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# Isolation of salivary epithelial cells from human salivary glands for in vitro growth as salispheres or monolayers

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**TITLE:**

# Isolation of Salivary Epithelial Cells from Human Salivary Glands for In Vitro Growth as Salispheres or Monolayers

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**KEYWORDS:**

salivary glands, primary culture, salisphere, organoid, Sjögren's syndrome, human cytomegalovirus, models for viral replication, saliva

**SUMMARY:**

We present a method for isolating and cultivating primary human salivary gland-derived epithelial cells. These cells exhibit gene expression patterns consistent with them being of salivary epithelial origin and can be grown as salispheres on basement membrane matrices derived from Engelbreth-Holm-Swarm tumor cells or as monolayers on treated culture dishes.

**ABSTRACT:**

The salivary glands are a site of significant interest for researchers interested in multiple aspects of human disease. One goal of researchers is to restore function of glands damaged by radiation therapies or due to pathologies associated with Sjögren's syndrome. A second goal of researchers is to define the mechanisms by which viruses replicate within glandular tissue where they can then gain access to salivary fluids important for horizontal transmission. These goals highlight the need for a robust and accessible in vitro salivary gland model that can be utilized by researchers interested in the above mentioned as well as related research areas. Here we discuss a simple protocol to isolate epithelial cells from human salivary glands and propagate them in vitro. Our protocol can be further optimized to meet the needs of individual studies. Briefly, salivary tissue is mechanically and enzymatically separated to isolate single cells or small clusters of cells. Selection for epithelial cells occurs by plating onto a basement membrane matrix in the presence of media optimized to promote epithelial cell growth. These resulting cultures can be maintained as three-dimensional clusters, termed "salispheres", or grown as a monolayer on treated plastic tissue culture dishes. This protocol results in the outgrowth of a heterogenous population of mainly epithelial cells that can be propagated for 5–8 passages (15–20 population doublings) before undergoing cellular senescence.

## INTRODUCTION:

In mammals the salivary glands are organized into 3 pairs of major salivary glands: the parotid, submandibular, and sublingual glands. There also exists a series of minor glands located on the tongue and scattered throughout the oral cavity[1]. The major glands are responsible for the bulk volume of saliva produced[2]. In addition to providing antimicrobial protection through secreted factors, the saliva is important in the process of chewing and lubricating food as it passes through the esophagus[2]. As such, salivary gland dysfunction represents a medical issue where sufferers who are unable to produce enough saliva are more prone to developing maladies such as dental cavities or oral candidiasis[3]. Additionally, reduced saliva results in greater difficulty eating and digesting food having a significant impact on quality of life.

Salivary gland dysfunction is primarily found in patients suffering from Sjögren's syndrome, an autoimmune disorder, or in patients with head and neck cancer who have undergone irradiation therapy[3–6]. Damaged secretory acini within the glands as a result of autoimmunity or radiation therapy do not undergo self-renewal, which results in a patient living with irreversible damage[6]. The study of salivary glands has also garnered the attention or researchers interested in mechanisms of viral pathogenesis. Some pathogens, such as human cytomegalovirus (HCMV), persistently replicate within the salivary glands, which then allows for transmission of nascent virus to a new host via saliva[7,8]. Thus, there is an unmet need to develop robust and reproducible in vitro models of salivary gland tissue to tackle and understand a diverse array of medical problems.

Several models of the murine salivary gland system have been used as a starting point to understand and characterize the different epithelial cells that form the salivary glands[9,10]. There exist obvious and major differences between human and murine salivary glands[11]. Most notably the human parotid gland is larger in relation to the submandibular gland whereas the mouse submandibular and parotid are much similar in size[1,2,11]. While immortalized human submandibular cell lines exists, there are major concerns that the immortalized cells do not accurately maintain the phenotype of the primary tissue and secondary concerns that the immortalized cells are not actually derived from salivary epithelium itself[12]. For these reasons there is an interest and a need to study primary salivary tissue from humans.

