Journal of Visualized Experiments

Extra Cellular Matrix-Based and Extra Cellular Matrix-Free Generation of Murine Testicular Organoids

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61403R2
Full Title:	Extra Cellular Matrix-Based and Extra Cellular Matrix-Free Generation of Murine Testicular Organoids
Section/Category:	JoVE Bioengineering
Keywords:	testis; testicular organoid; extracellular matrix; 2D; 3D; assembly; tubule; endocrine function
Corresponding Author:	Maxwell E Edmonds Northwestern University Feinberg School of Medicine Chicago, IL UNITED STATES
Corresponding Author's Institution:	Northwestern University Feinberg School of Medicine
Corresponding Author E-Mail:	maxwell.edmonds@northwestern.edu
Order of Authors:	Maxwell E. Edmonds
	Micah D. Forshee
	Teresa K. Woodruff
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Chicago, Illinois, United States

Cover Letter

NORTHWESTERN UNIVERSITY FEINBERG SCHOOL OF MEDICINE

Northwestern University
Feinberg School of Medicine

Department of Obstetrics and Gynecology
303 East Superior Street
Room 10-119
Chicago, Illinois 60611

Teresa K. Woodruff, PhD
Thomas J. Watkins Professor of Obstetrics and Gynecology
Vice Chair of Research
Dept of Obstetrics and Gynecology
Director, Oncofertility Consortium
Director, Center for Reproductive Research
Director, Women's Health Research Institute
Professor of Molecular Biosciences

tkw@northwestern.edu
Phone 312-503-2503
Fax 312-503-0219

feinberg.northwestern.edu
www.woodrufflab.org
oncofertility.northwestern.edu
womenshealth.northwestern.edu

NORTHWESTERN UNIVERSITY

February 27, 2020

Kyle Jewhurst, Ph.D.
Science Editor
JoVE

Dear Dr. Jewhurst:

I am enclosing our manuscript entitled: "**ECM and ECM-free Methods for Generating Murine Testicular Organoids,**" that we are submitting to *JoVE* for consideration as a methods paper.

The application of bioengineering, biomaterial, and biofabrication methods towards the reconstruction of *de novo* testicular tissue is relatively new for the study of male gonadal biology and fertility. Specifically, testicular organoids have seen a quick escalation in research interest over the past two years. Previously, our group has pioneered an expertise in the engineering of female gonadal tissues, the female reproductive tract, and its reproductive endocrine axis *in vitro* (*Laronda et al, Biomaterials, 2015; Shuo et al, Nature Communications, 2017; Laronda et al, Nature Communications, 2017; Xiao et al, Toxicological Sciences, 2017*). In the current article and recent work (Edmonds, 2020, *in review)*, we have expanded our purview to the testis, in exploring fundamental paradigms within testicular tissue self-assembly as organoids. In this methods manuscript, we have directly compared testicular organoid generation from murine primary cells between ECM-free and ECM culture methods, in 2D and 3D, and directly compared them for outcomes across three predetermined tissue model benchmarks: (a) cellular self-assembly, (b) the inclusion of major cell types (Sertoli, Leydig, germ, and peritubular cells), and (c) appropriately compartmentalized tissue architecture.

With the methods detailed in this manuscript, the reader will have four different culture techniques available to them for assembling testicular organoids in both ECM and ECM-free environments. Importantly, all four methods allow the researcher to non-invasively observe organoid self-assembly over time through time-lapse imaging or video recording, and to noninvasively collect conditioned media for analysis of secreted hormones and cytokines, without disturbing organoids in culture. Furthermore, we demonstrate the utility of 2D ECM and 3D ECM-free models for studying *de novo* morphogenesis of tubule-like structures exhibiting multiple hallmarks of native seminiferous tubules. Additionally, organoids exhibited long-term durable endocrine function, productive of testosterone and inhibin B hormones over 12-weeks of culture, with preserved differential responses to gonadotropin hormone stimulation. Collectively, these data demonstrate that testicular organoids provide a robust model for studying testicular tissue morphogenesis and development and might soon provide a viable research tool for studying *in vitro* spermatogenesis, developing future assisted reproductive technologies, and designing new drugs and contraceptives. Detailed and accessible methods publications will allow for these translational developments to be universally disseminated across the reproductive science community, through which we hope they will increase the field's ability to achieve next-generation advances in biomedical discovery and innovation.

We believe our work on testicular organoid generation, with a focus upon direct comparisons and functional outcomes, is an ideal fit for *JoVE* and will be of broad interest to your readership. All animal research was performed under appropriate regulatory approval and conducted according to regulation. None of the authors have financial disclosures and all of the authors have contributed substantially to this manuscript and approved its submission.

We look forward to your timely and favorable consideration of our work. Please do not hesitate to contact me if you have any questions or require additional information.

Sincerely,

Teresa K. Woodruff, PhD

TITLE:

Extra Cellular Matrix-Based and Extra Cellular Matrix-Free Generation of Murine Testicular Organoids

AUTHORS AND AFFILIATIONS:

Maxwell E. Edmonds[1], Micah D. Forshee[1], Teresa K. Woodruff[1]

[1]Department of Obstetrics and Gynecology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

Corresponding author:
Teresa K. Woodruff (tkw@northwestern.edu)

Email addresses of co-authors:
Maxwell E. Edmonds (maxwell.edmonds@northwestern.edu)
Micah D. Forshee (micah.forshee@bcm.edu)

KEYWORDS:

testis, testicular organoid, extracellular matrix, 2D, 3D, assembly, tubule, endocrine function

SUMMARY:

Here, four methods for generating testicular organoids from primary neonatal murine testicular cells are described i.e., extracellular matrix (ECM) and ECM-free 2D and 3D culture environments. These techniques have multiple research applications and are especially useful for studying testicular development and physiology in vitro.

ABSTRACT:

Testicular organoids provide a tool for studying testicular development, spermatogenesis, and endocrinology in vitro. Several methods have been developed in order to create testicular organoids. Many of these methods rely upon extracellular matrix (ECM) to promote de novo tissue assembly, however, there are differences between methods in terms of biomimetic morphology and function of tissues. Moreover, there are few direct comparisons of published methods. Here, a direct comparison is made by studying differences in organoid generation protocols, with provided outcomes. Four archetypal generation methods: (1) 2D ECM-free, (2) 2D ECM, (3) 3D ECM-free, and (4) 3D ECM culture are described. Three primary benchmarks were used to assess the testicular organoid generation. These are cellular self-assembly, inclusion of major cell types (Sertoli, Leydig, germ, and peritubular cells), and appropriately compartmentalized tissue architecture. Of the four environments tested, 2D ECM and 3D ECM-free cultures generated organoids with internal morphologies most similar to native testes, including the de novo compartmentalization of tubular versus interstitial cell types, the development of tubule-like-structures, and an established long-term endocrine function. All methods studied utilized unsorted, primary murine testicular cell suspensions and used commonly accessible culture resources. These testicular organoid generation techniques provide a highly accessible and reproducible toolkit for research initiatives into testicular organogenesis

and physiology in vitro.

INTRODUCTION:

Testicular organoids are a pioneering technique for studying testicular development, spermatogenesis, and physiology in vitro[1–4]. Several methods have been explored for organoid generation; these include a variety of extracellular matrix (ECM) and ECM-free culture systems, in both two-dimensional (2D) and three-dimensional (3D) orientations. Different generation methods can promote distinct cellular assembly strategies; this results in a high level of morphological and functional variability between published organoid models. The purpose of this article is to discuss the current state of in vitro testicular models, and to serve as a template for future investigators, when designing testicular organoid experiments. Within the present study, four different culture system archetypes are defined and characterized in experimental process and biological outcome. These include: 2D ECM-Free, 2D ECM, 3D ECM-Free, and 3D ECM culture methods. The strategies presented herein are intended to be simple, accessible, and highly reproducible between different laboratories and research groups.

Historically for the testis, the designation “in vitro”, has been used for several different culture methods of testicular tissues and cells. These include organotypic tissue/organ culture methods (i.e., explant culture)[5], isolated seminiferous tubule culture[6], testicular cell culture[7], and methods of de novo tissue morphogenesis (i.e., biological constructs and organoids)[1]. The first investigations into in vitro spermatogenesis were performed approximately 100 years ago, with the culture of rabbit testis explants in 1920[8], and later in 1937 with mouse explants[9]. Within these initial experiments spermatogonia were observed to largely degenerate across the first week of culture, though some meiotically differentiating cells were identified. Reminiscent of these historical reports, testis explant culture was revived and optimized in 2011 to become a feasible technique for studying the testis[10]. Since 2011, explant culture has produced fertility competent sperm in multiple reports[11–13]. Yet, due to explant culture’s reliance upon pre-existing native testis tubules, these recent advances are more accurately described as examples of “ex vivo” testicular function and spermatogenesis, tissue function that was maintained or resumed upon removal from an organism’s body. Despite its prevalence in the literature, long-term germ cell maintenance and differentiation within testicular explants is challenging to replicate[14–18], especially over timeframes long enough to fully observe in vitro spermatogenesis (~35 days in mice[19] and 74 in humans[20]). It is intriguing to appreciate that many of the same challenges experienced 100 years ago, are still experienced within ex vivo spermatogenesis today.

Different than ex vivo approaches, testicular organoids are de novo assembled microtissues generated entirely in vitro from cellular sources (i.e., primary testicular cells). Testicular organoids provide a creative strategy to circumvent the field’s historical reliance upon pre-existing native tissue, and to recapitulate testicular biology completely in vitro. There are multiple requirements shared by most organoid tissue models; these include (1) in vivo-mimetic tissue morphology or architecture, (2) multiple major cell types of the represented tissue, (3) self-assembly or self-organization in their generation, and (4) the ability to simulate some level of the represented tissue’s function and physiology[21–24]. For the testis, this can be captured in four major hallmarks: (1) the inclusion of major testicular cell types, germ, Sertoli, Leydig, peritubular,

and other interstitial cells, (2) cell-directed tissue assembly, (3) appropriately-compartmentalized cell types into separate tubular compartments (germ and Sertoli) and interstitial regions (all other cell types), and (4) some degree of tissue function (e.g., reproductive hormone secretion or tissue responses, and germ cell maintenance and differentiation). Considering the historic challenges in maintaining germ cell differentiation ex vivo and in vitro, the recapitulation of in vivo-mimetic testicular architectures (i.e., structures resembling seminiferous tubules) with additional markers suggesting simulation of testicular physiology (e.g., endocrine function), are priority milestones towards generating organoids which might one day sustain in vitro spermatogenesis.

The majority of published testicular organoid methods take advantage of commercially available extracellular matrices (ECM, e.g., collagen or proprietary ECM formulations)[25–27] or custom-sourced ECMs (i.e., decellularized testis ECM-derived hydrogels)[28–30]. Exogenous ECM promotes de novo tissue formation through providing an assembly-supportive scaffold for tissue generation. ECM methods have afforded an impressive level of tissue formation, including some germ cell presence and tissue-mimetic morphology[25,28]. However, the ECMs they utilize are not always universally available (i.e., decellularized ECM-derived hydrogels), and some methods require sophisticated gel and cell seeding orientations (e.g., 3-layer gradients of ECM and 3D printing)[25,31,32]. Scaffold-free methods (e.g., hanging drop and nonadherent culture plates)[33–35] have also generated robust and highly reproducible organoids without the need of ECM gels or scaffolds. However, the tissue morphology of these scaffold-free organoids is often dissimilar to in vivo testes, and most of these reports incorporate a biochemical ECM additive to promote tissue formation[33,34,36], or alternatively, rely upon centrifugation for forced cell aggregation and compaction[34], making them less ideal for studying cell-directed migration and self-organization.

The four organoid generation methods presented in this manuscript include both ECM-dependent and independent strategies, each using simple cell seeding that enables the observation of cell-driven organoid self-assembly. All four techniques can be performed from the same cell suspensions or can make use of custom and cell-type enriched populations. A strength of these methods is the ability to observe organoids self-assemble in real-time, and to directly compare how testicular structures self-assemble between different culture microenvironments. The phenotypic differences between these four culture methods should be considered for their impact on the research question or subject of the investigator. Each method produces biological constructs or organoids within 24 h or less. In conclusion, the methods presented here provide a toolkit of organoid assembly techniques for studying testicular organoid assembly, tissue development, and testicular physiology in vitro.

PROTOCOL:

All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Northwestern University, and all procedures were performed under IACUC-approved protocols.

1. Preparation of enzymatic tissue-dissociation solutions

1.1. Use two different enzymatic solutions (Solution 1 and Solution 2), both made using a basal culture medium solution (BM).

1.2. To prepare BM, add serum and penicillin-streptomycin to minimum essential medium to final concentrations of 10% and 1% respectively (see **Table of Materials** for specific reagents). Then sterile filter the BM through a 0.22 µm filter. Before use with cells, pre-equilibrate sterile BM to a neutral pH by dispensing into a culture dish and placing within a humidified, 5% CO_2 incubator at 37 °C for a minimum of 1 h.

NOTE: BM can be stored at 4 °C for up to 1 week, after which fresh BM should be made.

1.3. To prepare collagenase I stock solutions, first dissolve 100 mg of collagenase I into 1 mL of sterile embryo grade H_2O (final concentration 10% m/v), invert or swirl to dissolve, and store 20 µL aliquots at -20 °C for later use. Aliquots should be thawed only once.

1.4. To prepare deoxyribonuclease I (DNase I) stock solutions, add 20 mg of DNase I into 1 mL of sterile embryo grade H_2O (final concentration 2% m/v), invert or swirl to dissolve (do not vortex), and store 20 µL aliquots at -20 °C for later use. Aliquots should be thawed only once.

1.5. For hyaluronidase stock solutions, add 30 mg into 1 mL of sterile phosphate buffered saline (PBS; final concentration 3% m/v hyaluronidase in PBS containing Ca^{++}/Mg^{++}), invert or swirl to dissolve, and store 100 µL aliquots at -20 °C for later use. Aliquots can be thawed and re-frozen several times without loss of enzymatic activity.

1.6. To prepare dissociation **Solution 1**, add 10 µL collagenase I and 10 µL DNase I into 1 mL of sterile, pre-equilibrated BM (Final concentrations: 1 mg/mL collagenase I and 5 µg/mL DNase I). Triturate gently with a pipette to mix the solution, and pre-warm to 37 °C before use with the tissue.

NOTE: Solution 2 is prepared by adding 33 µL of hyaluronidase (prewarmed to 37 °C) per 1 mL of Solution 1, (to a final concentration of 1 mg/mL). This occurs mid-way through enzymatic dissociation of testis tissue at step 2.5 below.

2. Testis tissue dissociation

NOTE: All mice were housed within polypropylene cages and provided with food and water ad libitum. Animals were fed irradiated chow which does not contain phytoestrogens. Juvenile CD-1 mice, 5 days post-partum (dpp), were used for all experiments and anesthetized prior to euthanasia and tissue collection, within an anesthesia chamber attached to an isoflurane vaporizer (2.5 L/min in O_2). Mice were confirmed for full anesthesia via the absence of a response to toe-prick, after which mice were euthanized via decapitation.

2.1. Anesthetize mice in an isoflurane chamber, ensure anesthesia via a toe-prick, and then decapitate the mouse using a sharp scissor. Place the euthanized mouse supine on a dissection

mat and sterilize the abdomen with 70% ethanol. Tent the skin of the lower abdomen with forceps and open the abdomen with scissors.

2.2. Locate the testes in the lower left and right inguinal regions of the abdomen. Cut their connections to the vas deferens and any anchoring connective tissue, then lift the entire testis (with epididymis still attached) from the animal. Place testes in a Petri dish of pre-equilibrated BM.

2.3. Under a dissection microscope and within a sterile field, make a small incision in the tunica albuginea on one end of each testis with either a small microdissection scissor or by tearing gently using two fine forceps.

