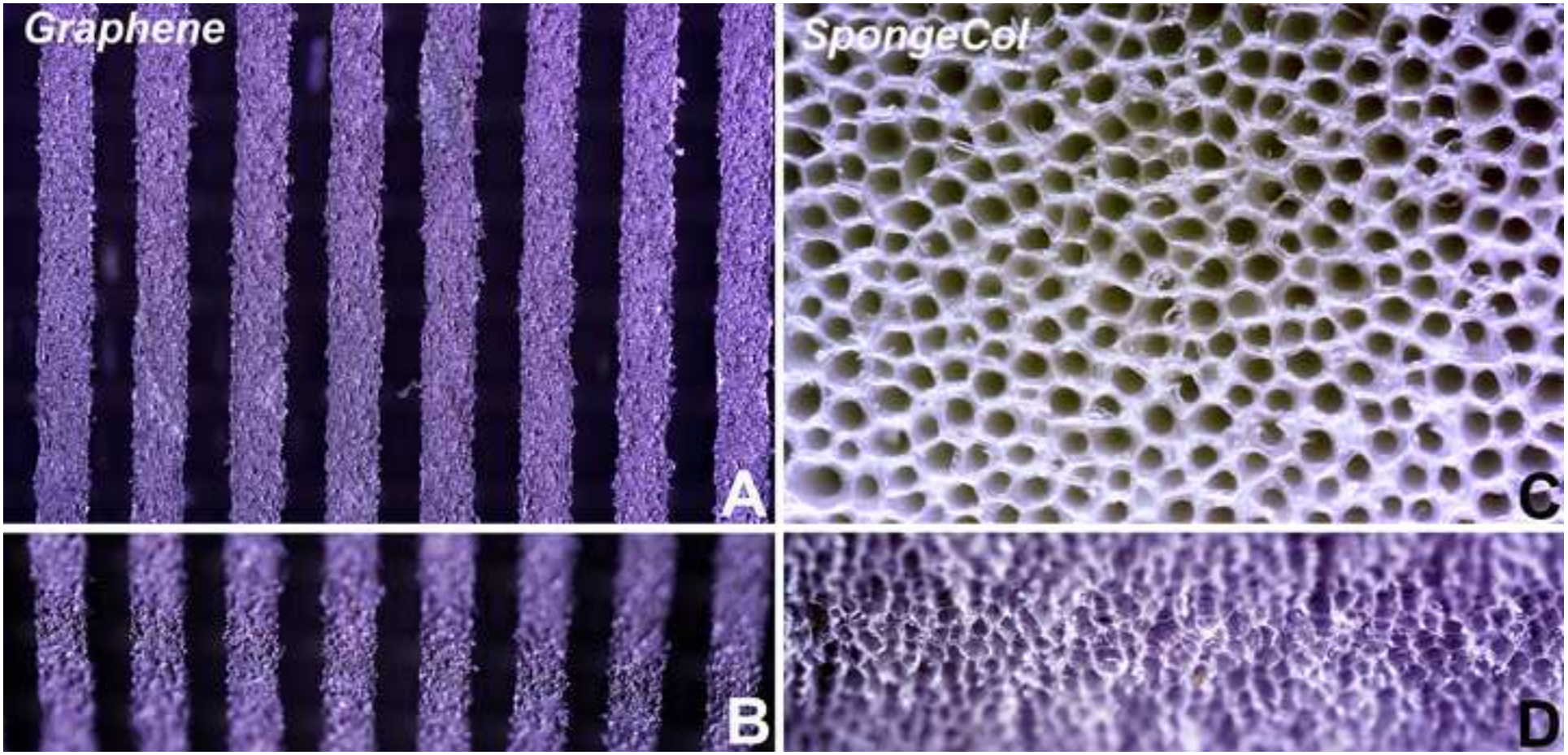
Figure

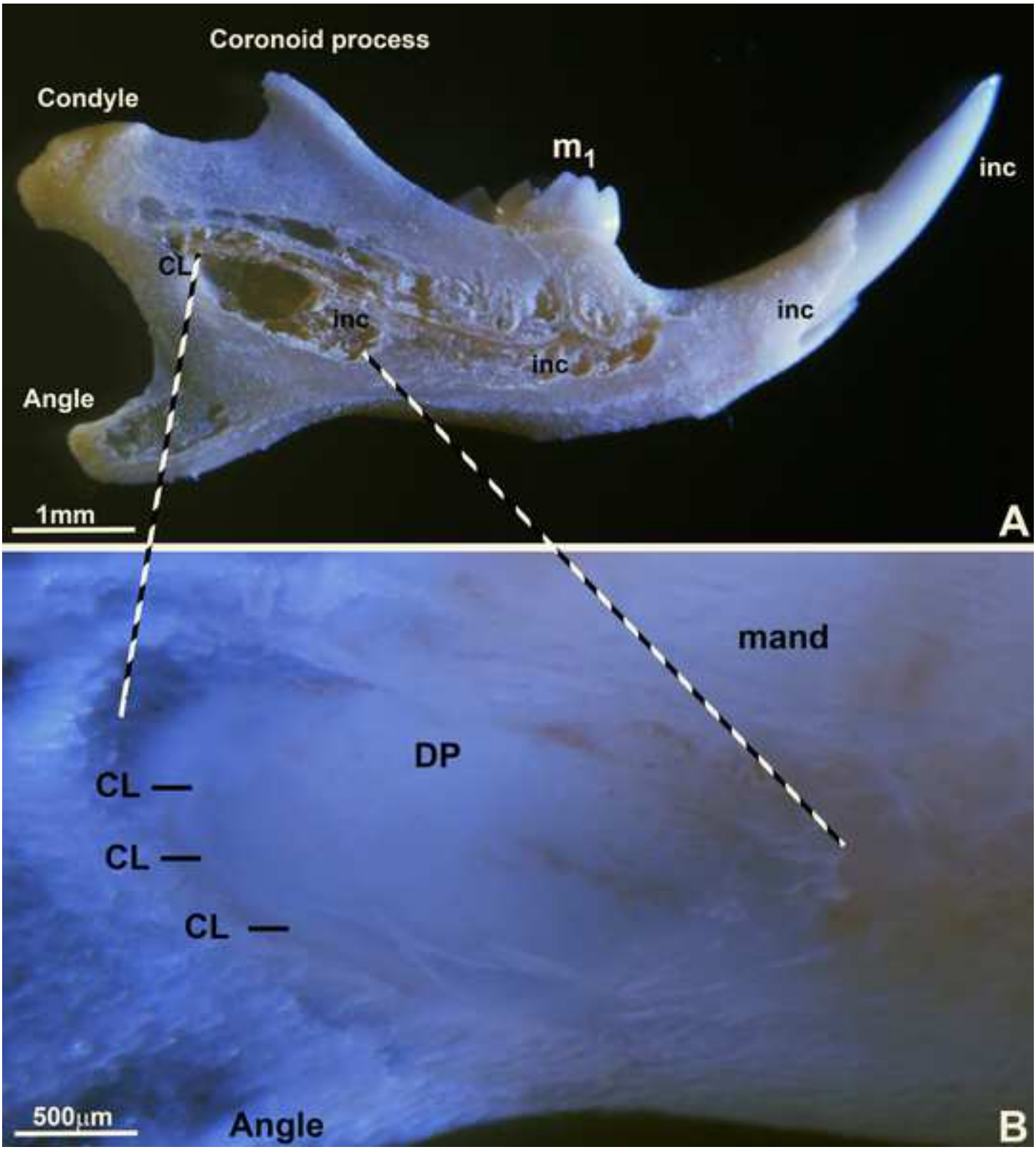


Figure

Click here to access/download;Figure;Figure 2 Surfaces L.tif

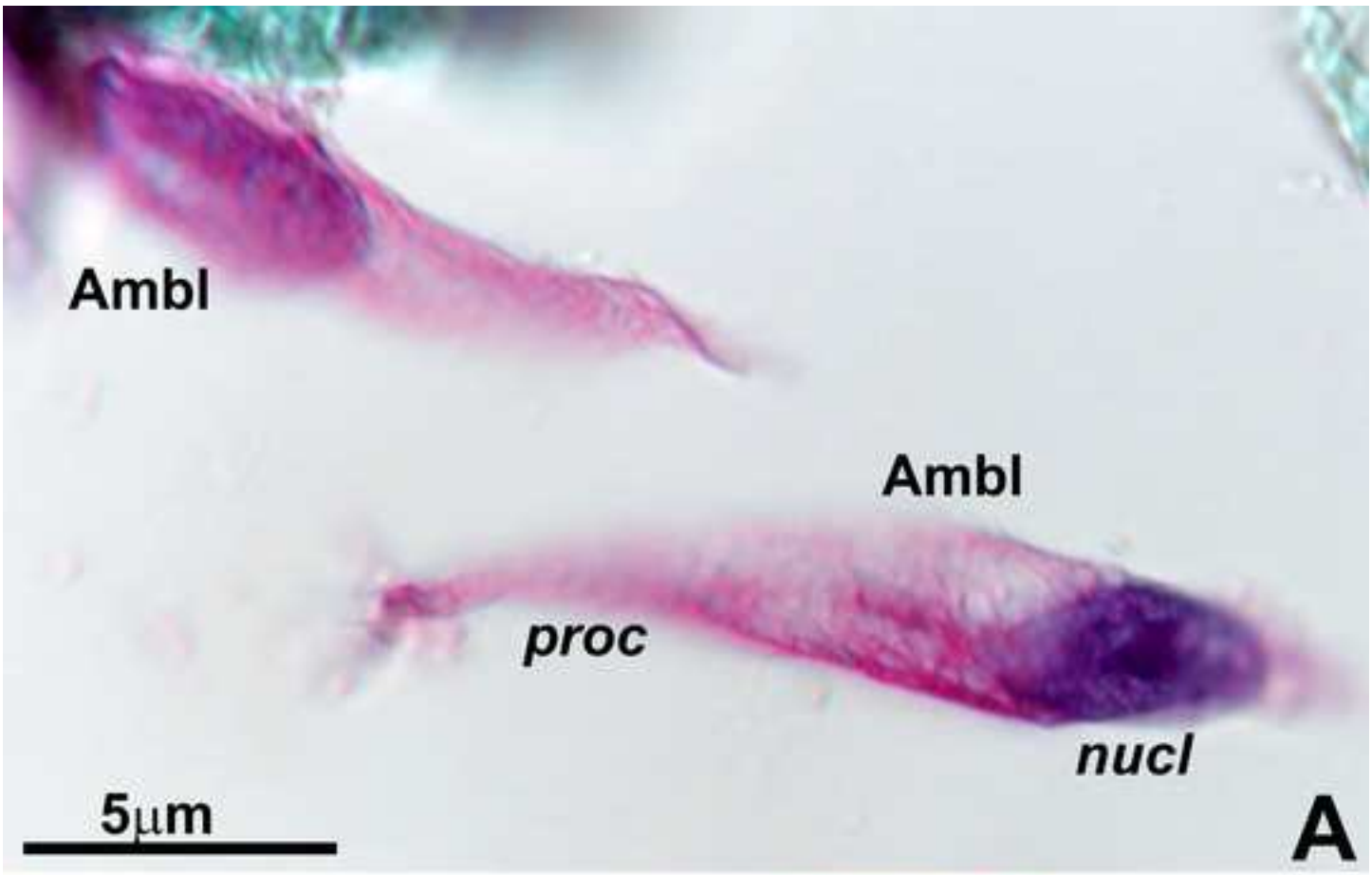


Figure



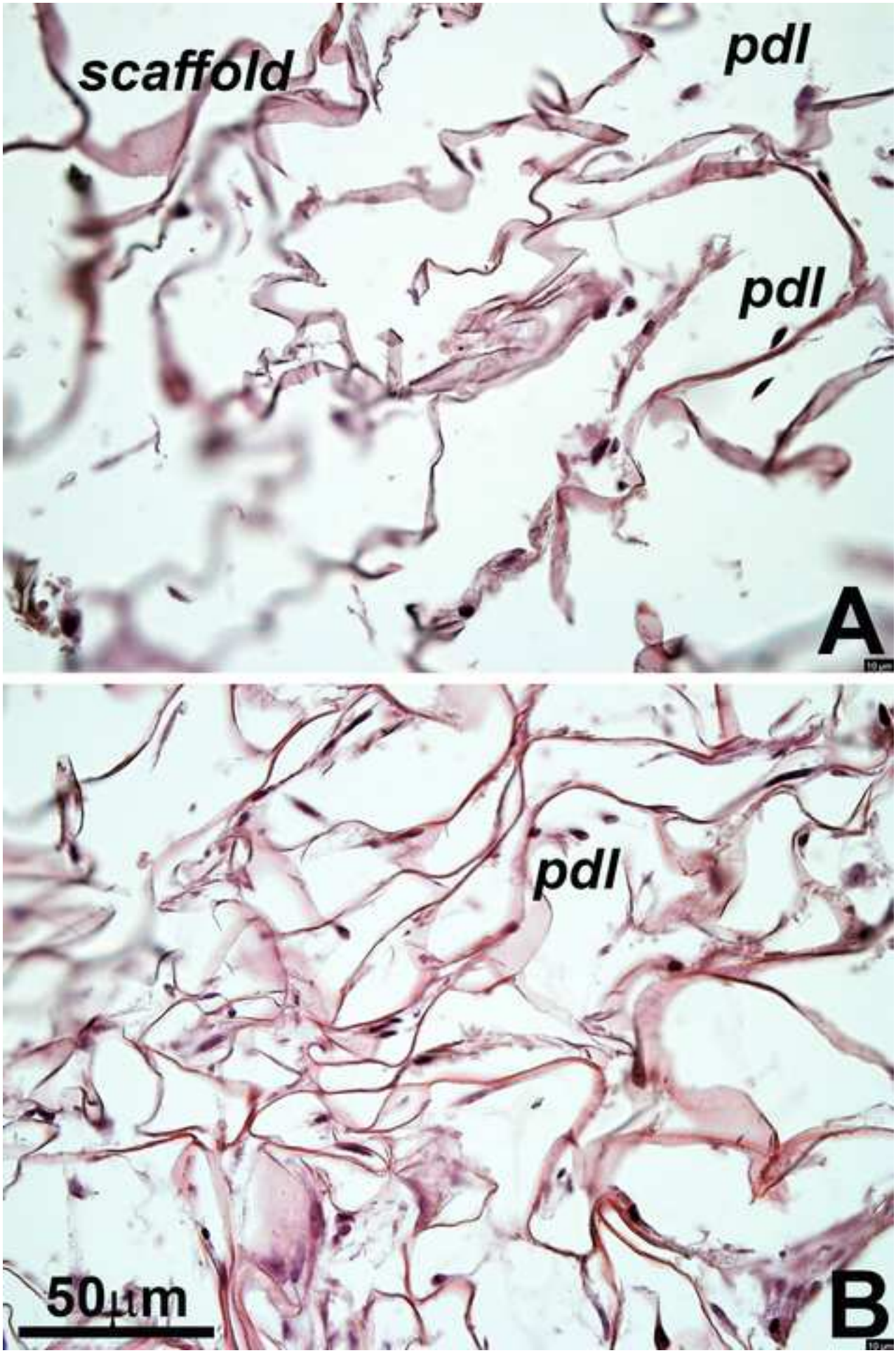
Figure

Click here to access/download;Figure;Figure 4 Cervical Loop L.tif



Figure

Click here to access/download;Figure;Figure 5 PDL cells SpongeCol Galanin L.tif



Figure

Click here to access/download;Figure;Figure 6 Lung Tissue L.tif

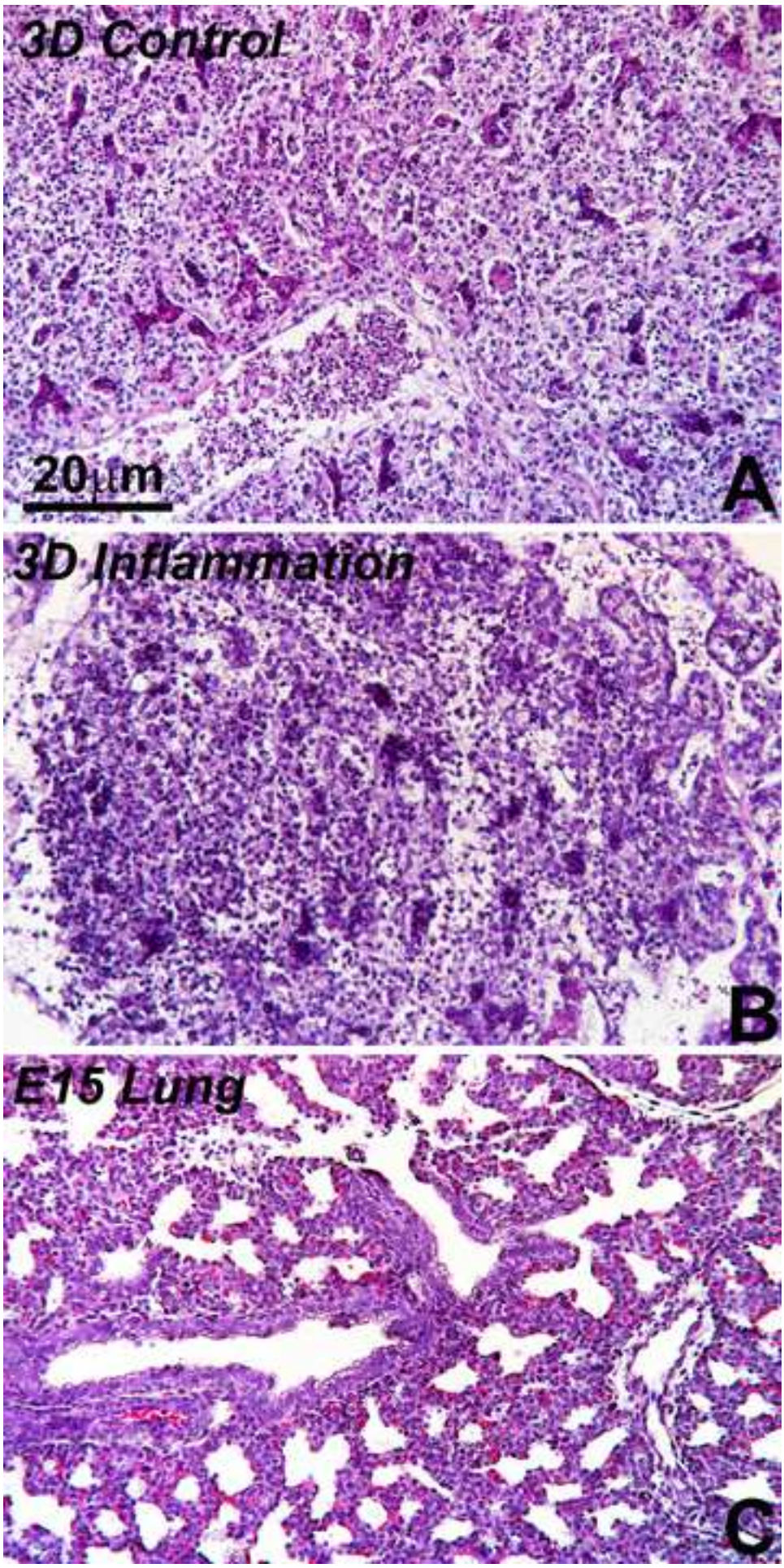


Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Antibiotic-antimycotic	ThermoFisher Scientfic	15240096
Ascorbic Acid	Sigma Aldrich	A4544
BGJb Fitton-Jackson Modification m	ThermoFisher Scientfic	12591
BIOST PGA scaffold	Synthecon	Custom
BMP-2	R&D Systems	355-BM
BMP-4	R&D Systems	314-BP
DMEM Media	Sigma Aldrich	D6429-500mL