Here we describe a protocol to isolate primary epithelial cells from human salivary glands for tissue culture. Our protocol is based on the works of Pringle et al. and Tran et al. with modifications made to generally simplify their procedures[13,14]. First, while Tran et al.'s protocol calls for a long five-hour incubation for enzymatically digesting the salivary tissue, our modified protocol has been successful with as little as one-hour incubation, similar to that in Pringle et al. Second, we use different enzymes for tissue digestion, most notably we don't use trypsin as is used in the original Tran et al. protocol, but instead use a mixture of dispase and collagenase. We prefer to avoid trypsin in the initial digestion steps as a recent study suggested that initial digestion of the salivary tissue by trypsin results in reduced cell viability, possibly by the loss of a rare stem cell population[15]. Lastly, we culture the salispheres on the surface of basement membrane matrix (BMM) using a commercially available media originally formulated for the propagation of bronchial epithelial cells. Our protocol can be used to isolate salivary cells from

parotid, submandibular, and sublingual glands. These cells express salivary amylase and other salivary specific genes indicating that they maintain a phenotype similar to that of salivary acinar epithelial cells[7]. We have successfully generated salivary cultures from >50 primary human tissue samples using this methodology. Moreover, the primary salivary cells can easily be cryopreserved for use at a later date.

**PROTOCOL:**

These protocols and studies have been reviewed and approved by the Institutional Review Board at the University of Cincinnati (Federal-wide Assurance #00003152, IRB protocol 2016-4183). Salivary tissue is commonly resected in many head and neck surgical procedures and is usually uninvolved by the malignant process. Typically, freshly resected salivary gland tissue is used within 2–4 h after removal from the patient (**Figure 1A**).

**1. Reagent preparation**

1.1 Bronchial epithelial cell growth medium (BEGM)

1.1.1. Add components from the kit provided by manufacturer (see the **Table of Materials**).

1.1.2. Wash each tube once with the base media to ensure full transfer of components. Then add charcoal stripped fetal bovine serum to make a final concentration of 4% serum.

1.2. Red blood cell (RBC) lysis buffer

1.2.1. To make 10x stock, add 40.15 g of $NH_4Cl$, 5.0 g of $NaHCO_3$, and 0.186 g of ethylenediaminetetraacetic acid (EDTA) and bring up to a final volume of 200 mL using $dH_2O$. Then filter sterilize and store at 4 °C.

1.2.2. Dilute to a working concentration of 1x in sterile $dH_2O$ prior to use.

1.3. Dissociation solution

1.3.1. To a 100 mL bottle of dispase solution (see the **Table of Materials**) add 0.15 g of collagenase type III. After collagenase has fully dissolved, filter sterilize the new solution and aliquot. Store at -20 °C.

**2. Tissue digestion**

2.1. Using a 100 mm tissue culture plate, mechanically mince the tissue into fine pieces ~1–2 mm in size using autoclave-sterilized dissecting scissors and surgical forceps (**Figure 1B,C**). Connective tissue between pieces should be cut to ensure proper separation and ease in further steps.

2.2. Add 6 mL of the dispase/collagenase solution to the minced tissue. Then incubate at 37 °C for approximately 30 min to 1 h.

2.3. Disrupt the tissue by pipetting with a 5 mL serological pipette 15–20 times.

2.4. Incubate at 37 °C for another 30 min to 1 h.

2.5. Repeat steps 2.3 and 2.4 two-three more times or until the tissue resembles a slurry and can easily pass through the pipette opening (**Figure 1D**).

NOTE: The starting size of the tissue specimen and how fineness of the tissue mincing will determine how many times one needs to repeat steps 2.3 and 2.4. Typically, we receive tissue approximately 1 cm x 1 cm in size. Additionally, one can monitor tissue dissociation using a standard inverted tissue culture microscope to track the progress of cell dissociation from the tissue. Small clusters of cells are ideal for initial outgrowth of salivary cells as salispheres.

**3. Coating wells with basement membrane matrix**

NOTE: Basement membrane matrix (BMM) should be thawed overnight at 4 °C the day before it will be used. Once thawed, BMM should be constantly maintained on ice or at 4 °C as it will rapidly solidify (i.e., form a gel) at warmer temperatures. Additionally, care should be taken if samples have been chilled on ice prior to plating on BMM as cold samples will "melt" the BMM and expose cells to the plastic dish.