2.3.1. Then, while holding the testis from the opposite end of the incision, gently squeeze the testis with fine forceps and push in a gentle sweeping motion towards the hole in the tunica; this will release the testicular tissue as one cohesive piece.

2.4. Cut the testes into smaller pieces ($\leq$ 2 mm^3) and place them into 1 mL of pre-warmed (37 °C) dissociation Solution 1.

2.4.1. Incubate at 37 °C for 10 min.

2.4.2. For more than 10 testes, increase the total dissociation solution volume by 1 mL, ensuring a minimum of 1 mL of dissociation solution per 10 testes (e.g., 2 mL for 20 testes, 3 mL for 30 testes, etc.).

2.4.3. Gently triturate the testis pieces 50 times (50x) in solution 1 using a P1000 pipette. Ensure that the tubules separate from one another and from interstitial tissue at this point. If clumps remain, incubate for an additional 5 min and triturate once more (50x).

2.5. Add 33 µL of hyaluronidase stock solution (pre-warmed at 37 °C, from step 1.5) per 1 mL of solution 1 dissociation mixture (containing the partially dissociated testicular tissue and tubules). After adding hyaluronidase, this is called solution 2.

2.5.1. Triturate (50x) using a P1000 and incubate at 37 °C for 5 min.

2.5.2. Triturate (50x) using a P200 pipette.

2.5.3. Ensure that at this point no visible tubules or clumps of cells are present. If clumps persist, incubate for up to 5 more min, with further trituration using a P200 pipette (50x).

2.6. Quench the dissociation enzymes by adding fetal bovine serum (FBS) to 10% of the total volume of solution 2. Triturate several times using a P200 pipette to ensure no clumps remain, and filter through a 40 µm cell strainer to produce a single-cell suspension.

2.7. Centrifuge cells at 100 *x g* for 7 min, discard the supernatant, and resuspend the cells in fresh BM.

2.8. Count the total and viable cell concentrations using trypan blue exclusion on a hemocytometer. Add 10 µL of 1:1 diluted, cell suspension: trypan blue solution, into the hemocytometer cell counting chamber (see **Table of Materials**).

2.8.1. Re-centrifuge cells at 100 *x g* for 7 min and resuspend in fresh BM.

NOTE: Only use viable cells for calculating cell concentration and number. Only use cell suspensions of ≥ 80% viability for generating organoids.

2.8.2. Prepare the single cell suspension into cell concentrations as described in order to aliquot 280,000 cells given the volumes used in the protocol specific steps below in section 3: 2D ECM-Free – 0.56 x 10^6 cells/mL, 2D ECM – 0.56 x 10^6 cells/mL , 3D ECM-Free – 4.66 x 10^6 cells/mL, 3D ECM – 2.8 x 10^6 cells/mL.

NOTE: All culture experiments presented here start with 280,000 cells seeded per culture well. These numbers are matched to the representative data in **Figures 1-Figure 4**.

3. Preparation of organoid culture dishes and seeding of cells

NOTE: To ensure a homogenous ECM, pre-thaw frozen aliquots of ECM overnight before experimentation. ECM aliquots should be submerged within a bucket of ice within a 4 °C refrigerator or cold room to guarantee a slow, gradual increase in temperature. All ECM is used at a 1:1 final dilution in BM for culture. Keep thawed ECM and 1:1 diluted ECM on ice until immediately before use, otherwise the ECM might polymerize prematurely.

3.1. For 2D ECM-free culture, no special preparation is necessary, plate single cell suspensions (500 µL of 0.56 x 10^6 cells/mL in BM) directly onto 4- well chamber slides, and place into a 35 °C incubator for culture.

NOTE: Cells should adhere to the bottom of the culture dish within the first 24 h of culture and may exhibit some small 3D cell clusters within this same time.

3.2. For 2D ECM culture, dispense 100 µL of cold 1:1 diluted extracellular basement matrix medium (e.g., Matrigel) into a 4 well chamber slide, ensuring the gel covers the entirety of the dish bottom.

3.2.1. Place the chamber slide in a 35 °C incubator for a minimum of 30 min to allow the ECM to polymerize into a gel.

3.2.2. Add the cell suspension (500 µL of 0.56 x 10^6 cells/mL in BM) directly on the top of the 2D gel once it has polymerized.

NOTE: Cells should cluster together to form small 3D clusters within the first 24 h of culture.

3.3. For 3D ECM-free culture, prepare agarose 3D Petri dish inserts before starting the cell culture.

3.3.1. First, autoclave 1.5 g agarose powder in a 100 mL beaker, then add 75 mL sterile, distilled water and microwave to produce molten 2% agarose for 3D Petri dish casting.

3.3.2. Within a sterile workspace, dispense molten agarose into the 3D Petri dish mold until the meniscus is level with the sides of the mold.

3.3.3. Allow the agarose to cool and solidify. When solid, turn the mold upside down and gently flex repeatedly until the agarose 3D Petri dish falls free from the mold.

NOTE: At this point, one can prepare many agarose 3D Petri dishes and store them in sterile H_2O or DPBS at 4 °C for upwards of one month.

3.3.4. Prior to culturing, place agarose 3D Petri dishes into a 24 well culture dish, and cover them with 1 mL of BM. Let the 3D Petri dishes equilibrate in BM for at least 30 min within a 37 °C culture incubator. Discard the BM, and repeat the equilibration once more with 1 mL fresh BM. After equilibration of 3D Petri dishes in BM, they will appear the same color as the BM (i.e., pink).

3.3.5. To prepare for cell seeding, remove all BM from the well and dispense 200 µL of fresh BM around, but not inside the center recess of the 3D Petri dish. Also, collect any remaining BM from inside the center cell-seeding recess of the microwell insert.

3.3.6. Dispense the single cell suspension (4.66 cells/mL in 60 µL of BM) into the center recess of the agarose 3D Petri dish. Gently triturate up and down to mix cells and guarantee a single cell suspension at the start of culture.

3.3.7. Place into in a humidified 35 °C incubator for culture. The following day, remove the 200 µL of BM from around the microwell insert, and replace with 1 mL of fresh BM. This will bring the liquid level above the plane of the insert, submerging the entire culture.

3.3.8. Slowly and carefully remove/add media from outside of the agarose 3D Petri dish. The organoids should have compacted overnight, allowing them to rest at the bottom and enabling media changes to leave organoids undisturbed.

3.4. For 3D ECM culture, prepare a single cell suspension by combining, in equal parts, the cell suspension in BM with cold, pre-thawed ECM (final concentration = 2.8×10^6 cells/mL).

3.4.1. Immediately dispense the cell-ECM mixture into a 4 well chamber slide, ensuring the mixture covers the entire bottom of the plate.

3.4.2. Place the chamber slides at 35 °C in an incubator and allow its contents to polymerize. This should take at least 30 min. After the polymerization, add 500 µL of BM on the top of the culture.

NOTE: Cells should have clustered together to form small 3D aggregates within the first 24 h of culture.

4. Organoid maintenance

4.1. Culture all organoid model types at 35 °C. For All culture types exchange half of their media with fresh BM every 2 days. To ensure that organoids are not accidentally collected while exchanging medium, always collect media slowly from a corner of the chamber slide dish, and from an external point outside of agarose 3D Petri dishes. All media can be stored at -20 °C for use with immunoassays or other analyses later (i.e., quantification of secreted reproductive hormones or cytokines).

4.2. After 7 days in culture, use BM containing follicle stimulating hormone (final concentration 20 mIU/mL) and human chorionic gonadotropin (final concentration 4.5 IU/mL). This applies to all organoid culture types.

4.3. Routinely image all organoid cultures (i.e., time-lapse imaging) for characterizing organoid formation and quantifying metrics of self-assembly, development, and growth over time.

5. Organoid Collection

NOTE: All organoids can be fixed with 4% paraformaldehyde in PBS for downstream immunolabeling and histological analyses. Fix for 2 h at room temperature with rotation, or overnight at 4 °C.

5.1. For 2D ECM-free cultures, first rinse the sample with fresh PBS, and then add fixative directly on top of the adhered constructs.

5.2. For ECM (2D and 3D) culture methods, rinse once with PBS, and then either add fixative directly on the top of the ECM-organoid sample (to fix the ECM gel and organoids together), or alternatively, gently pipette the organoids up and down to free them from the surrounding ECM, and transfer to a separate tube for fixation.

5.3. For 3D ECM-free culture, gently pipette the organoids up and down within the center recess of the agarose 3D Petri dish; this will flush the organoids out facilitating their easy collection with a pipette. Then transfer organoids to a separate tube for fixation.

5.4. Before processing into paraffin, embed many organoids ($\geq$ 20) within a small volume (~ 30 µL) of tissue processing gel; this helps orient and concentrate organoids into a small area within paraffin blocks, facilitating easier observation when sectioning and easier visual identification

within paraffin sections.
NOTE: Organoids can be challenging to identify after paraffin embedding and sectioning upon a microtome.

REPRESENTATIVE RESULTS:

Organoid generation was considered unsuccessful if testicular cells did not self-assemble within 72 h of culture, however, all methods presented here assemble within 24 h when using juvenile (5 dpp) murine cells. Failure of biological construct generation presented as a continuation of freely suspended cells (0 h column in **Figure 1**) even after extended culture (72 h). In the absence of tissue self-assembly, any apparent cell clusters easily dispersed into individual cells upon even gentle manipulation (i.e., pipetting). Successfully generated tissues were initially observed as 3D cell "clusters" (yellow arrows in 6 h column of **Figure 1**). Within ECM-free environments (2D and 3D), these constructs visibly appeared to "compact" across the first 24 h of culture, especially when in 3D agarose Petri dishes (**Figure 1A,C**). In ECM environments (2D and 3D) cell clusters possessed clear margins between the cluster and their surrounding environment (**Figure 1B,D**). Cell clusters were also observed to migrate across the ECM and fuse together forming larger clusters (red arrows in **Figure 1B**). The time required to appreciate separate self-assembled structures was measured, and there was no significant difference in time required between both 2D and 3D ECM-free conditions and 2D ECM, however, 3D ECM culture required significantly more time to assemble de novo structures than all other culture methods (**Figure 1E**). 2D ECM-free and 3D ECM culture generated cell clusters with significantly smaller sizes than 2D ECM and 3D ECM-free culture methods; 3D ECM-free culture produced the largest clusters with a single large and compact cluster within each well of the 3D agarose Petri dish (**Figure 1C,F**). In summary, these data demonstrate the ease with which to produce testicular biological constructs from juvenile mouse primary cells in four archetypal culture environments and highlight different cell-directed assembly-phenotypes within these different culture environments.

A goal of all organoid models is to recapitulate an inner morphology mimetic of native tissue. To assess for this outcome, biological constructs assembled within each culture condition were cultured for 72 h and then probed for cell-specific markers and visualized with immunofluorescence (**Figure 2**). Variability in tissue morphology was observed between different culture methods. 2D ECM-free organoids presented as clusters of Sertoli cells (SOX9 and βCatenin) with some germ cells (DDX4, a pan germ cell marker) adhered on top of a 2D basal confluent layer containing many somatic cells, including Sertoli, peritubular (αSMA), and Leydig cells (3βHSD) (**Figure 2A-D**). Note that **Figures 2B-D** are epifluorescent images of the entire 2D ECM-free sample, not a 5 µm section; this enables visualization of both the basal somatic cell layer and the superiorly oriented aggregates of Sertoli and germ cells. In contrast, 2D ECM culture presented with a largely different phenotype, as clear biological structures with distinct borders were easily discerned on top of the basal ECM gel (**Figure 2E**). These structures possessed a complex inner morphology with *de novo* compartmentalization of tubule vs. interstitial cell types of the testis, and so were deemed successful organoids. Tubular regions contained Sertoli, peritubular, and germ cells, and interstitial regions contained Leydig, peritubular cells and non-labeled cells (**Figure 2F-H**). Similarly, 3D ECM-free organoids also possessed a compartmentalized inner morphology and so were deemed successful organoids. In particular, 3D ECM-free

organoids were distinguished by tubular regions containing peritubular cells that specifically oriented around groups of Sertoli and germ cells with high fidelity (**Figure 2I-L**). 3D ECM assembled structures did not possess a sophisticated morphology, but instead were observed to be clusters of Sertoli cells, with occasional germ and Leydig cells. In these samples, many Sertoli and un-labeled cells remained suspended in between organoids in a "stroma-like" orientation (**Figure 2M-P**). Together, these data highlight the variability in morphological phenotypes that different organoid generation methods produce and supports the use of two specific organoid assembly environments, 2D ECM and 3D ECM-free, for the generation of testicular organoids with an inner morphology highly mimetic of testicular compartmentalization.

To further assess testicular organoid function, 3D ECM-free assembled organoids were selected for a deeper long-term analysis. For this study, these organoids were cultured for 14 days and then probed for cell and structure specific markers. Upon immunofluorescent analysis at 14 days, 3D ECM-free organoids were observed to contain tubule-like-structures (TLS) and a tissue architecture remarkably similar to in vivo testes (**Figure 3A-D**). Interstitial cells were appropriately located in separate regions from TLS. Tissue sections were then probed for the pan germ cell marker, DDX4, spermatogonial stem cell marker, SALL4, and meiosis marker, SCP3 (**Figure 3E-G)**. Rare DDX4-positive and SALL4-positive cells were observed, however, no SCP3 signal was identified. Upon deeper characterization of TLS, they were observed to contain a lumen-appearing space surrounded by polarized Sertoli cells and an external monolayer of peritubular cells (**Figure 3I-L**). Sertoli cells also exhibited tight junctions between one another, as visualized with transmission electron microscopy and with labeling against ZO-1, a junctional protein and component of the blood-testis barrier (**Figure 3H,L**). Next, 3D ECM-free organoids were studied for endocrine function in 12-week, long-term culture with gonadotropin stimulation (**Figure 4**). Testosterone and inhibin B, reproductive hormones from Leydig and Sertoli cells respectively, were both identified and quantified from organoid conditioned medium (**Figure 4A**). Over 12-weeks of culture, both hormones were measured to significantly respond to the supplementation of gonadotropins FSH and hCG (red arrow denotes the beginning of supplementation, during weeks 2 – 12). At completion, a test was performed to determine if endocrine responsiveness was preserved. Gonadotropins were removed for 48 h, during which both testosterone and inhibin B concentrations significantly decreased in concentration. After 48 h, gonadotropins were returned to culture and both hormone concentrations significantly increased again over a final 24 h, demonstrating proper endocrine-responsiveness (**Figure 4B**). Collectively, these histological and endocrine assay results demonstrate that testicular organoids are a useful model for studying testicular development (i.e., de novo compartmentalization and tubulogenesis) and somatic cell testicular function in vitro (e.g., tight junction formation and endocrine function).

FIGURE AND TABLE LEGENDS:

Figure 1: Organoids self-assemble in 2D and 3D, ECM and ECM-free culture conditions. 5 dpp murine testicular cells were cultured in four different conditions: 2D ECM-free (row **A**), 2D ECM (row **B**), 3D ECM-free (row **C**), and 3D ECM (row **D**). Graphics depicting the culture method are provided in the left-hand column. Representative image montages were assembled from time-

lapse images captured during the live culture. Time points for each image are labeled at the top margin. Time 0 h is before any organoid assembly has occurred, time 3 h is during ongoing cell-driven organoid assembly, and times 6 h and 9 h demonstrate representative, successfully formed organoids. Yellow arrows mark cell clusters and red arrows mark locations where separate cell clusters migrated and merged together. All scale bars = 1 mm. (**E**) Time required before separate cell clusters could be visibly appreciated was recorded for each condition. 2DF = 2D ECM-free; 2DE = 2D ECM; 3DF = 3D ECM-free; 3DE = 3D ECM. (**F**) Area per cluster was measured for each culture condition. Images were selected from n= 3 – 5 separate experiments. One-way ANOVA with Tukey's multiple comparisons test was used to determine significance in 1E and 1F; graphs were assembled from the means of n=4 separate experiments. This figure has been modified from Edmonds and Woodruff[37].