FBS	ThermoFisher Scientfic	16140071
Fibricol	Advanced Biomatrix	5133-20mL
Fibronectin	Corning	354008
Galanin	Sigma Aldrich	G-0278
Gelatin disc	Advanced Biomatrix	CytoForm 500
Graphene sheets	Advanced Biomatrix	CytoForm 300
hEGF	Peprotech	AF-100-15
hFGF	ThermoFisher Scientfic	AA1-155
Hydroxyapatite disc	Advanced Biomatrix	CytoForm 200
Il-6 protein	PeproTech	200-06
Keratinocyte SFM media (1X)	ThermoFisher Scientfic	17005042
Laminin	Corning	354259
LRAP peptide	Peptide 2.0	Custom made sequence: MPLPPHPGSPGYINLSYEVLTPLKWY QSMIRQPPLSPILPELPLEAWPATDKT KREEVD
Matrigel	Corning	354234
Millipore Nitrocellulose membrane	Merck Millipore	AABP04700
RCCS Bioreactor	Synthecon	RCCS 4HD
SpongeCol	Advanced Biomatrix	5135-25EA
Syring valve one way stopcock w/swivel male luer lock	Smiths Medical	MX5-61L
Syringes with needle 3cc	McKESSON	16-SN3C211
Trypsin EDTA (0.25%)	ThermoFisher Scientfic	25200056