3.1. Slowly pipette BMM (see the **Table of Materials**) into each well to be coated. Evenly and slowly distribute the BMM over the well.

NOTE: Generally, we use 500 µL per each well of a six-well plate, but this volume can be adjusted for use on other variants of plates such as 12-well or 24-well, or as desired for thicker or thinner hydrogels.

3.2. Incubate the coated plates at 37 °C for at least 15 min prior to use to allow the BMM time to solidify.

**4. Filtering of undigested tissue, RBC lysis, and cell plating**

4.1. Transfer tissue homogenate from step 2.5 to a 15 mL conical tube. Wash plate once with 6 mL of Dulbecco's phosphate buffered saline (DPBS) to transfer remaining cells.

4.2. Filter the homogenate into a 50 mL conical tube through a 70 µm nylon mesh cell strainer to remove undigested tissue. Centrifuge strained cells for 5 min at 500 x *g*.

4.3. Dilute 10x RBC lysis buffer in sterile $dH_2O$ to make a 1x working solution.

4.4. Aspirate the supernatant. Then resuspend the cell pellet in 10 mL of 1x RBC lysis buffer. Incubate for 5 min at 37 °C.

4.5. Add 20–25 mL of DPBS to neutralize the RBC lysis buffer and minimize lysis of salivary cells. Centrifuge for 5 min at 500 x *g*.

NOTE: After treatment with RBC lysis buffer, the cell pellet should be white. Red color in the pellet indicates that not all RBC have been fully lysed. Repeat steps 4.3 through 4.8 if needed for complete lysis of all RBC.

4.6. Aspirate the supernatant. Resuspend the pellet in 1–2 mL of BEGM per well then place resuspended cells onto BMM-coated wells. Incubate at 37 °C.

4.7. Once plated on BMM, cells will form into spherical structures or "salispheres" over a 2–3-day period. Cells can be maintained as salispheres for about 5–7 days before the BMM begins to degrade allowing the cells to access and adhere to the plastic and grow as a monolayer.

**5. Subculturing the salispheres and maintenance on BMM**

5.1. Aspirate media from wells, then add 1 mL of dispase/collagenase solution to each well and incubate at 37 °C for ~15 min or until BMM has mostly dissolved.

5.2. Transfer cells to a 15 mL conical tube and wash wells once with DPBS to obtain remaining cells. Centrifuge for 5 min at 500 x *g*.

5.3. Aspirate the supernatant. Resuspend the pellet in 2 mL of trypsin then incubate at 37 °C for 15 min.

5.4. Neutralize the trypsin using any complete media containing 10% serum. Centrifuge for 5 min at 500 x *g*.

5.5. Aspirate the supernatant. Resuspend the cells in DPBS to remove residual media, trypsin, and serum. Centrifuge for 5 min at 500 x *g*.

5.6. Aspirate the supernatant. Resuspend in BEGM. Plate the resuspended cells from step 5.9 onto solidified BMM-coated culture dishes. Feed with fresh BEGM every 2–3 days.

5.7. Culture the cells in a humidified incubator maintained at 37 °C with 5% $CO_2$.

**6. Subculturing the salispheres on treated plastic tissue culture dishes**

NOTE: If maintaining cells as a monolayer on plastic, start at this step. The following protocol applies to cells growing in a 100 mm dish.

6.1. Aspirate the media. Wash once with DPBS to remove residual media.

6.2. Add 2 mL of trypsin then incubate at 37 °C for 15 min, or until cells have fully detached.

6.3. Neutralize trypsin using 4 mL of any complete media containing 10% serum. Transfer cells to a 15 mL conical tube.

6.4. Wash the plate once with approximately 5 mL of DPBS to collect remaining cells. Centrifuge for 5 min at 500 x *g*.

6.5. Aspirate the supernatant. Resuspend cells in 6–10 mL of DPBS to remove residual media.

6.6. Centrifuge for 5 min at 500 x *g*.

6.7. Aspirate the supernatant and resuspend in BEGM. Plate cells onto treated plastic tissue culture dishes. Feed with fresh BEGM every 2–3 days.