Figure 2: 2D ECM and 3D ECM-free cultured organoids exhibit compartmentalization of tubular and interstitial cell types. Representative brightfield and immunofluorescent images of self-assembled organoids after 72 h of culture. 2D ECM-free samples were imaged whole mount, all other samples were imaged in 5 µm tissue sections. (**A – D**) 2D ECM-free culture. (**E – H**) 2D ECM culture. (**I – L**) 3D ECM-free culture. (**M – P**) 3D ECM culture. Cell-specific markers used for analysis are labeled on the top margin: Sertoli cell nuclei (SOX9) and cell bodies (βCatenin), germ cells (DDX4, a pan germ cell marker), Leydig cells (3βHSD), and peritubular cells (αSMA). All fluorescent samples were co-stained for DNA with DAPI. Yellow arrows point to DDX4-marked germ cells in the second column from the left, red arrows point to Sertoli cell clusters in the right two columns. Bright field scale bars = 400 µm, fluorescent scale bars = 100 µm. This figure has been modified from Edmonds and Woodruff[37].

Figure 3: Organoids develop tubule-like structures populated by rare germ cells. 3D-ECM-free assembled organoids were cultured for 14 days. (**A–D**) Representative H&E and immunofluorescent images detailing cell types and tissue features around tubule-like structures (TLS); the same organoid is depicted in adjacent tissue sections enabling side-by-side comparison of morphological features: Leydig cells (3βHSD), peritubular cells (αSMA), Sertoli cell bodies (βCatenin), collagen membrane (COL IV), and Sertoli cell nuclei (SOX9); scale bars = 100 µm. (**E – G**) Immunofluorescent labeling was performed against germ cells, including a pan germ cell marker (DDX4), spermatogonial stem cell marker (SALL4) and meiotically active spermatocytes (SCP3). Highly magnified insets are outlined by yellow panels in 3E and 3F. Green triangles point to DDX4-labeled cells; Red arrows point to SALL4-labeled cells. (**H**) Representative transmission electron micrograph (TEM) of a tight junction between Sertoli cells within an organoid; TEM scale bar = 100 nm. (**I – L**) High magnification representative images of a TLS labeled for key features of the seminiferous epithelium including tight junctions (ZO1) and laminin. The same TLS is depicted in adjacent tissue sections in panels I – L. All fluorescent samples were co-stained for DNA with DAPI. Images were selected from n=7 separate biological experiments. This figure has been modified from Edmonds and Woodruff[37].

Figure 4: Organoids secrete testosterone and inhibin B over 12-weeks of culture in response to gonadotropins FSH and hCG. Conditioned media collected from 3D-ECM-free organoids was measured for testosterone and inhibin B via enzyme-linked immunosorbent assay (ELISA). (**A**) Organoids were cultured for twelve weeks, with FSH and hCG supplementation during weeks 2 – 12 (beginning of supplementation is marked with a red arrow on the x-axis); all values were compared to day 7 of culture for statistical tests. (**B**) Magnified graph of a "re-stimulation test" to determine if endocrine responsiveness was preserved after 12 weeks. The period of the test is outlined by the gray box in 4A. At the completion of 12-weeks in culture, gonadotropins were removed for 48 h and then re-supplemented for a final 24 h of culture. Hormones were measured at 0, 2, 6, 12, and 24 h after gonadotropin re-stimulation; red-shaded areas designate time periods during culture with FSH and hCG, non-shaded areas designate time periods without FSH and hCG; noted p-values are relative to 2 h after re-stimulation. Two-way ANOVA with Tukey's multiple comparison's test was used to determine significance for all endocrine data, n=5 separate biological experiments. This figure has been modified from Edmonds and Woodruff[37].

DISCUSSION:

With the completion of this organoid generation protocol, the user will have four different culture techniques available to them for assembling testicular constructs and organoids in either ECM or ECM-free environments. Importantly, all four methods allow the researcher to non-invasively observe organoid self-assembly over time through time-lapse imaging or video recording, and to noninvasively collect conditioned media for analysis of secreted hormones and cytokines, without disturbing tissues during culture. In all methods, over the course of 24 h, an experimenter can generate as many as several hundred organoids / testicular constructs, as cell numbers allow. These methods promote tissue self-assembly into constructs with different sizes and morphologies; organoid size depends on the cell number and concentration used in culture, as seen in other organoid reports[34]. Reducing the organoid size or diameter might help reduce the development of inner regions of necrosis which are sometimes presents in larger organoids. A particular strength of 2D ECM and 3D ECM-free protocol methods are their ability to generate morphologically mimetic testicular organoids, containing de novo compartmentalization of tubular versus interstitial cell types. Furthermore, 3D ECM-free assembled organoids provide a model for de novo tubulogenesis of seminiferous TLS, with appropriately compartmentalized and oriented Sertoli and peritubular cells. This is an important phenotype for studying testicular organoids, and is still a variable outcome amongst different testicular organoid reports; multiple other reports lack tubule versus interstitium compartmentalization and some even develop an "inside-out tubule" phenotype[32–34,38]. While none of the organoid generation methods presented in the present manuscript were characterized to maintain large germ cell populations over extended days of culture, as germ cells were rarely observed as early as 72 h, both 2D ECM and 3D ECM-free methods might provide a useful tool to study in vitro tubulogenesis and the somatic cell component of a spermatogonial niche environment. With this goal in mind, testicular organoids provide a potential platform for optimizing future protocols to improve in vitro germline stem cell maintenance, meiotic progression, and differentiation.

Another advantage of these protocols is the ability to customize and scale organoid generation.

Customized, drug-treated, or engineered cell populations can replace, or be used in addition to, primary mouse testicular cells[37]. If cell suspensions are of a low viability (<80 %), methods should be taken to improve cell viability of the cellular suspension. These can include reducing the time spent in dissociation media and minimizing titration during tissue digestion (steps 2.4.1 – 2.5.2), increasing the number of washes after dissociation, or removing dead cells after dissociation with cell-sorting or dead-cell labeling kits. However, none of these steps were necessary to produce the representative data shared in this report. For 2D and 3D ECM culture methods, these protocols can be used with other structural protein-based biomaterial matrices in addition to those used for the studies presented here. These other biomaterials include collagen, gelatin, commercially available ECM extracts, and custom-made decellularized ECM-derived hydrogels[25,26,28]. There are a few pointers for trouble-shooting ECM gel dispensing and creating high-quality agarose 3D Petri dish inserts. When casting ECM gels onto chamber plates, be sure to work quickly and use cold pipette tips to prevent premature polymerization of the ECM within a tube or pipette tip prior to dispensing in the culture dish. When casting molten agarose into the 3D Petri dish molds (for 3D ECM-free culture), use only hot and not warm agarose to ensure high quality casting of the inserts with minimal variation, and check that the agarose has fully cooled to room temperature and solidified before attempting to remove from the mold. Agarose 3D Petri dishes are best handled gently with fine forceps. When collecting organoids for fixation, be sure to work under a dissection microscope to visualize the collection of all organoids. Organoids can stick to the plastic side of culture dishes and the inside of pipette tips; glass pipettes exhibit less organoid adherence than plastic. Fixing organoids while still encapsulated within or on top of ECM is a more challenging and delicate process than removing them from ECM prior to fixation. Tissue processing gel can be cast above the ECM-organoid construct prior to removal from the culture chamber to help reinforce the gel before fixation[39]. Fixation should be performed at room temperature to retrieve ECM hydrogels as they are likely to de-polymerize if lowered to 4 °C. Additionally, 0.1 % - 1.0 % glutaraldehyde can be added to the 4 % PFA solution to help further cross-link the ECM; however, this method increases background autofluorescence of the sample.

There are considerable germ cell-specific limitations to the results achieved by the organoid generation methods presented above, which represent priority areas for future innovation. Germ cells are poorly supported over extended culture, are only easily observed during the first few days of culture and are rarely observed by the end of the first week of culture and at later time points. While undifferentiated spermatogonia can be maintained within in vitro cell culture while maintaining their ability to restore spermatogenesis upon transplantation into in vivo tubules[40], the tubule somatic microenvironment (i.e., direct Sertoli cell interactions) is hypothesized to be a prerequisite for differentiating pre-meiotic germ cells into and through meiosis and spermiogenesis in vitro[1,5,40,41]. Testicular organoids containing spermatogonia at early time points within a structurally mimetic TLS might enable the field to non-invasively study somatic-somatic, and somatic-germ cell interactions entirely in vitro. Optimization of media additives and cell preparation prior to culture (e.g., incorporation of agents used for in vitro SSC culture)[42,43] might increase the yield of germ cells in future studies, especially over culture periods longer than several days. Inversely, methods to re-introduce spermatogonia after TLS have formed, such as through microinjection, pose an interesting opportunity to restore germ cells within organoids,

and test the capability of in vitro organized somatic environments to maintain germ cells. Other biological factors have been observed to affect ex vivo explant culture, including age/maturity[45] and genetic background differences[17]. These same variables have yet to be directly investigated within the field of testicular organoid biology. However, it is plausible that different assembly, morphology, and functional phenotypes might result when organoids are generated from cells isolated from differently aged animals (i.e., neonatal, juvenile, adult), or animals of varying genetic background (e.g., different mouse strains).

In summary, the techniques shared in this manuscript provide four useful models for studying cell-driven testicular construct self-assembly, the generation of two separate structurally mimetic testicular organoid models, and the important achievement of TLS development and hormone-responsive endocrine function in vitro. These models encompass 2D and 3D spatial orientations in ECM and ECM-environments with minimally complex, completely defined culture medias. Each method is highly reproducible and uses only commonly available culture resources. These methods may prove advantageous for studying testicular morphogenesis in vitro and optimizing future culture conditions for in vitro spermatogenesis. More so, 2D ECM and 3D ECM-free methods provide a novel tool for studying the process of the de novo tissue compartmentalization unique to the testis, in vitro tubulogenesis, and somatic-somatic and somatic-germ cell interactions. Testicular organoids provide a flexible and scalable opportunity to investigate the development and regulation of somatic testicular physiologic hallmarks; including the blood-testis barrier and endocrine production and response; and, also, a useful tool for developing next-generation translational research tissue models. These include incorporating reproductive hormones into larger systems-level models, such as tissue-on-a-CHIP and micro-physiologic platforms[45,46]. Other translational goals towards which testicular organoids might one day be applied include reproductive toxicology and immunologic barrier testing, male contraceptive development, and assisted reproductive technology innovation.

ACKNOWLEDGMENTS:

This work was funded by the National Institutes of Health, National Institute of Child Health and Human Development (NICHD) F31 HD089693, the National Institute for Environmental Health Sciences / National Center for Advancing Translational Sciences (NIEHS/NCATS) UH3TR001207 and 4UH3ES029073-03, and the Thomas J. Watkin's Memorial Professorship.

The authors would like to thank Eric W. Roth for their assistance with transmission electron microscopy. This work made use of the BioCryo facility of Northwestern University's NU*ANCE* Center, which has received support from the Soft and Hybrid Nanotechnology Experimental (SHyNE) Resource (NSF ECCS-1542205); the MRSEC program (NSF DMR-1720139) at the Materials Research Center; the International Institute for Nanotechnology (IIN); and the State of Illinois, through the IIN. It also made use of the CryoCluster equipment, which has received support from the MRI program (NSF DMR-1229693). Graphics in Figure 1 were designed using BioRender.com.

DISCLOSURES:

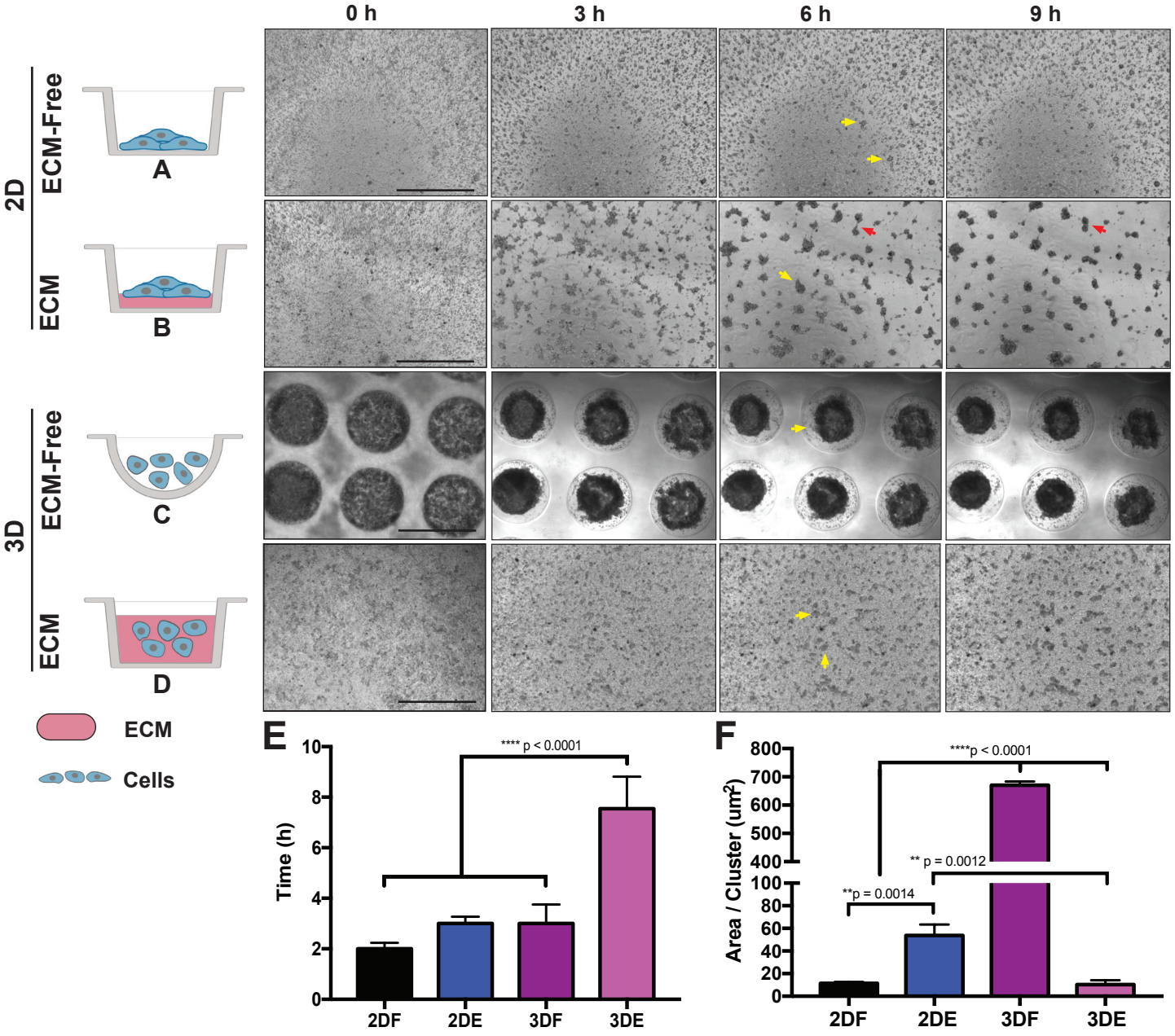
The authors have nothing to disclose.