Comments/Description

Available from the company through a custom order

Texas A&M University College of Dentistry
Department of Periodontics

05/07/2021

Vineeta Bajaj, Ph.D.
Review Editor, JoVE

Revision of our manuscript, JoVE62690 "Growing Cells and Tissues in Microgravity: Cervical Loop, Ameloblasts, Periodontal Progenitors, and Lung Alveoli"

Dear Dr. Bajaj,

Thank you very much for your thorough review of our manuscript. We appreciate your and the reviewer's comments and have now revised the manuscript according to your suggestions. Please find below our response to individual concerns.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have now thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please revise the title to be more concise and avoid punctuations.
We have now revised the title to read "Propagation of dental and respiratory cells and organs in microgravity"

3. Please provide an email address for each author.
These have now been provided.

4. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "This protocol presents…"
We have now rephrased the summary according to your guidance.

5. Please define all abbreviations during the first-time use.
We have now introduced abbreviations at the time of first-time use.

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Synthecon RCCS bioreactor (Synthecon, Houston, TX), Advanced Biomatrix, SpongeCol, Millipore, Fitton-Jackson, etc.
We have now removed all commercial language from the manuscript and placed these in the Table of Materials and Reagents.

7. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
We have now used your recommended format.

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8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
We have modified the protocol text to match the imperative tense style.

9. The Protocol should contain only action items in complete sentences that direct the reader to do something.
We have now modified the protocol accordingly.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
We have now made sure that the protocol only contains discrete steps.

11. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
We have now added additional detail to address answers to "how" questions.

12. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
We have now highlighted 3 pages for the filmable content.

13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
We have not reused any figures.

14. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
The four critical steps of our protocol have now been described and discussed in the first four paragraphs.
b) Any modifications and troubleshooting of the technique
These are included in the discussion of the four critical steps.
c) Any limitations of the technique
d) The significance with respect to existing methods

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Limitations and significance over existing methods have now been mentioned in paragraph 5 of the discussion.

e) Any future applications of the technique

These are no listed in paragraph 6 of the discussion.

Reviewers' comments: Reviewer #1:

Manuscript Summary: In this manuscript, Pandya et al., describes the utilization of rotary cell culture bioreactor and showed specific applications for different cell types.

Major Concerns:

* In general, I expected some material to in video format because the journal is particularly suitable for such media. I recommend authors to use this availability, if possible, which would communicate their protocol better.