6.8. Culture the cells in a humidified incubator maintained at 37 °C with 5% $CO_2$.

**7. Cryopreservation of cells**

NOTE: Cryopreservation of cells can be accomplished from a single cell suspension originating from either salisphere or monolayer grown cells.

7.1. Count the cells on a hematocytometer to calculate total amount of cells present.

7.2. Centrifuge for 5 min at 500 x *g*.

7.3. Aspirate the supernatant. Resuspend the pellet in BEGM with 10% dimethyl sulfoxide (DMSO). Concentration of cells should be 2 x $10^6$ cells/mL to 10 x $10^6$ cells/mL.

7.4. Aliquot cells into sterile cryogenic storage tubes. Place the tubes in an insulated foam rack and incubate at -80 °C overnight to slowly freeze the cells.

7.5. On the next day, place tubes in liquid nitrogen for long term storage.

NOTE: Cells should be rapidly thawed at 37 °C. Upon thawing, cells should be pelleted at 500 x *g* and the DMSO-containing media removed prior to plating.

**REPRESENTATIVE RESULTS:**

Two–three days after plating cells from digested tissue onto BMM, cells will readily form small clusters that will continue to expand in size up to 15–20 cells per cluster (**Figure 2A**). Cellular debris and detached dead cells are typically seen and should be removed by aspirating and replenishing with fresh media. Cells will continue to proliferate as salispheres for about 3–10 days, or as long as the BMM layer remains intact. Occasionally, due to partial breakdown of the BMM, cells will become attached to the underlying plastic and begin to proliferate as a

monolayer. Salispheres grown on BMM will exhibit variability in size and structural complexity. Salispheres can be maintained by passing them onto freshly prepared BMM as described in the protocol. Within 2–3 days after passing onto fresh BMM, the cells will reform into spheres. Salisphere cells can also be plated onto cell culture-treated plastic and grown as a monolayer where they exhibit morphology consistent with cells of epithelial origin as shown in **Figure 2B**. While the salisphere cells grown on BMM as salispheres are likely to maintain a phenotype more characteristic of salivary tissue, the cells grown as a monolayer can be advantageous for some experimental protocols. More extensive characterization will need to be performed to determine to what extent the cells maintain a salivary phenotype when grown on a monolayer as compared to cells grown as salispheres.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Initial processing of human submandibular glands for salisphere preparation.** Excised salivary gland tissue approximately 1 cm in diameter (**A**) is mechanically minced into smaller pieces using sharp scissors (**B**) until the gland is separated into digestible pieces of approximately 1–2 mm in size (**C**). The glandular pieces are then incubated in the dispase/collagenase solution for 30–90 min with periodic passage through serological pipettes until the sample is digested to mostly single cells or small clumps of cells (**D**).

**Figure 2: Primary human salisphere cells can be cultured as spheres on the basement membrane matrix or as a monolayer on treated cell culture dishes.** Following initial digestion of salivary tissue, the digested glands are plated directly onto BMM and cultured for 3–10 days. Cells are fed every 2–3 days to provide fresh nutrients. Once the primary salispheres develop, the matrices can be digested in dispase/collagenase solution, trypsinized to generate single cells, and re-plated onto fresh BMM where they reform into spheres (**A**) or plated onto cell culture dishes where they readily form a cobblestone morphology typical of an epithelial monolayer (**B**).

**DISCUSSION:**

Salivary dysfunction represents a concern for the quality of life for those suffering from Sjögren's syndrome as well as those undergoing radiation therapy for cancers adjacent to the salivary glands[3–5,16]. One proposed therapy to treat these patients is to grow functional salivary stem cells or organoids in vitro, which can then be inserted into damaged salivary gland to replace affected tissue[9]. Additionally, salivary glands are often a site for persistence and transmission of human pathogens. For example, human herpesviruses such as human cytomegalovirus (HCMV) are known to infect and use the salivary glands and saliva to transmit virus to new hosts[7,8,17]. The molecular mechanisms underlying viral persistence in the salivary gland and movement to saliva remain unknown. The development of in vitro salivary cell systems will provide an essential model system to explore this mechanistic information.