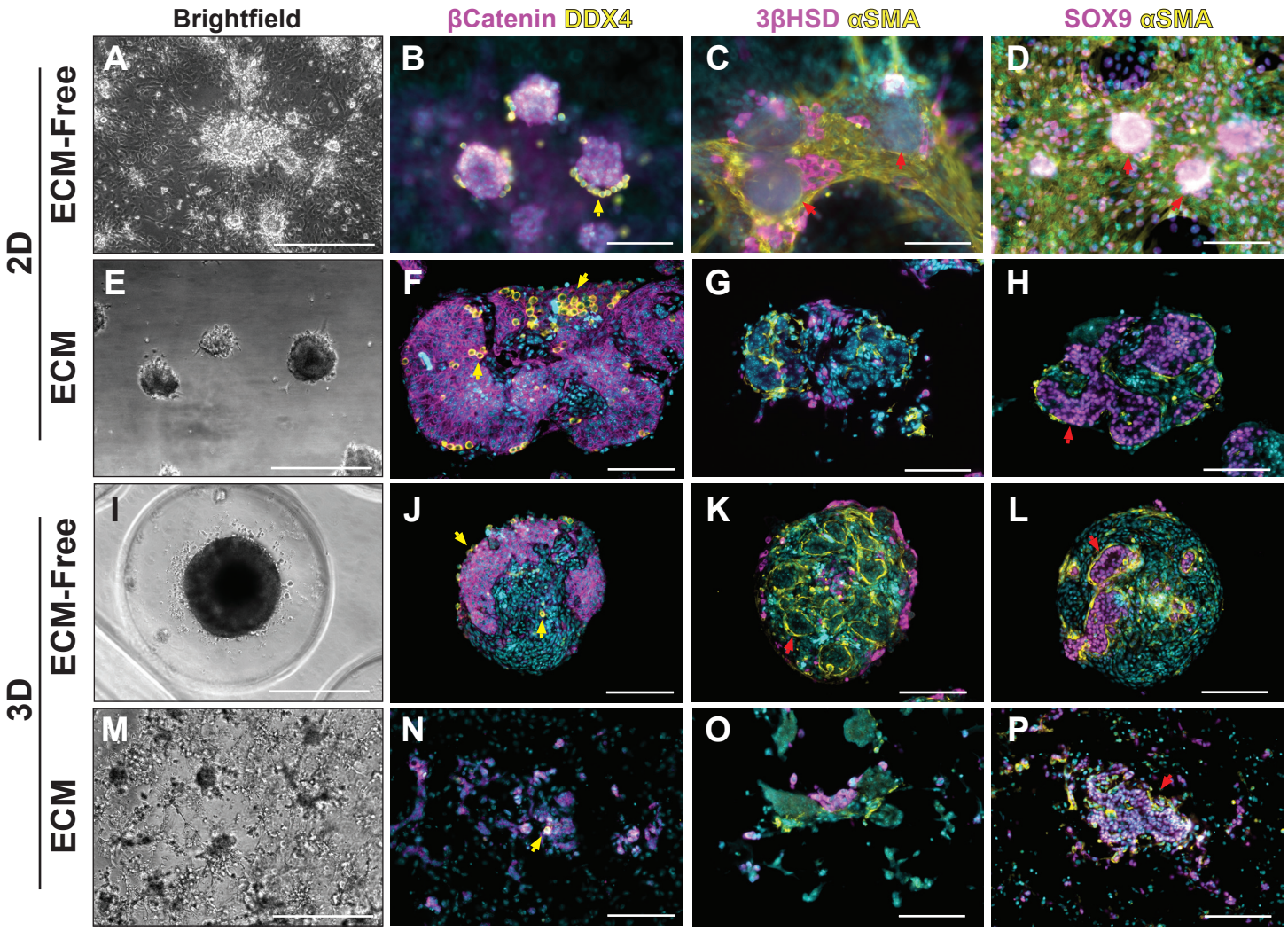
REFERENCES:

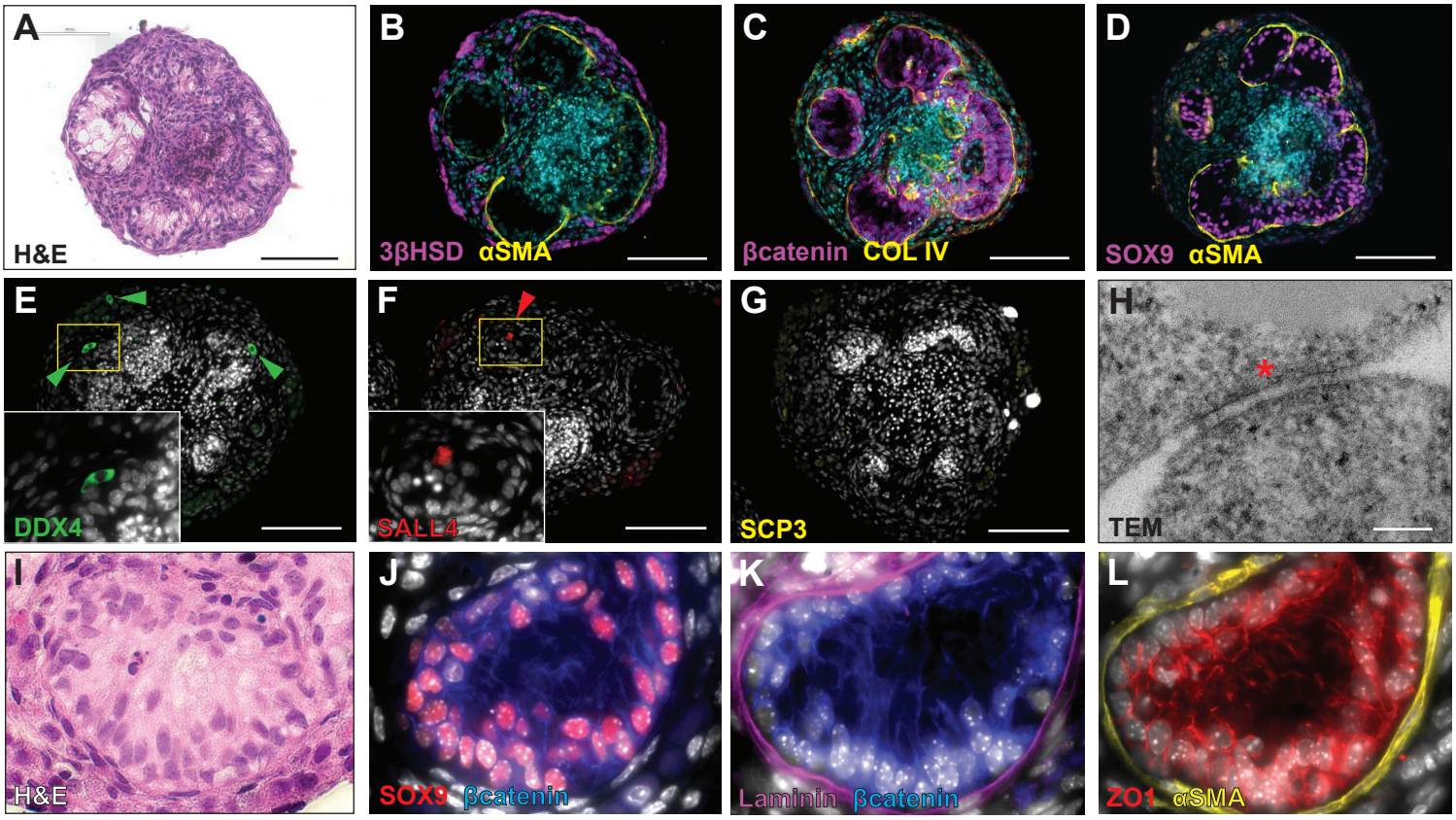
1. Alves-Lopes, J. P., Stukenborg, J. -B. Testicular organoids: a new model to study the testicular microenvironment in vitro? *Human Reproduction Update*. **24** (2), 176–191 (2018).
2. Komeya, M., Sato, T., Ogawa, T. In vitro spermatogenesis: A century-long research journey, still half way around. *Reproductive Medicine and Biology*. **17** (4), 407–420 (2018).
3. Sakib, S., Goldsmith, T., Voigt, A., Dobrinski, I. Testicular organoids to study cell–cell interactions in the mammalian testis. *Andrology*. andr.12680 (2019).
4. Gargus, E. S., Rogers, H. B., McKinnon, K. E., Edmonds, M. E., Woodruff, T. K. Engineered reproductive tissues. *Nature Biomedical Engineering.* **4** (4), 381-393 (2020).
5. Sato, T. et al. In vitro production of functional sperm in cultured neonatal mouse testes. *Nature*. **471** (7339), 504–507 (2011).
6. Eddy, E. M., Kahri, A. I. Cell associations and surface features in cultures of juvenile rat seminiferous tubules. *The Anatomical Record*. **185** (3), 333–357 (1976).
7. Dietrich, A., SCholten, R., Vink, A., Oud, J. Testicular cell suspensions of the mouse in vitro. *Andrologia*. **15**, 236–246 (1983).
8. Champy, C. De la méthode de culture des tissus. VI. Le testicule. *Archives de Zoologie Experimentale Generale*. **60**, 461–500 (1920).
9. Martinovitch, P. The development in vitro of the mammalian gonad. Ovary and ovogenesis. *Proceedings of the Royal Society B of Biological Sciences.* **125**, 232–249 (1938).
10. Sato, T. et al. In vitro production of fertile sperm from murine spermatogonial stem cell lines. *Nature communications*. **2**, 472 (2011).
11. Komeya, M. et al. Long-term ex vivo maintenance of testis tissues producing fertile sperm in a microfluidic device. *Scientific Reports*. **6** (1), 21472 (2016).
12. Sanjo, H. et al. In vitro mouse spermatogenesis with an organ culture method in chemically defined medium. *PLoS One*. **13** (2), e0192884 (2018).
13. Komeya, M. et al. In vitro spermatogenesis in two-dimensionally spread mouse testis tissues. *Reproductive Medicine and Biology*. **18** (4), 362–369 (2019).
14. Reda, A. et al. In vitro differentiation of rat spermatogonia into round spermatids in tissue culture. *Molecular Human Reproduction*. **22** (9), 601–612 (2016).
15. Reda, A. et al. Knock-Out Serum Replacement and Melatonin Effects on Germ Cell Differentiation in Murine Testicular Explant Cultures. *Annals of Biomedical Engineering*. **45** (7), 1783–1794 (2017).
16. Chapin, R. E. et al. Lost in translation: The search for an in vitro screen for spermatogenic toxicity. *Birth Defects Research Part B - Developmental and Reproductive Toxicology*. **107** (6), 225–242 (2016).
17. Portela, J. M. D. et al. Strains matter: Success of murine in vitro spermatogenesis is dependent on genetic background. *Developmental Biology*. **456** (1), 25–30 (2019).
18. Gholami, K. et al. The air-liquid interface culture of the mechanically isolated seminiferous tubules embedded in agarose or alginate improves in vitro spermatogenesis at the expense of attenuating their integrity. *In Vitro Cellular & Developmental Biology. Animal*. **56** (3) 261-270 (2020).

19. Oakberg, E. F. Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. *American Journal of Anatomy*. **99** (3), 507–516 (1956).
20. Amann, R. P. The cycle of the seminiferous epithelium in humans: A need to revisit? *Journal of Andrology*. **29** (5), 469–487 (2008).
21. Clevers, H. Modeling Development and Disease with Organoids. *Cell*. **165** (7), 1586–1597 (2016).
22. Lancaster, M. A., Knoblich, J. A. Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science*. **345** (6194), 1247125 (2014).
23. Rossi, G., Manfrin, A., Lutolf, M. P. Progress and potential in organoid research. *Nature Reviews Genetics*. 19 (11):671-687 (2018).
24. Takahashi, T. Organoids for Drug Discovery and Personalized Medicine. *Annual Review of Pharmacology and Toxicology*. **59**, 447–62 (2019).
25. Alves-Lopes, J. P., Söder, O., Stukenborg, J. -B. Testicular organoid generation by a novel in vitro three-layer gradient system. *Biomaterials*. **130**, 76–89 (2017).
26. Lee, J. H., Kim, H. J., Kim, H., Lee, S. J., Gye, M. C. In vitro spermatogenesis by three-dimensional culture of rat testicular cells in collagen gel matrix. *Biomaterials*. **27** (14), 2845–2853 (2006).
27. Zhang, J., Hatakeyama, J., Eto, K., Abe, S. I. Reconstruction of a seminiferous tubule-like structure in a 3 dimensional culture system of re-aggregated mouse neonatal testicular cells within a collagen matrix. *General and Comparative Endocrinology*. **205**, 121–132 (2014).
28. Vermeulen, M. et al. Development of a Cytocompatible Scaffold from Pig Immature Testicular Tissue Allowing Human Sertoli Cell Attachment, Proliferation and Functionality. *International Journal of Molecular Sciences*. **19** (1), 227 (2018).
29. Baert, Y. et al. Derivation and characterization of a cytocompatible scaffold from human testis. *Human Reproduction*. **0** (0), 1–12 (2014).
30. Baert, Y., Rombaut, C., Goossens, E. Scaffold-Based and Scaffold-Free Testicular Organoids from Primary Human Testicular Cells. *Methods in Molecular Biology.* **1576**, 283-290 (2019)
31. Alves-Lopes, J. P., Söder, O., Stukenborg, J.-B. Use of a three-layer gradient system of cells for rat testicular organoid generation. *Nature Protocols*. **13** (2), 248–259 (2018).
32. Baert, Y., Dvorakova-Hortova, K., Margaryan, H., Goossens, E. Mouse in vitro spermatogenesis on alginate-based 3D bioprinted scaffolds. *Biofabrication*. **11** (3), 035011 (2019).
33. Pendergraft, S. S., Sadri-Ardekani, H., Atala, A., Bishop, C. E. Three-dimensional testicular organoid: a novel tool for the study of human spermatogenesis and gonadotoxicity in vitro. *Biology of Reproduction*. **96** (3), 720–732 (2017).
34. Sakib, S. et al. Formation of organotypic testicular organoids in microwell culture. *Biology of Reproduction*. **100** (6) 1648-1660 (2019).
35. Cameron, D. F. et al. Formation of Sertoli Cell-Enriched Tissue Constructs Utilizing Simulated Microgravity Technology. *Annals of the New York Academy of Sciences*. **944** (1), 420–428 (2006).
36. Strange, D. P. et al. Human testicular organoid system as a novel tool to study Zika virus pathogenesis. *Emerging Microbes & Infections*. **7** (1), 1–7 (2018).
37. Edmonds, M. E., Woodruff, T. K. Testicular organoid formation is a property of immature

somatic cells, which self-assemble and exhibit long-term hormone-responsive endocrine function. *Biofabrication*. doi: https://doi.org/10.1088/1758-5090/ab9907 Accepted.(2020).
38. Baert, Y. Primary Human Testicular Cells Self-Organize into Organoids with Testicular Properties. *Stem Cell Reports*. **8** (1), 30–38 (2017).
39. Pinto, M. P., Jacobsen, B. M., Horwitz, K. B., Maggiolini, M. An immunohistochemical method to study breast cancer cell subpopulations and their growth regulation by hormones in three-dimensional cultures. **2**, 15 (2011).
40. Brinster, R. L. Germline stem cell transplantation and transgenesis. *Science (New York, N.Y.)*. **296** (5576), 2174–2176 (2002).
41. Hue, D. et al. Meiotic Differentiation of Germinal Cells in Three-Week Cultures of Whole Cell Population from Rat Seminiferous Tubules. *Biology of Reproduction*. **59** (2), 379–387 (1998).
42. Zhou, Q. et al. Complete Meiosis from Embryonic Stem Cell-Derived Germ Cells in Vitro. *Cell Stem Cell*. **18** (3), 330–340 (2016).
43. Kanatsu-Shinohara, M. et al. Long-Term Proliferation in Culture and Germline Transmission of Mouse Male Germline Stem Cells. *Biology of Reproduction*. **69** (2), 612–616 (2003).
44. Helsel, A. R., Oatley, M. J., Oatley, J. M. Glycolysis-Optimized Conditions Enhance Maintenance of Regenerative Integrity in Mouse Spermatogonial Stem Cells during Long-Term Culture. *Stem Cell Reports*. **8** (5), 1430–1441 (2017).
45. Sato, T. et al. In vitro spermatogenesis in explanted adult mouse testis tissues. *PLoS One*. **10** (6), e0130171 (2015).
46. Xiao, S. et al. A microfluidic culture model of the human reproductive tract and 28-day menstrual cycle. *Nature Communications*. **8** (1), 1–13 (2017).
47. Skardal, A. et al. Drug compound screening in single and integrated multi-organoid body-on-a-chip systems. *Biofabrication*. **12** (2), 025017 (2020).