We will submit video format material as a second step following the manuscript.

* For the microgravity protocols used for tissue engineering, I advise authors to mention work from RPM bioreactors (numerous to select from, perhaps include 3D Clinostats as well) and also novel magnetic levitation studies (with scaffold: https://doi.org/10.1002/adhm.201500092; scaffold-free: https://doi.org/10.1002/bit.27631 AND https://doi.org/10.1038/s41598-018-25718-9)

We have now also mentioned work from RPM bioreactors and magnetic levitation studies.

* Information for the scaffolds used in the study comes at the results section, unexpectedly. It might be beneficial to dedicate a short paragraph in the introduction.

We have now added information about the scaffolds used in the introduction.

Minor Concerns:

* I failed to see the reason for highlighting the odd protocols.

Highlighting was performed to identify relevant section for the video script.

* In the results section bioreactor is introduced as "RCCS-4D" though it is not clear if this refers to a model or a specific working mode. The Table of Materials section also does not have the information. I suggest giving a brief description.

RCCS-4D is the name of the model. This is listed in the Table of Materials section.

* In results 1 section, authors argue that scaffolds allow "for cells to attach to scaffold surfaces and form cytoskeletal" networks. Alterations in the cellular cytoskeleton is not directly shown in the study, therefore I would either use citations to the claim, or revise.

We have now listed references in support of our claim.

* In results 5 section, authors mention "a significantly higher level of extracellular matrix", a statement that generally requires quantification and statistical tests.

We have now removed this statement as it is part of another study.

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Reviewer #2:

I have agreed to review this manuscript because I was intrigued by the title, but already reading the abstract, and even more the text, I found something different from what I expected.

We apologize if this reviewer found the title misleading. We have now revised the title of the manuscript.

It is not clear what the authors pursue: to study the effects of microgravity or to create 3D cultures for in vitro studies?

I think I understand that the authors propose the RCCS bioreactor as a tool for the establishment of 3D cultures and their characterization. The authors' study proposal could be interesting, even if not entirely new, but formulated in a confused way and hidden by many arguments that are not always pertinent and sometimes incomplete.

This manuscript is written to follow the format of a JoVE Methods paper as required by the Journal guidelines. The technology described in the manuscript serves as a means for readers to generate 3D cultures in microgravity for the study of unique cell populations in a gravity-free environment.

In my opinion, the manuscript should be re-written in order to focus and emphasize the real aim of the authors. The authors showed two models but their usefulness is not clearly described. The description of results is not always supported by the data shown in the figures. For example the description of results coming from fig. 5 (lines 254-259) are not supported by only the images, the author wrote: ..... The cells survived a two-week culture period in the 3D culture.....(are there vitality assay?)..... the galanin-coated scaffold group demonstrated a significantly higher proliferation rate....(are there proliferation curves and statistical analyses?)..... The cells in the experimental group also demonstrated a significantly higher level of extracellular matrix containing connective tissue fibers....(are there a specific staining?).

These are excellent suggestions. The author team is afraid that they go beyond the scope of JoVE, which by default focuses on establishing and sharing Methods with their readers. However, these are outstanding ideas for future studies.

Some suggestions.

If the aim is to establish 3D cultures, the description of the effects induced by microgravity should be avoided, also because the functioning principle of RCCS is to create a free-fall environment simulating microgravity and allowing cells to interact to each other or to scaffolds. They presented two models: dental-derived cell co-cultures in presence of scaffolds and lung organ cultures. The use of this second model is only roughly described. The quality of the images in the figures (for example fig. 1 and 4, but not only) should be improved. In particular the fields acquired appeared to be out of focus. The English language and style should be accurately revise also to avoid typing errors.

Micrographs are in focus and highlight an optical cross-section through a 3-dimensional environment. We have now thoroughly revised the use of language and style.

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We appreciate the thorough review of our manuscript and look forward toward your response.

Sincerely,

Thomas G.H. Diekwisch, DMD, Ph.D., Ph.D.

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