We report here a robust protocol for generating primary salivary "salispheres" that can be grown in vitro either as clusters on BMM or as monolayers on plastic. The ability to culture and propagate primary human salivary gland epithelial cells represents an important platform on which researchers can (1) potentially develop replacement therapies for patients with salivary

gland deficiencies for whom no other options exist; (2) better understand the basic physiology underlying salivary gland development, proliferation, secretion, etc.; and (3) define mechanisms used by pathogens to replicate and spread to new hosts.

Using these salivary cell cultures, we have reported that HCMV requires the pentameric glycoprotein complex for primary infection and also that the virus persists for an extended period, reminiscent of what occurs with HCMV in vivo[7]. Moreover, our functional studies revealed that the salivary cultures do not contain contaminating fibroblasts as fibroblast tropic, lab adapted viruses fail to infect and replicate in the primary salivary derived cells[7]. While we have used this protocol to generate cells for the evaluation of HCMV replication and spread in a primary salivary system, this protocol could readily be adapted to generate salivary cells useful for the wide range of research topics mentioned above. Additionally, this protocol could be adapted to suit the needs and budget of each individual researcher. While we have not validated other conditions ourselves, others have reported success growing salivary epithelial cells under different media conditions. For example, Dulbecco's modified Eagle's medium (DMEM):F12 mixture supplemented with epidermal growth factor and fibroblast growth factor-2 has been reported to promote robust growth of salivary cells[18]. Alternatively, Nam et al. use minimal essential media supplemented with fetal bovine serum before transferring the cells to a serum-free version of a keratinocyte growth media and have reported robust growth of salivary cells on plastic[15]. Thus, it does not appear that the salivary epithelial cells exhibit an absolute requirement for one particular media type, but rather appear to grow and proliferate on a number of commercially available media types.

In addition to media formulation, different basement membrane formulations could be evaluated for their abilities to support robust salivary epithelial cell growth. Growth of cells on commercially available basement membrane-containing matrixes readily yield salispheres and provides a simple, albeit expensive culturing system[7,19]. Similar to that discussed above concerning choice of media for cell growth, researchers have reported growth and development of salispheres on a number of different matrices and hydrogel systems. Hydrogels made from hyaluronic acid have the added benefit in creating an extracellular matrix crosslinked with desired chemical constituents to study their interactions and effects on cells. Using primary human tissue derived salivary cells, Srinivasan et al. reported success in growth and maintenance of a salivary progenitor cell population in hyaluronic acid hydrogels; increased progenitor cell growth was further observed when the hydrogels were incorporated with peptides derived from the basement membrane proteins such as perlecan and laminin[20]. Another system recently developed by Foraida et al. report using "electrospinning" to develop a nanofiber scaffold made of poly(lactic-co-glycolic acid) (PLGA). The PLGA hydrogels with added elastin were found to readily promote the development of a polarized submandibular cell system, which may be useful in ascertaining how cell polarization influences salivary biology and biochemistry[21].

In this protocol, we have presented a simple method for the isolation and growth of primary salivary gland-derived epithelial cells. This protocol results in the rapid outgrowth of an epithelial cell population that can be maintained on average for 5–8 passages. It is critical to monitor the tissue during treatment with dispase/collagenase to ensure appropriate digestion as mentioned

in the protocol. Incomplete digestion will severely diminish the number of salispheres that survive. It is also important to monitor the cultures for the outgrowth of fibroblasts that have an elongated shape and are very distinct from the polygonal epithelial cells that typically grow in discrete patches or clumps. We have used this protocol for the isolation of epithelial cells from humans and mice, but it seems likely that it could be adapted for use with other mammalian systems. Moreover, this protocol could be further modified by using additional purification steps based on cell surface marker expression and flow cytometry that could lead to the generation of more homogenous initial cell populations. Similarly, this protocol could be used to determine how different media or matrix/hydrogels formulation lead to the outgrowth of specific cell types when first generating the salispheres. In conclusion, this protocol should serve as a robust starting platform for the isolation and growth of primary salivary cells which can readily be adapted for a wide variety of experimental protocols and research areas.