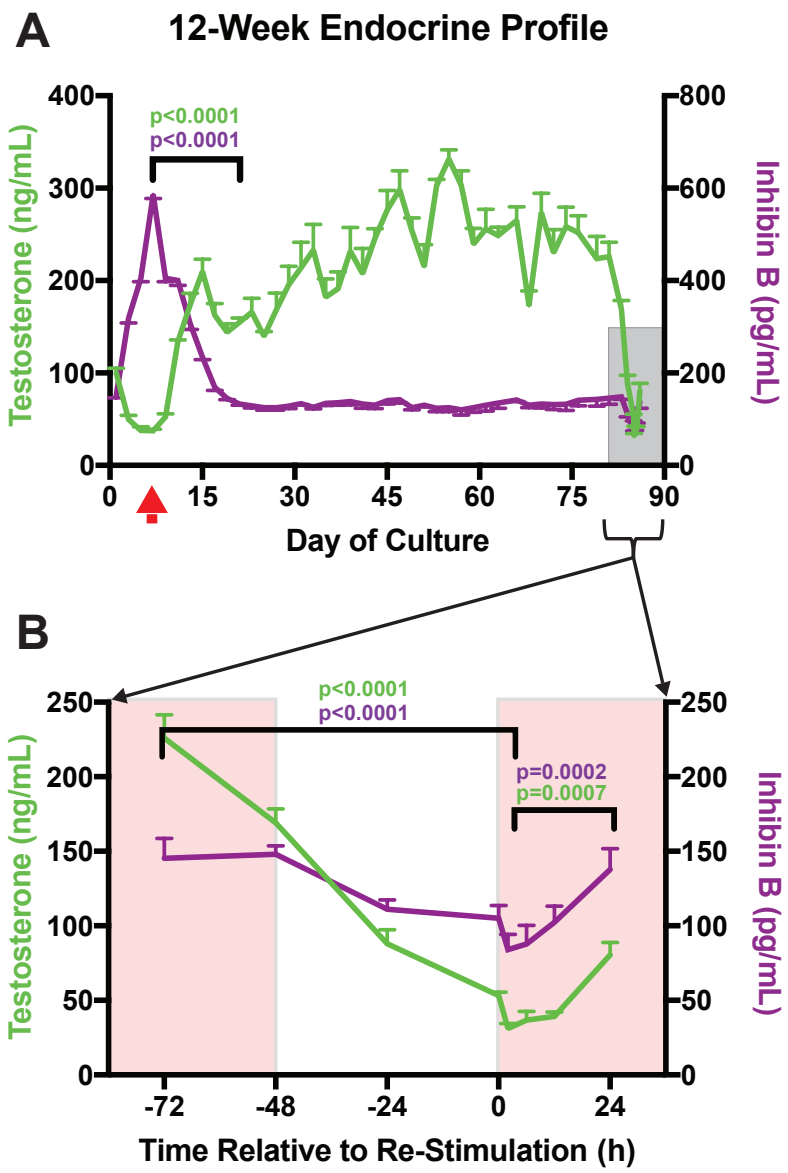


Table of Materials

Click here to access/download;Table of Materials;Edmonds_Table of Materials_04.04.20.xlsx

Name of Material/ Equipment	Company	Catalog Number
0.22 um Media Sterile Filters	Millipore Sigma	scgpu05re
3βHSD primary antibody	Cosmo Bio Co	K0607
AlexaFluor 568 α-Mouse	Thermo Fisher Scientific	A-21202
AlexaFluor 568 α-Rabbit	Thermo Fisher Scientific	A10042
Alpha Minimum Essential Medium	Thermo Fisher Scientific	11-095-080
Collagenase I	Worthington Bio	LS004197
Corning Matrigel Membrane Matrix, LDEV-free	Corning	354234
Countess Cell counter	Thermo Fisher Scientific	C10227
Countess Cell Counting Chamber Slides	Thermo Fisher Scientific	C10228
DDX4 primary antibody	Abcam	138540
Deoxyribonuclease I (2,280 u/mgDW)	Worthington Bio	LS002140
DPBS 1X, + CaCl + MgCl	Thermo Fisher Scientific	14040182
Dulbecco's Phosphate Buffered Saline +Ca/+Mg	Thermo Fisher Scientific	14040117
Embryo Grade H2O	MIllipore Sigma	W1503
Fetal Bovine Serum	Thermo Fisher Scientific	16000044
Follicle stimulating hormone	Abcam	ab51888
Human chorionic gonadotropin	Millipore Sigma	C1063
Hyaluronidase, from bovine testes	Millipore Sigma	H4272
Inhibin B Enzyme-linked Immunosorbent Assay	Ansh Labs	AL-107
KnockOut Serum Replacement	Thermo Fisher Scientific	10828-028
MicroTissues 3D Petri Dish micro-mold spheroids (24-35, 5x7 array)	Millipore Sigma	Z764051
Nunc, Lab Tek II Chamber Slide System, 4-well	Thermo Fisher Scientific	12-565-7
Penicillin/Streptomycin	Thermo Fisher Scientific	15-140-122
Richard-Allan Scientific; Histogel, Specimen processing gel	Thermo Fisher Scientific	HG-4000-012
SOX9 primary antibody	Millipore Sigma	AB5535
Tedklad Global Mouse Chow (Breeder)	Teklad Global	2920
Tedklad Global Mouse Chow (Maintenance)	Teklad Global	2916
Testosterone Enzyme-linked Immunosorbent Assay	Calbiotech	TE373S
Trypan Blue Solution, 0.4%	Thermo Fisher Scientific	15250061
αSMA primary antibody	Millipore Sigma	A2547

βCatenin primary antibody	BD Biosciences	610154

Comments/Description
For sterile filtering media
Leydig cell marker, 1:500 dilution
Fluorescence-tagged secondary antibody
Fluorescence-tagged secondary antibody
Base of culture media
For dissociation solution 1
Extracellular matrix used for casting 2D and 3D ECM culture gels
Autmated cell counter (hemacytometer machine)
Hemacytometer slide for use with Countess automated counter
Spermatogonia marker, 1:500 dilution
For dissociation solution 1
For reconstituting Hyaluronidase
PBS
For reconstituting Collagenase I and Dnase I
For quencing enzyme dissocation solutions
For long-term organoid culture
For long-term organoid culture
For dissociation solution 2
Inhibin B ELISA Kit
Serum source for Basal media
For 3D ECM-Free organoid fabrication
For 2D ECM-free, and 2D, 3D ECM culture
Antibiotic for media
For aiding paraffin embedding
Sertoli Marker, 1:500 dilution
Mouse food without phytoestrogens
Mouse food without phytoestrogens
Testosterone ELISA Kit
For cell counting
Peritubular marker, 1:500 dilution

Sertoli Cytoplasm marker, 1:100 dilution

Dr. Xiaoyan Cao and the JoVE Editorial Team,

We thank you for the opportunity to re-submit our revised article to your journal after peer review. We have found the critiques from review to be very helpful and have compiled an updated manuscript which incorporates much of the criticisms, suggestions, and feedback from each reviewer.

The manuscript text document details all revisions using Word software track-changes. To ease the process of review, we have provided two copies of the manuscript text, first without track changes and then with track changes, and two copies of the figures, first as compressed PDFs, secondly as full vector .ai files. The comments from each reviewer are independently addressed below in a copy of the review comments. The original comments are in black font. Our responses are in red font. Line numbers correspond to the current revised article (without track changes), and references to updated figures are all explained where applicable to the specific reviewer comment. Overall, we have made significant additions to the introduction and discussion sections of the manuscript, and adjusted language and included more details within the step by step protocol section. The original three figures have been expanded to cover four separate figures, with increased quantification provided in Figure 1, additional images provided in Figure 3, and reformatted endocrine data for Figure 4. These additions and revisions greatly strengthen this manuscript, and its use for other researchers in the fields of testicular biology, reproductive engineering and *in vitro* modeling, and organoid generation. We express our sincere gratitude to all reviewers of this manuscript.

Please do not hesitate to contact us should you require anything further to complete the review of this article. Thank you for your consideration of our work.

Sincerely,

Maxwell Edmonds and co-authors,

MD/PhD Candidate
Laboratory of Teresa K. Woodruff
Northwestern Medicine
Maxwell.edmonds@northwestern.edu
401-480-1194

Dear Dr. Edmonds,

Your manuscript, JoVE61403 "ECM and ECM-free Methods for Generating Murine Testicular Organoids," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **Apr 07, 2020**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Xiaoyan Cao, Ph.D.
Review Editor
JoVE
617.674.1888
Follow us: Facebook | Twitter | LinkedIn
About JoVE

_____

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
The manuscript has been thoroughly proofread and revised for grammar.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: KnockOut, Teklad Global, Matrigel, microwell, etc.
Thank you for bringing this to our attention. We have removed or replaced all of the above mentioned terms, and all terms with commercial connotations. Please see replacement terms below:
Matrigel → replaced with "ECM"
Microwell → replaced with "agarose 3D Petri dish"

3. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.
Thank you, we have proofread and eliminated all instances of personal pronouns and colloquial phrases.

4. 2.1: Please specify the concentration of isoflurane and mention how proper anesthetization is confirmed.
We have added in the concentration of isoflurane and provided a description of how anesthetization is confirmed prior to euthanasia.

5. 2.8.1: Please specify the cell concentration.
We have included the cell concentrations for each of the four methods described.

6. Figure 1: Please change the time unit "hrs" to "h". Please replace "Matrigel" with a generic term.
All "Matrigel" terms have been replaced with "extracellular matrix / ECM." We have replaced all mention of "hrs" to "h."

7. Figure 3: Please abbreviate liters to L (mL, µL) to avoid confusion.
We have changed all volume units to mL or µL.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This is a well-written manuscript by a respected group in the field of female reproductive biology. The topic is of interest to the reproductive biology community, but the results are too preliminary to be of interest or usefulness to the male reproductive biology field at this time. This reviewer recommends the authors continue refining culture conditions to enhance spermatogonial survival (should be quite possible by using the media formulations already in use for SSC cultures) and then to add components that support spermatogonial differentiation and then meiotic initiation (e.g. retinoic acid). The following are a list of minor and major concerns, (roughly listed in order of appearance in the manuscript). It is this reviewer's sincere hope that this critique will help improve the study going forward:
Thank you for your perspective.

* Well-written
Thank you.

* Would be useful for the authors to clearly describe the different types of in vitro culture - this reviewer has seen in vitro as a term to describe what are more accurately explant cultures (e.g. Sato et al., 2011) as well as what is described here organoids from single cell suspensions
Thank you, this is great advice and will be very valuable for future readers. We have revised the introduction to briefly address historical methods referred to as “in vitro” testicular models, and then have stated a definition for “organoid” which is used throughout the remainder of the manuscript (paragraphs 2 & 3 of the introduction, lines 62-97).

* 68-9: Germ should be lower-case
“germ” cell has been made to lower case in all instances where it was capitalized. Thank you.

* Should also reference the first in vitro spermatogenesis reports - Champy, 1920 (rabbits) and Martinovich, 1937 (mice) - it gives readers a historical context, as well as a sense of how much progress has been made in the past ~100 years (truthfully not that much) in terms of in vitro spermatogenesis
This is a fantastic suggestion. We have added reference to both of these historical publications within the newly added second paragraph of the introduction (line 67).

* 71-2: has maintenance of germ cells really been that much of a challenge in explant cultures?
Yes, this has been a challenge for the field at large. While explants have been successful in producing fertilization-competent sperm on occasion (Sato 2011, recent publications by Takehiko Ogawa Laboratory), there are some drawbacks: (1) These techniques have been very challenging to replicate, especially over timeframes longer than 3-5 days, which is too short a timeframe to fully assess *in vitro* spermatogenesis. There is a lack of corroborating studies by other groups within the published literature, and this has been commented on by other authors in the field as well (Chapin 2016, Reda et al 2017). Our own experience with these published methods has provided very minimal germ cell persistence or development within as little as 3-7 days (Woodruff unpublished data not shown). This observation is also with the use of specialized microfluidic culture systems, a method highlighted for its benefit to explant culture by the Ogawa group, but also unable to be replicated comparably in our own hands (Komeya 2017, Woodruff unpublished data not shown). Historically, there has been a large impediment in taking spermatogonia into and through meiosis *in vitro*, as well as maintaining spermatogonia populations longitudinally over time in explants (Komeya review 2018). Many authors have hypothesized that haploid germ cells identified after *in vitro* explant culture are cells which had already committed to meiosis before culture, or were preexisting, and therein were only ‘maintained’ across culture. Other than the Ogawa group, it has been postulated that *in vitro* explant culture might only enable spermatocytes already involved in the first prophase to finish meiosis, or, haploid round spermatids to further differentiate into elongating spermatids *(*E. & A. Steinberger 1964, 1966, 1967*)*. Maintenance and recruitment of spermatogonia towards meiosis might be achievable in sporadic events, however even in the Ogawa publications, germ cell presence is often sporadic across a tissue section’s area or only limited to a sub-marginal area, and is often assessed through non-invasive measures (i.e. visualization of Acrosin-GFP using their transgenic mouse line), without confirmed quantification via histological methods. The work

of Ogawa is important as is publications like this one which provide rigorous step-by-step information that provides a fundamental starting point for all members of the field.

* 73: "instructed"?
This wording has been removed and re-clarified. Thank you.

* Why the space between 4, 35, etc and °?
Convention for the journal. JoVE requests that a space be left between all numbers and their corresponding unit designation.

* Curious - why maintain cells at 35°C instead of 33° or 34°C, which are more common in the literature?
35°C is also a commonly used temperature for testicular organoid culture. Alves-Lopes 2017 use 35°C (rat model). Baert & goosens 2017 also use 35°C (primary human testicular cells). Topraggaleh 2019 also uses 35°C (mouse). In contrast, because neonatal and juvenile testes are often undescended, there is some logic in actually using 37°C for the beginning of culture; Sakib 2019 uses 37°C (mouse organoids). In that case, it perhaps makes sense to start at 37°C and then lower the temperature after organoid morphogenesis has occurred, or after germ cell maintenance is established.
34°C is commonly used in tissue explant culture by the Ogawa group (Sato 2011 and others), but other successful explant modeling reports using prepubertal mouse tissues use 37°C (Ellie Smart…Norah Spears 2018). Ultimately, we chose a middle temperature, 35°C, because of its frequency of use for organoids, and to have a baseline relevant to other organoid publications in the field.

* Figure 2B-D: what is the green staining? Also, should stain with some other germ cell markers to distinguish types of germ cells - e.g. ZBTB16 for undiff spermatogonia, STRA8 for diff spermatogonia and preleps, SYCP3/REC8/H1T for spermatocytes, lectin for spermatids, etc
There was no green marker used in Figure 2, however, when yellow and red overlap they produce a greenish color (e.g. αSMA [yellow cell bodies of peritubular cells] over SOX9 [red nuclei of Sertoli cells]). Figure 2B-D is epifluorescence of a thicker sample instead of a 5 µm section (Figure 2 F-H, J-L, and N-P). Please see the next comment for responses to germ cell differentiation.

* How long can the organoids be maintained? How long do the germ cells survive? And more importantly, to what stage of development do they progress?
We have not tested organoids longer than 12 weeks, however, 3D ECM-free organoids are viable and maintain hormone-responsiveness and function throughout this entire period. Germ cells were rarely observed at 14 days into culture, but have not been observed longer than this. Organoids at 14 days have been screened with SALL4, a spermatogonial stem cell marker (Valli 2014), as well as SCP3 for pachytene spermatocytes. Rare SALL4 positive germ cells were observed, however no SCP3 signal was identified across 7 separate experiments. These new data have been added into Figure 3.

* 406: DDX4 is a pan germ cell marker, and not specific to spermatogonia (although this reviewer realizes spermatogonia are the only germ cell type present in the 5 dpp testis)
Thank you for pointing this out. We have adjusted the text of the manuscript (lines 381, 408-409, 454,) to clarify this point.

* Fig 2-3: given the large numbers of spermatogonia in the 5 dpp testis, it seems most are absent even after 72 hrs of culture; how many remain after 14 days? This reviewer does not see any in the image shown in Fig. 3C. Should stain these samples with a germ cell marker, e.g. DDX4/VASA or TRA98/GCNA1 to identify germ cell content of these organoids. If none remain, this reviewer wonders what the utility of this organoid system is to study spermatogenesis (when it's not occurring)? In this reviewer's opinion, there is currently little interest in the field for a system to study endocrinology in this context (with no germ cells)
Thank you for your comments, this is an interesting point relevant to the field of *in vitro* testicular modeling. We have probed for DDX4, SALL4, and SCP3 to investigate spermatogonia, spermatogonial stem cell, and spermatocyte presence and have found rare instances of DDX4 and SALL4, but no occurrences of SCP3 labelling (Current Figure 3). The maintenance of germ cells is a challenge in our system, and in other published testicular organoid systems (Baert 2017, Baert 2018, Pendergraft 2017, Sakib 2019, von Kopylow 2018). However, is it widely hypothesized that germ cells cannot differentiate without Sertoli-interactions or a supportive tubular niche environment. We propose that an organoid model containing spermatogonia at early time points, will enable the field to non-invasively study somatic testicular cells in a structurally and endocrine-functionally mimetic organoid. This could be used towards optimizing conditions for improved germ cell maintenance (i.e. with different media additives or microinjection of germ cells after tubule formation). Furthermore, an endocrine-functional model enables the incorporation of the testis and testicular hormones (i.e. testosterone & inhibin B) into larger systems-level Tissue-on-a-CHIP studies (Xiao 2017, Skardal 2020). This additional biological system modeling opportunity has been added into the discussion of the manuscript.

* 442-4: it has been known for a long time that rudimentary ring-like structures self-assemble between Sertoli cells even in 2-D cultures, and yet this reviewer is unaware of any researchers using that system to study tubulogenesis - I guess this reviewer is questioning the level of interest the male reproductive field has in such a system - the fetal testis has proven to be a much more useful and physiologically-relevant system for this type of work, as done in labs of Blanche Capel and her previous trainees (Humphrey Yao and Tony DeFalco, respectively)
The reviewer is correct that there is a long and successful history of studying fetal testicular morphogenesis, sex cord, and tubulogenesis. Organoids can be produced in high abundance from a small number of animals, making them higher throughput than using entire fetal gonads which require an entire fetus and a mother animal for each experiment. Furthermore, a larger goal of the organoid field is to produce technologies that will translate towards human-based utility or application in the future. Access to human fetal testicular tissue is incredibly rare and an ethically-challenging resource for basic science. For this reason, utilization of postnatal cell sources must be investigated. This ideally will include the development of human iPS derived testicular cell sources in the future.

* 448: inappropriate to cite a manuscript that is under review
We have removed mention of this, thank you.

* 469-471: to more fairly describe the results presented in Fig 2, would replace the following adjectives: "some" with "considerable"; "moderately" with "poorly"; "numerous" with "few"
Thank you for your thoughts on this word selection. We have adjusted the word choice in the manuscript to ensure the reader does not misunderstand the challenges of supporting

germ cells with the exact protocols provided in this manuscript. However, as the reviewer themselves have astutely stated in comments above, germ cell maintenance and full differentiation *in vitro* has been a challenge for the field over the past 100 years, and therefore is beyond the scope of this specific organoid-generation methods paper.

* 476-7: This is not really true - spermatogonia can be grown in culture for many months, while retaining their ability to seed spermatogenesis in germ cell-deficient testes following transplantation; would recommend the authors employ the culture conditions used for successful spermatogonial culture (e.g. those used by Oatley, Orwig, Shinohara, Brinster labs, etc); and the reference provided is not an ideal one - that study (ref 12) followed up on work from Paul Cooke's group, who showed that Sertoli cell number can be increased or decreased in vivo by extending or shortening their proliferative period by manipulating thyroid hormone signaling, and thus correspondingly change the size of the germ cell population (since each Sertoli cell can support a finite number of germ cells) - the cited Oatley lab paper (ref 12) extended those findings to show there were increased numbers of SSCs within that larger germ cell population
Thank you for pointing this out, we have clarified the statement on spermatogonia maintenance (line 554-555). We have also adjusted the cited manuscripts to more directly support the statement that germ cells require Sertoli cell interactions in order to progress through meiotic differentiation *in vitro*.

* 478-483: This may well be due to technical issues, that may be resolved by improving culture conditions; not fair to speculate about adult somatic cells' inability to support germ cells, when neonatal ones barely do, as presented in this manuscript
Thank you for your comments, we have removed these lines from the manuscript. These statements were referring to somatic cell observations in self-assembling and producing tissue-mimetic architecture, not the ability to maintain germ cells specifically (unpublished data not shown, *Woodruff 2020)*. In lieu of these comments, we have replaced the statements with mention of age and strain differences observed in *ex vivo* explant culture, and suggest that these need to be directly studied for testicular organoids as well (lines 565-571).

* 481-3: why mention "embryonic", when the previous sentence described postnatal?
Thank you. We have removed these statements (see comment above). Otherwise, we have simplified the language in this paragraph to “neonatal, juvenile, and adult.”

* 485: would replace "useful" with "preliminary" - what is the utility, when the germline is not maintained? What types of studies would benefit from these models?
Thank you for your feedback on this statement. We have chosen to keep “useful” instead of “preliminary,” for specific reasons: (1) The development of an *in vivo* mimetic architecture (i.e. tissue compartmentalization and tubule-like structures) is an important outcome, and a necessary phenotype to be accurately termed an “organoid” by the organoid field at large (i.e outside of reproduction), and one worth sharing to other research groups. Moreover, biomimetic phenotypes are not ubiquitous throughout published testicular organoid models, even for other mouse-based organoids (compare examples in Pendergraft 2017, Baert 2017, and Sakib 2019). This has been added in the discussion (lines 507-512). Moreover, our own representative results demonstrate that structurally mimetic organoids are not achieved in all four methods, but are preferentially observed in 2D ECM and 3D ECM-free methods. (2) The goal of this manuscript is to provide easy and accessible protocols for

developing and studying organoid self-assembly, not specifically to establish robust and long-lasting spermatogenesis *in vitro*. As we and the reviewer agree, *in vitro* maintenance of a germline is the long-term goal, however the achievement of structurally-mimetic and endocrine functional constructs is an iterative advancement in line with this shared goal in the field. (3) We have updated additional opportunities for endocrine functional and somatic cell dominant organoids in the last paragraph of the discussion (see lines 583-590). (a. Tissue-on-a-CHIP systems modeling, b. reproductive toxicology and barrier function, c. optimization of germ cell maintenance [however not specifically within the scope of this methods paper], d. assisted reproductive technology innovation.

Reviewer #2:

Manuscript Summary:
This report describes four methods (2D ECM-free, 2D ECM, 3D ECM-free, 3D ECM culture) to derive testicular organoids from prepubertal mouse testicular cells. All four methods described here are relatively simple and do not require specialized techniques or ingredients. Out of all the methods described, only one (3D ECM-free) appears to result in organoids that have morphological similarity to testis in vivo. The methods are well described and detailed enough to allow replication by other groups.
Thank you for your comments on the quality of our manuscript. We greatly appreciate your feedback.

Minor Concerns:

Line 91: Why would gravity settlement of cells yield a more biomimetic tissue assembly compared to cells that have been settled by centrifugation? Centrifugation should not affect cellular processes.
Thank you for your comments. Let us clarify: the methods proposed in this manuscript utilize gravity settlement so that cell-autonomous self-assembly (i.e. cellular migration, aggregation, organization) can be visualized and observed during live culture (i.e. timelapse imaging). This is according to commonly shared definitions of organoids in the field, (see third paragraph of introduction and/or lines 84-88). Centrifugation would not allow the observation of cell migration leading to assembly. Therefore, in the methods shared in this manuscript, all structures and organoids generated are cell-directed, and not the result of artificially imposed compaction from centrifugation. There are other methods published which centrifuge cells into an agarose microwell (similar to the 3D ECM-free system in our manuscript) (Sakib 2019, cited in-text). The phenotype of Sakib's organoids do not develop tubule-like structures, but have an inside-out orientation. Organoid phenotype differences are cited in the discussion (lines 507-512).

Line 101: An organoid would need to have a biomimetic morphology. Not all the structures produced by the authors really fit the definition of an organoid. According to Fig 2, only ECM-free 3D cultures seem to have resemblance to in vivo testes. The next closest biomimetic structure is the 2D ECM-free. The rest lack well defined structure.
We agree that organoids should possess a biomimetic morphology; not all published testicular organoids achieve such a phenotype (see citations on line 512 in text). However, we determined that 2D ECM and 3D ECM-free both have biomimetic morphology. We determined this by the compartmentalization of tubular versus interstitial cell types. While

2D ECM-free also shows Sertoli and germ cells together, they lack a tubule-like orientation, and are not singular organoids but outcroppings on top of a confluent somatic cell layer. For these reasons 2D ECM-free was not determined to produce full organoids.

Line 148: Could this protocol be applied to mice older than day 5?
Yes. We have explored using cells isolated from mice of 12 dpp, 21 dpp and 8 weeks (unpublished data, *Woodruff 2020*). We observed that self-assembly was delayed (compared to 5 dpp) when using juvenile mice (cells isolated from 12 or 21 dpp animals), and moreover assembly was entirely absent in adult cells (8 weeks). That being said, the direct purpose of this manuscript was to share the process of replicating these organoid generation techniques (which use exclusively 5 dpp mice), not the representative data, and so we believe a direct comparison of these techniques between several age groups is best covered in a separate article. However, a brief discussion of potential age-differences in *in vitro* modeling has been added to the discussion (Lines 565-571).

Line 204: Do cell preparations with viability less than 80% not form organoids? Under the digestion protocol described by the authors, what is the average viability? If the viability is low, what strategies would the authors suggest to improve that?
These are great questions, and will be useful for future users of the protocol. We have not tested if cell preparation with < 80% viability will produce organoids; this is because we aim to incorporate as few dead and dying cells as possible into our developing organoids. Our digestion protocol is very similar to others used in the field for many years (for an example see Gassei and Schlatt 2006); we achieve 91% viability on average (N=8) as determined with Trypan Blue exclusion assay. We have added text into our discussion on options for ameliorating low viability of cell preparations (lines 522-526), this is a great addition to the manuscript, thank you.

Line 207: It would be more useful, if the authors also mentioned a range of cell numbers required for these protocols. What is the minimum number of cells required for successful formation of organoids? What is the maximum number of cells that can be used to form organoids, without causing a necrotic core?
These are also great questions. We have do not have direct answers, however can postulate given our own experience and familiarity with the literature. Sakib et al 2017 explored the limits of producing mouse testicular organoids from minimal number of cells and identically aged animals (5 dpp). They could produce organoids with as few as 125 cells (Sakib 2017). We have added this to our discussion (added in context on lines 503). All organoids aim to prevent or avoid necrosis, however this is beyond the specific scope of this manuscript. However, we have addressed the observations of necrotic-appearing locations in some organoids (in text line 503-504).

Line 282: Did the authors evaluate the viability during formation? Cells embedded in Matrigel along with 500uL of media on top may experience impaired gas exchange and nutrient transfer. This could account for lack of biomimetic morphology (Fig. 2). What steps can be taken to address such outcomes?
Thank you for your comments. Embedding singular cells within 3D ECM (including Matrigel) has been widely used throughout the testicular organoid field (Alves-Lopes 2017, Vermeulen 2019) and in other organoid models as well (i.e. intestinal and prostate organoids of Hans Clevers). However, in our own studies, we did not assess viability of cells/organoids during active formation (but all were performed in pre-gassed and warmed

media during cell culture incubation). Beyond this, we cannot directly comment on this possibility within our own culture systems. We appreciate your insights into the differences in nutrient/gas exchange accessibility between the four generation methods.

Line 259, 269: 280,000 cells suspended in 60uL even for 24hrs, seems like a fairly high density. How soon is the media used up by these cells? When the 200uL media is removed with 1mL, what is the overall distance between the cell cluster in the insert and the media meniscus and how does it compare to the 2D ECM, 2D ECM free and 3D ECM organoids? Is the 3D ECM free maintained at gas liquid interphase? If so, then these organoids may have better gas exchange than the 2D ECM, 2D ECM free and 3D ECM organoids. Could that account for lack of biomimetic structure in those experiments?
Thank you for your comments, please let us clarify. 3D ECM-free organoids are not cultured at an interphase, and are completely submerged under 1 mL of media after the initial 24 h of culture (and not nearby a meniscus). We have clarified this on lines 292-294. During the initial 24 h when there is only 260 uL of total media for this method, we have never observed a gross change in color of the media suggesting acidity (as would be expected with an aMEM-based medium), nor in any of the 4 methods. All four methods are completely submerged in media by 24 h of culture, the only difference being the 2D/3D and ECM/ECM-free environment.

Line 343: The 3D ECM group does not seem to have any testes specific morphology.
We agree. The text is updated to state that 3D ECM-free and 2D ECM methods promote superior biomimicity (lines 385-394 and 504-507).

Line 373: The evidence of Sertoli cell polarization is not satisfactory. B-catenin seems to be primarily localized in the Sertoli cell nucleus and does not show the cell body.
While Bcatenin is observed adjacent to the Sertoli cell nucleus in many cells, it is not observed “in” the nucleus. Bcatenin is not specific to the nuclear membrane (the nucleus is visualized via DAPI), but is a member of plasma membrane junctional complexes and is particularly well observed at locations of Sertoli-Sertoli junctions (Mruk & Cheng 2015). This can be observed well in Figure 3J-L, as Sertoli cells are extending into the center of the tubule-like-structure along with tight junctional protein ZO-1, but their nuclei are not, they are aligned along the basal membrane on the exterior of the tubule like structure.

Fig. 2: B, C, D lack clarity. Should be replaced with higher resolution images. M, N, O, P images could benefit from higher magnifications.
Thank you for your comments. Figures 2B-D are epifluorescent images of the entire 2D ECM-free sample, not only a 5 um section. We selected these images because they provide a less biased and more transparent perspective of these samples. 2D ECM-free samples largely form a confluent 2D layer of somatic cells, on top of which, some aggregations of Sertoli and germ cells can be observed (which are focused upon with the microscope in Figure 2B-D). A single plane image would not demonstrate this dynamic. (This has been updated in the representative data and is stated in the figure legend.) Additionally, we selected the current scale for images M-P so that they are identical with the other conditions, allowing direct side-by-side comparison by the reader. If they look drastically different (i.e. size and structure), and that is resulting from an un-skewed interpretation of identically processed data (and therefore real differences).

Reviewer #3:

Manuscript Summary:
In their study "ECM and ECM-free methods for generating murine testicular organoids" Edmonds and colleagues describe four different culture conditions useful for the generation and study of testicular organoids. The authors employ ECM and ECM-free approaches in 2D or 3D conditions and add a description regarding the analysis of the functionality. Analysis of the generated organoids is mainly based on morphology, immunofluorescence staining as well as hormonal assays describing the production of testosterone and inhibin b in one of the culture conditions for up to 12 weeks.
Overall, the study is well-written and focuses on a very interesting and important aspect of in vitro differentiation techniques useful to understand and study testicular physiology. However, some parts of the manuscript and experiments should be addressed more in detail to improve the impact of this study for the people interested in this topic.
Thank you for your feedback to improve this article.