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**DISCLOSURES:**

The authors report nothing to disclose.

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Figure 1



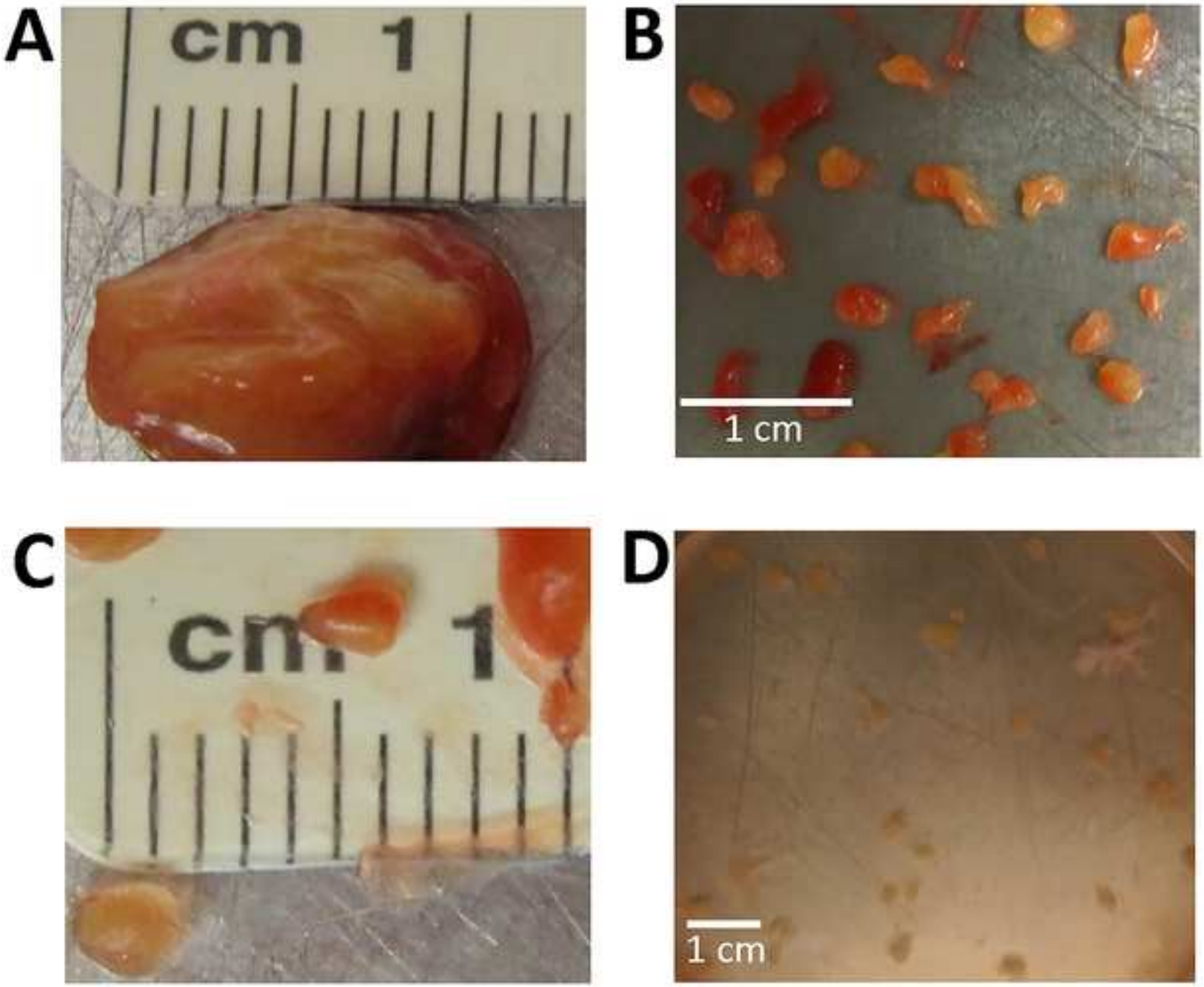

Figure 2



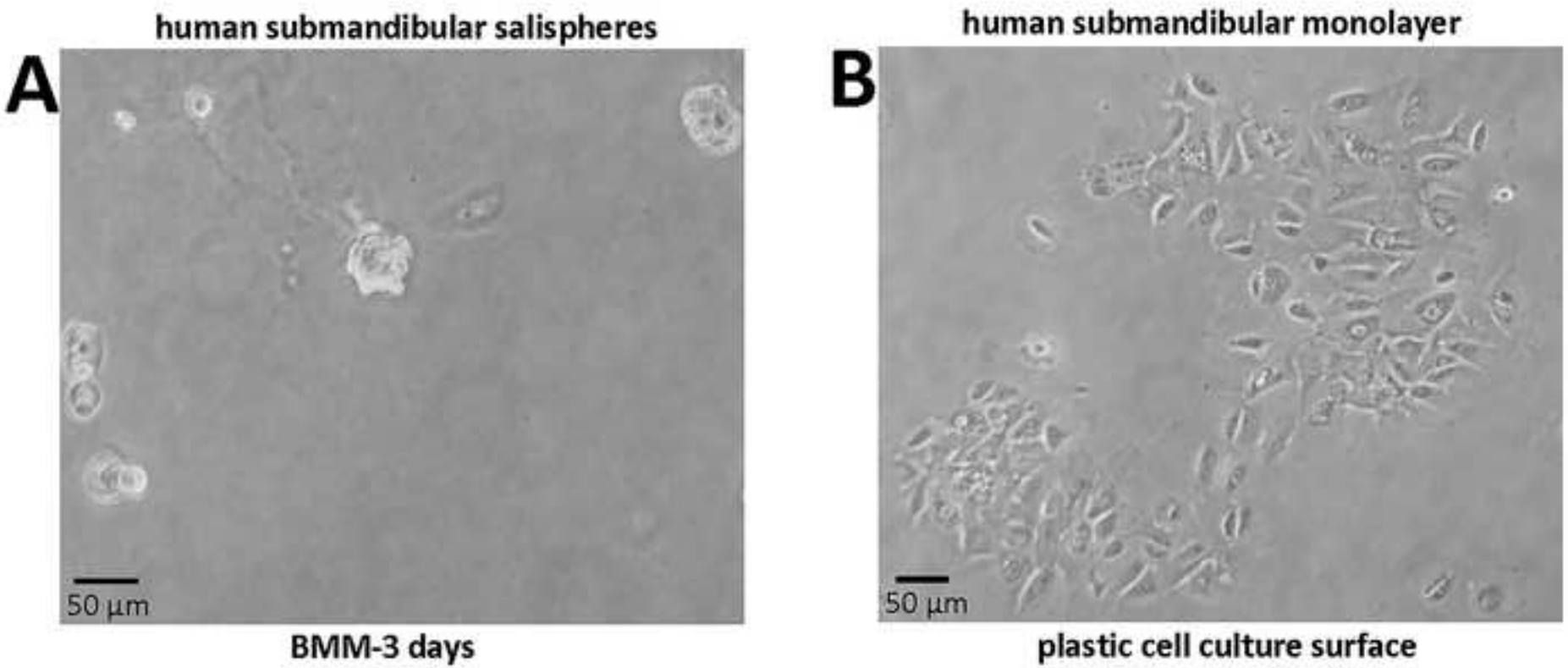

Table of Materials

Click here to access/download;Table of Materials;Beucler JoVE Revised Materials Final.xls