Major Concerns:

1) The introduction summarizes the field of testicular organoids by mentioning several techniques, however, the aspect of different age groups and the results obtained with other methods should be discussed. It would be interesting to see if the four approaches are showing testicular organoid formation, when applied to different age groups, e.g. newborn, pubertal, and adult tissue samples.
We agree with the reviewer's comments concerning interest in age-differences for *in vitro* modeling. We have updated the discussion (lines 565-571) include a reference to age differences in other *ex vivo* testicular culture techniques, as well as a statement expressing the need for age to be considered biological variable in organoid generation as well. However, the direct purpose of this manuscript was to share the process of replicating these organoid generation techniques, not the representative data itself, and so we believe a direct comparison of these techniques between several age groups is best covered in a separate article.

2) Please comment if a different strain might result in different outcomes in terms of organoid formation. This might be interesting in the light of a publication by Portela and colleagues last year in Dev Biology describing differences of two mice strains in terms of in vitro spermatogenesis using the explant tissue culture conditions. Additional experiments showing the reproducibility with at least one other mouse strain (e.g. C57/Bl6 or DBA) might be useful to show the robustness of the conditions described.
This is an interesting point, particularly because *in vitro* spermatogenesis (and spermatogonial stem cell culture) has been variable between mouse strains, and is particularly more effective when using DBA-background mice. However, we believe a direct comparison of mouse strain is beyond the scope of this specific methods papers and is best covered in detail in a separate primary research article. However, we have provided reference to Portela et al, and mention in the need to study this same "strain" variable for organoid culture as well (line 565-571). Thank you for this question, we believe it should have priority in future reports.

3) Point 2.4.2: "even more volume if needed" is a rather unspecific description for a detailed protocol. Please be more specific to help the reader to plan the experiments.

We agree with the reviewer's comments. We have revised the statements to give specific and conservative recommendations for volume per testis number when performing the enzymatic dissociation (1 mL per 10 testes). Please see lines (197-199).

4) Point 2.5: Does the solution 2 also needs to be pre-warmed? Or is it just the additive of hyaluronidase?
This step is the addition of only 33 uL hyaluronidase stock to the pre-existing Solution 1 (already containing partially dissociated tissue). It is not the addition of a separate 'solution 2'. i.e Solution 1 + hyaluronidase = Solution 2. There is a NOTE describing this definition in section 1 (lines 161-163). We have also added further description to avoid confusion at the step of adding hyaluronidase (lines 206-208).

5) Point 2.8: Please add the details of the "hemocytometer" and how much volume should be used.
We have added description to this, and also added reference to the Table of Materials (lines 224-226)

6) I suggest to add the concentration rather than the total cell number. This would allow the reader to check if different culture plates can be used.
Thank you for the recommendation. We have included both cell numbers used for the representative data (280,000, lines 233-237) and also added concentrations specific to each culture type (lines 248, 260, 288, and 301).

7) Point 3: Please add more details of the Matrigel already here; e.g. standard Matrigel or is it growth factor reduced Matrigel or do both types work?
The specific Matrigel we use is listed in the Table of Materials. It is standard Matrigel, not growth factor reduced, however, considering organoids also form in the absence of exogenous ECM (and with other ECMs published; see Sakib 2019, Alves-Lopes 2017, Vermeulen 2019), we have confidence that many types of Matrigel/ECMs will work for this protocol. However, comparing different commercially available ECMs was not the intention of this methods paper.

8) Point 3.3.2: Please explain what is meant by "green 3D Petri-Dish microwell…" I suggest to keep the names provided in the list of materials used. Otherwise this might lead to confusions.
We have replaced this term with "3D Petri dish mold" or "agarose 3D Petri dish" to limit confusion. The mold used for casting agarose 3D Petri dishes is a green color (mold supplier is listed in Table of Materials). However, we have removed this description as suggested, in order to limit confusion. There are highly detailed instructions for casting which come provided with the commercially available molds (that being said, it is a very simple process); the vendor is listed in the Table of Materials (Microtissues brand). Nevertheless, the instructions provided in this manuscript should be sufficient for a user to replicate 3D Petri dish fabrication, even without the commercially available instructions.

9) Can you really store the agarose microwells in sterile H2O or DPBS at 4C indefinitely? I suggest to state a less "long time period".
We agree with these comments and have changed to a conservative recommended storage period of 1 month at 4 ºC (line 276-277), although we have experience using them multiple months after fabrication.

10) Point 4.2: Please remove the statement "equivalent to 12 days post-partum in vivo", as there are no comparisons made to show this. Just calculating 5dpp plus 7 days in vitro does not result in a similar situation of 12 dpp. I understand that the authors like their systems, however, they are still in vitro systems, which might have different effects on cells compared to the complex situation in vivo.
Thank you for your comments, we have removed the above language.

11) Point 5.4.: Please add some more information here. It is not really clear what the indention of this point is.
Thank you for this perspective, this is an important point and we have added detail to clarify its intention (lines 347-351). Organoids can be very small and difficult to identify after paraffin embedding and microtome sectioning. Step 5.4 is advice on how to facilitate easier histological processing.

12) Results: Overall, to judge the reproducibility of the conditions, quantifying different cell types as well as additional measurements (e.g. organoids sizes) are needed. It is mentioned in the text that the size of the organoids formed can be influenced (line 437), however this is not shown and should be added if needed.
Thank you for your comments. We have added in quantification of organoid assembly dynamics as well as organoid sizes between the four different culture conditions in order to better demonstrate the differences between methods (Figure 1E & 1F). We have also removed reference to cell numbers changing organoid size for which data was not provided, but have provided reference to another paper which observed this ability for testicular organoids (Sakib 2017).

13) The cell clusters formed in all conditions are identified as testicular organoids, however, sticking to the nature of the word "organoid" and its definition, the functionality is only shown in detail for cell clusters formed in 3D-ECM-free conditions. I suggest to add a paragraph to the discussion regarding the definition of organoids as such as how close the described structures meet these criteria. In case assays needed to show the functionality are missing, those need to be added or the structures formed in vitro needed to be renamed.
We agree with the reviewer that this is an important topic for distinction. Many authors across multiple organ types and systems publish in vitro assembled tissues which are deemed “organoids” but actually do not have biomimetic structure or function. This is also true for testicular organoids, of which there is a large degree of morphological differences between different reports. We have added additional text in paragraphs 2 and 3 of the introduction to describe and explicitly state the organoid definition used in this manuscript (explicitly stated on lines 84-88). We determine organoids to *de novo* biological tissues which mimic the native tissue in morphology and some function (although full function; large processes [e.g spermatogenesis] can are ambitious goals). We viewed *de novo* compartmentalization of tubular versus interstitial cell types as meeting both our organoid requirements (2D ECM and 3D ECM-free models). Those that do not meet our criteria are termed “biological constructs or tissues.”

Minor Concerns:
1) The term "neonatal" suggests a slightly different age, as neonatal in mice can be more related to age of 1 to 2 days, maximum 3dpp, instead of 5dpp. I suggest to state "juvenile" instead. In principle, this is just a minor issue, as mice at a younger age might generate

similar results. However, since this has not been tested, the age should be mentioned more correctly to avoid confusions.
Thank you for your perspective. We have updated the manuscript to state either the exact age of mice used (5 dpp), or the terms “juvenile” or “immature” testes.

2) Point 2.4, please add the temperature to "pre-warmed". I assume this is 37C as stated above, however, a protocol should be written the way that you can follow every step by step, without checking the description provided before in the general text.
Thank you, we have updated the temperature.

3) As mentioned in the introduction, one of the purposes of the study is to provide an easy to use system, however, reading point 3.3.4 to 3.3.6 I was wondering, how easy is it to produce the agarose microwells and how stable are those for the handling. Maybe this can be discussed a bit more in the discussion part.
Thank you for your concern. Casting agarose to produce 3D Petri dishes is a straightforward protocol, with high-detailed additional instructions provided by the providing manufacturer (Microtissues.com/Sigma Aldrich, cited in the Table of Materials). We have provided full details in our protocol so that a user can fabricate the agarose 3D Petri dishes even without these additional instructions. Once fabricated, it is in fact very easy to handle the agarose 3D Petri dishes using fine forceps, as long as the agarose percentage is at least 2% and the user does not handle them roughly. We have updated the discussion section to state this (lines 535-539).

4) Please change the reference "Edmonds, 2020, in review" to "own unpublished data" unless it has been accepted.
We have made this change.

Figure 1 -AI

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure 1 revised_04.04.20.ai

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

This is an Adobe® Illustrator® File that was saved without PDF Content.
To Place or open this file in other applications, it should be re-saved from Adobe Illustrator with the "Create PDF Compatible File" option turned on. This option is in the Illustrator Native Format Options dialog box, which appears when saving an Adobe Illustrator file using the Save As command.

Author Profile: Maxwell E. Edmonds

Maxwell is a current MD/PhD student in the Medical Scientist Training Program at the Northwestern University Feinberg School of Medicine. He is completing his dissertational studies under the advising of Dr. Teresa K. Woodruff.

Author Profile: Micah D. Forshee

Micah is a former summer undergraduate fellow at the Northwestern University Feinberg School of Medicine. He is currently pursuing his PhD at the Baylor College of Medicine.

Author Profile: Teresa K. Woodruff

Teresa is the Thomas J. Watkins Memorial Professor of Obstetrics and Gynecology at the Northwestern University Feinberg School of Medicine. She is also the Associate Provost for Graduate Education, the Dean of the Northwestern University Graduate School, and the Vice Chair for Research in the Department of Obstetrics and Gynecology.

jove

Vineeta Bajaj <vineeta.bajaj@jove.com>

Fw: Re-print permissions - BF-102511.R1 - ab9907
1 message

Maxwell Ethan Edmonds <maxwell.edmonds@northwestern.edu>
To: Vineeta Bajaj <vineeta.bajaj@jove.com>

Hello Dr. Bajaj,

Please see the permissions from IOP publishing for our manuscript below. Again, I apologize for the delay in receiving this. I have one question:

1. Given their language in the permission, I believe we might need to add in the text : "**© IOP Publishing. Reproduced with permission. All rights rese** that we include DOI or first page # information when citing the original work. (See their descriptions below).

I'm not sure that the bolded text or page numbers are in the manuscript copy I uploaded earlier this month. Can you assist in checking/adding these statem send it back, and I can add them.

Thank you very much. I await your instruction.

Sincerely,

- Maxwell

Maxwell E. Edmonds, PhD
MD/PhD Candidate
Laboratory of Teresa K. Woodruff, PhD
Northwestern Medicine

From: Permissions <permissions@ioppublishing.org>
Sent: Monday, June 22, 2020 2:20 AM
To: Maxwell Ethan Edmonds
Subject: Re: Re-print permissions - BF-102511.R1 - ab9907

Dear Maxwell,

Thank you for your response. I apologise for the issue you have had using the CCC Republication Service. I will look into why the required email did not reach you, howev provided the requested permission manually below:

Thank you for your request to reproduce IOP Publishing material *in* ***"ECM and ECM-free Methods for Generating Murine Testicular Organoids"*** *to be published in* ***Journa Experiments.***

Regarding:

Figures 1, 5, 6, *&* ***Supplement 1*** *from* ***"Testicular organoid formation is a property of immature somatic cells, which self-assemble and exhibit long-term hormo function"***

We are happy to grant permission for the use you request on the terms set out below.

License to publish material published by IOP Publishing
Please provide the below to your new publisher as proof of permission.

Conditions

Non-exclusive, non-transferrable, revocable, worldwide, permission to use the material in print and electronic form will be granted **subject to the following conditions:**

- Permission will be cancelled without notice if you fail to fulfil any of the conditions of this letter.
- You will reproduce the following prominently alongside the material:
  - the source of the material, including author, article title, title of journal, volume number, issue number (if relevant), page range (or first page if this is the onl date of first publication. This information can be contained in a footnote or reference note; or
  - a link back to the article (via DOI); and
  - **if practical and IN ALL CASES for works published under any of the Creative Commons licences the words "© IOP Publishing. Reproduced with permissio**
- The material will not be used in any way which, in the opinion of IOP Publishing, could distort or alter the original intention(s) and meaning, be prejudicial to the h co-author(s) and/or imply endorsement by IOP Publishing.

- Payment of £0 is received in full by IOP Publishing prior to use.

This permission does not apply to any material/figure which is credited to another source in our publication or has been obtained from a third party. Express permission for such mater from the copyright owner.

Kind regards,

Sophie
Copyright & Permissions Team
Sophie Brittain - Rights & Permissions Assistant
Cameron Wood - Legal & Rights Adviser
Contact Details
E-mail: permissions@ioppublishing.org

For further information about copyright and how to request permission: https://publishingsupport.iopscience.iop.org/copyright-journals/
See also: https://publishingsupport.iopscience.iop.org/
Please see our Author Rights Policy https://publishingsupport.iopscience.iop.org/author-rights-policies/
Please note: We do not provide signed permission forms as a separate attachment. Please print this email and provide it to your publisher as proof of permission. **Please** made by IOP Publishing to the effect that authors do not need to get permission to use any content where IOP Publishing is not the publisher is not intended to constitut Authors must make their own decisions as to the suitability of the content they are using and whether they require permission for it to be published within their article.

From: Maxwell Ethan Edmonds <maxwell.edmonds@northwestern.edu>
Sent: 17 June 2020 13:59
To: Permissions <permissions@ioppublishing.org>
Subject: Re: Re-print permissions - BF-102511.R1 - ab9907

Hello Sophie,

Thank you for your response. Please see the correspondence I received when submitting the application. I was only given an order number, but no confirm received later as suggested.

Thank you, and let me know if I can provide you with anything further.

- Maxwell

Maxwell E. Edmonds, PhD
MD/PhD Candidate
Laboratory of Teresa K. Woodruff, PhD
Northwestern Medicine

From: no-reply@copyright.com <no-reply@copyright.com>
Sent: Friday, June 12, 2020 11:12 AM
To: Maxwell Ethan Edmonds
Subject: Thank You for Your Order on Marketplace™

Marketplace™

Dear Maxwell Edmonds,

Thank you for placing your order on Marketplace™.

Order Summary:
Order date: 12 Jun 2020
Order number: 1041361
No. of items: 1
Order total: 0.00 USD

Billing Summary:
Payment method: Invoice
An invoice will be generated and emailed within 24 hours.

To view your order details, click the following link, sign in, and search for your order:Manage Account.

Please do not reply to this message.

To speak with a Customer Service Representative, call +1-855-239-3415 toll free or +1-978-646-2600 (24 hours a day), or email your questions and comments to support@copyright.com.

Sincerely,

The CCC Marketplace Team

Maxwell E. Edmonds
MD/PhD Candidate
Laboratory of Teresa K. Woodruff, PhD
Northwestern Medicine

From: Permissions <permissions@ioppublishing.org>
Sent: Wednesday, June 17, 2020 7:21 AM
To: Maxwell Ethan Edmonds
Cc: Biofabrication; Teresa K Woodruff
Subject: Re: Re-print permissions - BF-102511.R1 - ab9907

Dear Maxwell,

Thank you for your email and I apologise for the delay in dealing with your request.. I have been unable to locate your application on the CCC system - would you be able (600XXXXXX)?

Kind regards,

Sophie

Copyright & Permissions Team
Sophie Brittain - Rights & Permissions Assistant
Cameron Wood - Legal & Rights Adviser
Contact Details
E-mail: permissions@ioppublishing.org

For further information about copyright and how to request permission: https://publishingsupport.iopscience.iop.org/copyright-journals/
See also: https://publishingsupport.iopscience.iop.org/
Please see our Author Rights Policy https://publishingsupport.iopscience.iop.org/author-rights-policies/
Please note: We do not provide signed permission forms as a separate attachment. Please print this email and provide it to your publisher as proof of permission. **Please** made by IOP Publishing to the effect that authors do not need to get permission to use any content where IOP Publishing is not the publisher is not intended to constitut Authors must make their own decisions as to the suitability of the content they are using and whether they require permission for it to be published within their article.

From: Maxwell Ethan Edmonds <maxwell.edmonds@northwestern.edu>
Sent: 16 June 2020 01:58
To: Permissions <permissions@ioppublishing.org>
Cc: Biofabrication <bf@ioppublishing.org>; Teresa K Woodruff <tkw@northwestern.edu>
Subject: Re: Re-print permissions - BF-102511.R1 - ab9907

Hello Sophie,

I am emailing to ask about the status of our reprint permissions application? It was submitted per your instructions a few days ago, and we were told to exp hours. We haven't heard anything yet.

Could you help us look into the status of our application? Thank you!

Sincerely,

- Maxwell

Maxwell E. Edmonds, PhD
MD/PhD Candidate
Laboratory of Teresa K. Woodruff, PhD
Northwestern Medicine

From: Permissions <permissions@ioppublishing.org>
Sent: Friday, June 12, 2020 5:33 AM
To: Maxwell Ethan Edmonds
Cc: Biofabrication; Teresa K Woodruff
Subject: Re: Re-print permissions - BF-102511.R1 - ab9907

Dear Maxwell,

Thank you for your email and for taking the time to seek this permission.

We have set up a service with CCC's Republication Service, Marketplace, to handle permissions requests for IOP Publishing owned journals.

Because your request is for content from an IOP Publishing owned journal, you should make your request through the CCC Republication Service. This can be done by go and searching for the journal title you wish to use content from in the Get Permissions search bar in the top right hand corner.

Please then use the 'Republish or Display Content' tab and select the appropriate way that you will be reusing the content.

Where you are the **original author of the content** you wish to reuse, please ensure you select 'Author of requested content' from the drop-down list "Who will republish

If you have any further queries please do not hesitate to contact us.

Kind regards,

Sophie
Copyright & Permissions Team
Sophie Brittain - Rights & Permissions Assistant
Cameron Wood - Legal & Rights Adviser
Contact Details
E-mail: permissions@ioppublishing.org

For further information about copyright and how to request permission: https://publishingsupport.iopscience.iop.org/copyright-journals/
See also: https://publishingsupport.iopscience.iop.org/
Please see our Author Rights Policy https://publishingsupport.iopscience.iop.org/author-rights-policies/
Please note: We do not provide signed permission forms as a separate attachment. Please print this email and provide it to your publisher as proof of permission. **Please** made by IOP Publishing to the effect that authors do not need to get permission to use any content where IOP Publishing is not the publisher is not intended to constitut Authors must make their own decisions as to the suitability of the content they are using and whether they require permission for it to be published within their article.

From: Maxwell Ethan Edmonds <maxwell.edmonds@northwestern.edu>
Sent: 05 June 2020 21:41
To: Biofabrication <bf@ioppublishing.org>
Cc: Teresa K Woodruff <tkw@northwestern.edu>
Subject: Re-print permissions

Dear Sarah Obertelli, Biofabrication, and IOP Publishing,

We were thrilled to see our work coming online on your platform earlier this week. (Edmonds et al 2020 *Biofabrication* https://doi.org/10.1088/1758-5090/ab990

As was stated in the submission for this manuscript, we have an additional manuscript under consideration at the Journal of Visualized Experiments (JoVE to request reprint permission from Biofabrication. Specifically this article will be using some, but not all, of the figures in the manuscript we have recently pu

Please let us know how we can proceed to to gain the reprint permission for JoVE. The JoVE article will be citing Biofabrication as the source for the reprin

Thank you, and best wishes.

- Maxwell

Maxwell E. Edmonds, PhD
MD/PhD Candidate
Laboratory of Teresa K. Woodruff, PhD
Northwestern Medicine

From: Biofabrication <bf@ioppublishing.org>
Sent: Monday, May 11, 2020 6:40 AM
To: Maxwell Ethan Edmonds
Subject: Re: Source Figures for Re-submission

Dear Maxwell E. Edmonds

Thank you for your email. Please can we suggest that you send us your figure files as a zipped file via email and I will upload them for you.

Best regards

Sarah Obertelli

Biofabrication
BF 2018 Impact Factor = 7.236

Publishing Team:
Publisher: Richard Kelsall

From: Maxwell Ethan Edmonds <maxwell.edmonds@northwestern.edu>
Sent: 09 May 2020 21:43
To: Biofabrication <bf@ioppublishing.org>
Subject: Source Figures for Re-submission

Dear Jonathan Keen and Editorial Staff,

We have recently re-submitted our manuscript via the IOP author portal to Biofabrication. The manuscript ID is: BF-102511.R1. However, there was not enough space or of our source Figure files. Please let us know how we might submit these source files to you, and we will do so right away.

Thank you again for your consideration of our manuscript.

Sincerely,

Maxwell

Maxwell E. Edmonds
MD/PhD Candidate
Laboratory of Teresa K. Woodruff, PhD
Northwestern Medicine

_____
From: Maxwell Ethan Edmonds
Sent: Wednesday, April 29, 2020 5:08 PM
To: bf@ioppublishing.org
Subject: Re: Our initial decision on your article: BF-102511

Dear Editor Keen,

We thank you for your consideration of our manuscript. We are very pleased on the feedback and reviews in your provided report. We are revising the manuscript curren the Author Centre before the due date of June 3rd.

Thank you once again for your consideration of our work. We look forward to publishing with you.

Sincerely,

Maxwell E. Edmonds, PhD
MD/PhD Candidate
Laboratory of Teresa K. Woodruff, PhD
Northwestern Medicine

_____
From: Biofabrication <onbehalfof@manuscriptcentral.com>
Sent: Wednesday, April 15, 2020 9:22 AM
To: Maxwell Ethan Edmonds; maxandla@gmail.com
Cc: Maxwell Ethan Edmonds; maxandla@gmail.com; Teresa K Woodruff
Subject: Our initial decision on your article: BF-102511

Dear Mr Edmonds,

Re: "Testicular organoid formation is a property of immature somatic cells, which self-assemble into tubular structures and exhibit long-term hormone-responsive endocri
Maxwell; Woodruff, Teresa
Article reference: BF-102511

We have now received the referee report(s) on your Paper, which is being considered by Biofabrication.

The referee(s) have recommended that you make substantial changes to your article. The referee report(s) can be found below and/or attached to this message. You ca your Author Centre, at https://eur02.safelinks.protection.outlook.com/?url=https%3A%2F%2Furldefense.com%2Fv3%2F__https%3A%2F%2Fmc04.manuscriptcentral.cc Dq0X2DkFhyF93HkjWTBQKhk!HbYHUgdWJsDVPC1mJqHcAhz7JD0GEzyrGXuwgHh4WhryjCot1uZgDzD454FcLMDT2nZM1DJI3kKD%24&data=02%7C01%7Cb 7C4e33fe03a01148d2f7f408d7f46201cf%7Cf9ee42e6bad04e639115f704f9ccceed%7C0%7C0%7C637246574097548811&sdata=YwCs%2BWl2IBGp34yQiALgxt9 MAU95LKmjM%3D&reserved=0

Please consider the referee comments and amend your article according to the recommendations. You should then send us a clean final version of your manuscript. Ple files) point-by-point replies to the referee comments and either a list of changes you have made or an additional copy of your manuscript with the changes highlighted (fo https://eur02.safelinks.protection.outlook.com/?url=https%3A%2F%2Furldefense.com%2Fv3%2F__https%3A%2F%2Fpublishingsupport.iopscience.iop.org%2Fquestion revised-article%2F__%3B!!Dq0X2DkFhyF93HkjWTBQKhk!HbYHUgdWJsDVPC1mJqHcAhz7JD0GEzyrGXuwgHh4WhryjCot1uZgDzD454FcLMDT2nZM1JRMHqr0%24 02%7C01%7Cbf%40ioppublishing.org%7C4e33fe03a01148d2f7f408d7f46201cf%7Cf9ee42e6bad04e639115f704f9ccceed%7C0%7C0%7C637246574097548811&amp 2Fr9GKWplczKU4pxuh4gfYnGpvaZFOlESkAXQdPXINAU%3D&reserved=0). This will aid our referees in reviewing your revised article. Please upload the final ver files to your Author Centre by 03-Jun-2020.

If we do not receive your article by this date, it may be treated as a new submission, so please let us know if you will need more time.

Please note that if the referee(s) and Editorial Board are not satisfied with the changes to your manuscript, it may still be rejected.

We look forward to hearing from you soon.

Yours sincerely

Johnathan Keen

On behalf of the IOP peer-review team:
Johnathan Keen - Editor
David Jones and Miriam Howland - Associate Editors
Sarah Obertelli - Editorial Assistant

IOP Publishing guidance on Covid19 (Coronavirus): https://eur02.safelinks.protection.outlook.com/?url=https%3A%2F%2Furldefense.com%2Fv3%2F__https%3A%2F%
2Fioppublishing.org%2Fnews%2Fcoronavirus-covid-19-faqs%2F__%3B!!Dq0X2DkFhyF93HkjWTBQKhk!HbYHUgdWJsDVPC1mJqHcAhz7JD0GEzyrGXuwgHh4WhryjC
LMDT2nZM1Gg8CkCG%24&data=02%7C01%7Cbf%40ioppublishing.org%7C4e33fe03a01148d2f7f408d7f46201cf%7Cf9ee42e6bad04e639115f704f9ccceed%7C
7C637246574097548811&sdata=qdPr4LLAtkt7KBwgsgAccR%2FDjmBnnK5PLDJqrS8u0dA%3D&reserved=0

Want to find out what is happening to your submission right now? Track your article here: https://eur02.safelinks.protection.outlook.com/?url=https%3A%2F%2Furldefens
2Fv3%2F__https%3A%2F%2Fpublishingsupport.iopscience.iop.org%2Ftrack-my-article%2F%3Futm_source%3DTrack*20my*20article%26utm_medium%3DEmail__%
Dq0X2DkFhyF93HkjWTBQKhk!HbYHUgdWJsDVPC1mJqHcAhz7JD0GEzyrGXuwgHh4WhryjCot1uZgDzD454FcLMDT2nZM1MULpwqE%24&data=02%7C01%7C
7C4e33fe03a01148d2f7f408d7f46201cf%7Cf9ee42e6bad04e639115f704f9ccceed%7C0%7C0%7C637246574097548811&sdata=e%2FpDR4d2uX7DTQqhMdfmK
4lnoTuRsvQCWE%3D&reserved=0

bf@ioppublishing.org

Wei Sun - Editor-in-Chief
Richard Kelsall - Publisher

IOP Publishing
Temple Circus, Temple Way, Bristol
BS1 6HG, UK

https://eur02.safelinks.protection.outlook.com/?url=https%3A%2F%2Furldefense.com%2Fv3%2F__http%3A%2F%2Fwww.iopscience.org%2Fbf__%3B!!Dq0X2DkFhyF9
HbYHUgdWJsDVPC1mJqHcAhz7JD0GEzyrGXuwgHh4WhryjCot1uZgDzD454FcLMDT2nZM1EPP_ftB%24&data=02%7C01%7Cbf%40ioppublishing.org%7C4e33
01cf%7Cf9ee42e6bad04e639115f704f9ccceed%7C0%7C0%7C637246574097548811&sdata=R%2FsUdPauW06lkoXQHWj8eyl1WSWV%2FWYn8hg28viziRw%3I

2018 Impact Factor: 7.236

REFEREE REPORT(S):
Referee: 1

COMMENTS TO THE AUTHOR(S)
Comments
- In general, the paper shows a good characterization of a3 testicular in vitro morphogenesis model. They showed marker of both progenitor and differentiated cells, a
cells source and the aging and mixing towards the self-assembling phenotype. I confirm after my reading that the encouraging results of the submitted manuscript, finely
histology and biochemical data, can be fundamental for further studies and during the choice of specific matrix scaffold and selection of subpopulation of primary cells, in
and similar 3D cultures. The produced organoids are responsive in terms of endocrine function in a long term window. 4In addition the role of stem cells in the generation
highlighted and and suggested to the experts in the tissue engineering and to the clinicians for traslating organoid technique to human.
I would like to congratulate the authors.

- Abstract is OK, words have been checked is fewer than 300 words as IOP journal guidelines.
- Introduction is clear and it is representative of the state of the art. The references about similar models of organoids are reported in the manuscript that appears upc
- The discussion is accurate and not repetitive, but provide new critical elements and enhance the results.
- My unique constructive criticism is the fact that only a protein mixture ECM like Matrigel was used in the study, limiting the contribution of the different chemical envi
agree with the choice of the authors to do not include biomechanics influence on the organoid formation because the “simplest” model could become complicated as wel
- Materials are well detailed. The methods are artfully conceived, unambiguous and making the experimental part of the paper reproducible for the next researchers.
- The figures are clear, they rigorously drawn and statistics is correctly displayed. The legends allow to understand the experiments and interpret them, cartoons are

A few of minor revisions are below requested

1) May you shorten the title? However, this is only a suggestion for attraction on Pubmed and not a mandatory request. In case you just want to delete some words, n
the following “into tubular structures”.

2) An increased time of assembly has been reported for the 3D ECM organoid morphogenesis. Actually, the 3D ECM setting was very likely the only one encountering
of the gel in comparison to the others conditions in which there is a liquid instead surrounding the cells. Can the authors justify the increased time of assembly because
the Matrigel than the interaction with matrix proteins or receptors? for instance, may the density barrier delayed the interaction or block the migrating cells forming those
reasons explained on page 16th line 13-16, are there any other motivations that made the growing rate of organoids slower than the other different tested microenvironm

3) Moreover, could the presence of Matrigel led to a limited accessibility of some orienting proteins in the media that are necessary for normal morphogenesis and spe
acquire the “right” physiological cell polarity? Please briefly discussi it. The discussion about of 3DF is not necessary because the tissue hallmarks are already well descr

4) In some legends, in particular regarding panels C and D of Figures 3, 4 and 5, I believe it could better to explain which statistical groups is denoted by the letters (a

5) The authors can write, briefly in a single sentence, if there are some kind of physicochemical interactions between the cells and the agarose in the 3DF model or de
adherent situation at all even before the discussion section. Perhaps, that will clarify to non-beginners in tissue engineering that 3DF is a spheroid-life formation evolving
assembling organoid.

Referee: 2

COMMENTS TO THE AUTHOR(S)
I uploaded my comments as a file below. [See 'Comments_BF-102511.pdf']

Referee: 3

COMMENTS TO THE AUTHOR(S)

EDITORIAL BOARD MEMBER'S REPORT
GENERAL
Through the extensive experiments, the authors revealed that not the microenvironment but the cell's intrinsic nature is the determining factor in the functional testicular c
may probably achieved by direct cell-to-cell interactions. Also, toward the invitro spermatogenesis, the authors results seem to greatly contribute. This observation is biol
it linked to the utilization of self- assembling capability to organize themselves into functional tissues. On the contrary, the way of writing and focuses are not so engineer
reviewer worry about the suitability of this study in publishing in Biofabtrication. However, if the authors revise the current MS, so that it matches and satisfies the concern
Biofabrication, the reviewer think it may finally be accepted for publication.

SPECIFIC
1. Throughout the text: Delete less important biological experiment and results, or the authors can put them into supplements. You may keep figures and relevant texts d
originality of this study, as shown in the Title, but others including Table 1 may be deleted. I feel the initial part where you compare the four different culture systems may
keeping minimum photos and descriptions.
2. Physiological cellular responses against some model toxicant or chemicals etc.: When we think some applications such as reproductive toxicity, it is ideal to show the
organoids against some typical hormone or testis-specific toxicants. If possible, please add some such results or if you already did, consider to include these experiment
readers possible interests.
3. Discussion: In particular in the last paragraph of the Discussion, the authors refer to the future research extension using microfluidic systems etc., to which the readers
stronger interests over basic biological phenomena. Nevertheless, the authors only cite the related publications with no actual description about Ref. No. 78 and 79. Plea
rationale to combine the organoids with microfluidic devices and what the expected contributions are.

Letter reference: DSMa01