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 100 mm culture dishes | Thermo Scientific | 172931 | |
| 15 mL conical tubes | Thermo Scientific | 339651 | |
| 50 mL conical tubes | Thermo Scientific | 339653 | |
| Bronchial Epithelial Cell Growth Media | Lonza | CC-3171 | Add bullet kit as per manufacturer's instructions |
| Cell strainer 70 µm nylon mesh | Fisher | 22-363-548 | |
| Charcoal stripped fetal bovine serum | Gibco | 12676-029 | |
| Collagenase type III | Worthington | LS004182 | Store at 4 °C. |
| Cryogenic Tube | Fisher | 5000-0020 | |
| Dispase | Cell Applications | 07923 | Dissolve collagenase to make a 0.15% (w/v) stocl |
| Dissecting scissors | Fisher | 08-940 | |
| Dulbecco phosphate buffered saline | Corning | 55-031-PC | |
| General Chemicals | Sigma | | |
| PathClear Basement membrane extract | Cultrex | 3432-005-01 | Thaw at 4 °C at least 24 hr prior to use. Always h |
| Six-well culture dishes | Falcon | 353046 | |
| Surgical forceps | Fisher | 22-079-742 | |
| Trypsin-EDTA solution | Corning | 25-052-CI | |

. Supplement with 20 mL of charcoal stripped serum.

k. Filter sterilize then store at -20 °C.

andle on ice.

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March 28, 2019

Dr. Alisha DSouza
Senior Review Editor
JoVE

Dear Dr. DSouza,

We are resubmitting a revised version of manuscript JoVE59868 entitled “Isolation of salivary epithelial cells from human salivary glands for in vitro growth as salispheres or monolayers”. We have made all of the changes suggested by the editors and reviewers

A detailed response to the Reviewers comments is appended below. Also, we include a marked up version of the manuscript in which all changes to the manuscript are tracked to facilitate review of changes made. Thank you for your willingness to consider the revised manuscript and we hope that our revised manuscript will now be acceptable for publication in JoVE.

Sincerely,

William E. Miller, Ph.D.
Professor
Department of Molecular Genetics

**Response to Editorial Comments:**

*1. Please take this opportunity to thoroughly proofread……*
We have thoroughly checked for grammatical and spelling errors.

*2. Protocol Detail……..*
We have added detailed comments on media and culture conditions as suggested.

*3. Protocol Highlights……..*
We have highlighted ~2.5 pages of the text to identify which steps should be visualized.

*4. Discussion……*
We have revised the discussion in several places to ensure that the discussion addresses each of the 5 points raised by the editor.

*5. Figures….Add scale bars to the right panels…….*
We have added scale bars to the right panels.

*6. Commercial language…….*
We have replaced all commercial sounding language with generic names that are not company specific.

*7. Abbreviations……*
All abbreviations have been defined

*8. If your tables and figure are not original……..*
All tables and figures are original and have not been published previously.

**Response to Comments from Peer-Reviewers**

Reviewer #1.

Major Concern:
*1. The only real issue that I can point out is that there's no confirmation about the identity of cells isolated…*
In our recent publication in J. Virol, we have demonstrated that these cells are of epithelial origin and express amylase and other markers of salivary acinar epithelial cells. We have added a comment about these studies to the introduction (lines 92-93 of the marked up version) and also make specific reference to our published paper. We have also modified the language in the summary to clarify this point.

Minor Concerns:
*1. Line 132 - "2.5 Repeat steps 1.3 and 1.4 two-three more……..*
We have corrected this in the text to read steps 2.3 and 2.4 as suggested by the reviewer.

*2. A little more discussion about what the functional assessment……*

We have added a couple of sentences to the discussion adding language about our functional assessment as suggested by the reviewer. This discussion is in lines 336-340 of the marked up version.

Reviewer #2.

Major Concerns:
*1. In several places the authors use the phrase "mostly epithelial……"*
In our recent publication in J. Virol, we have demonstrated that these cells are of epithelial origin and express amylase and other markers of salivary acinar epithelial cells. We have added a comment about these studies to the introduction (lines 92-93 of the marked up version) and also make specific reference to our published paper. We have also modified the language in the summary to clarify this point.

*2. In particular, since fibroblasts are highly permissive for CMV……*
We know that our cultures contain little to know fibroblast contamination as fibroblast tropic strains of HCMV fail to replicate in our salivary cultures. We have added this language in lines 336-338 of the marked up version of the manuscript.