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SOP –UNITS-1.0 | Author: **E. Campaner, A. Zannini** | Approved: **G. Del Sal** | Issued: **12/04/2019** | Revised:

MOUSE MAMMARY ORGANOID CULTIVATION

Purpose

The SOP-UNITS-1.0 was issued to describe how to generate, plate and maintain organoids from mouse mammary tissue. In addition, this SOP contains the instructions for cryopreservation (freezing as well as thawing) of organoid cultures.

Scope

SOP 1.0 is intended to cover all resources, personnel and equipment needed to generate, maintain and cryopreserve organoids from mouse mammary tissue.

Introduction

The aim of this SOP is to provide protocols for the cultivation and long-term storage of mouse mammary organoids to be used as model for mammary gland development.

1. Cell culture media, reagents and solutions

- C57BL/6 female mice aged from 8 to 12 weeks
- Ethanol (Sigma Aldrich, cat. no. 32205-M)
- Penicillin-Streptomycin Solution 100X (Euroclone, cat. no. ECB3001D)
- 10X Collagenase/hyaluronidase in DMEM (Stem Cell, cat. no. 07912)
- Dispase 5U/mL (Stemcell, cat. no.07913)
- Trypsin 2,5% (Lonza, cat. no. BE17-160E)
- EDTA (VWR, cat. no. 20302.236)
- Deoxyribonuclease I from bovine pancreas (Sigma Aldrich, cat. no. DN25)
- HBSS Hank's Balanced Salt Solution modified: without Ca, Mg and Phenol Red (Lonza, cat. no. 04-315Q)

- Ammonium chloride Solution (Stemcell, cat. no. 07800)
- EpiCult-B Mouse Medium Kit (Stem Cell, cat. no. 05610)
- FGF basic (Peprotech, cat. no. 100-18B)
- EGF (Cell Guidance Systems, cat. no. GFH26)
- B27 supplement 50X (Gibco, cat. no. 17504-44)
- PBS-1× (Life Technologies, cat. no. 14190-094)
- DMSO (Sigma-Aldrich, cat. no. D8418)
- FBS (Euroclone, cat. no. ECS0180L)
- Advanced DMEM/F-12 (Ad-DF, Gibco cat. no. 12634-010)
- Hepes (Invitrogen, cat. no. 15630-056)
- Matrigel Growth Factor Reduced (BD, cat. no. 354230)
- Cell recovery solution (Corning, cat. no. 354253)
- TrypLE Express 1X (Gibco, cat. no. 12605-010)

2. Equipment

- Cell culture incubator with 5% CO_2, 37°C
- CKX31 Inverted Microscope, Olympus Life Sciences
- High-speed centrifuge (Eppendorf centrifuge 5810R)
- Pipette aid, serological pipettes (Euroclone cat no. EPS05N; EPS10N)
- Pipettes
- Pipette tips
- Conical centrifuge tubes (Euroclone, cat no. ET5015B; ET5050B)
- Microcentrifuge tubes (Euroclone, cat. no. ET3415)
- 100 mm Petri dish (Greiner Bio-one, cat. no. 633181)
- Carbon steel scalpel blades (Albion, cat. no. 03393)
- 24-Well adherent culture plate (Euroclone, cat. no. ET3024)
- 2-mL Cryogenic vials (VWR, cat. no. 479-0287)
- Corning CoolCell LX Cell Freezing Container (Sigma-Aldrich, cat. no. CLS432002-1EA)
- Ice

3. Reagent setup

- Transport medium: Ad-DF medium supplemented with 5% FBS, 1X Penicillin/Streptomycin, 10 mM Hepes.
- Digestion solution I: Dilute Collagenase/Hyaluronidase 1:10 (6 hours digestion) or 1:5 (3 hours digestion) in Transport medium. This solution should be freshly prepared and used immediately. For each sample at least 5mL of this solution should be prepared.
- Digestion solution II: 0.25% Trypsin, 0.01M EDTA in PBS.
- Digestion solution III: 0.1 mg/mL DNAse I in Dispase 5U/mL.
- Ad-DF basal medium: Ad-DF supplemented with 1X Glutamax, 1X Penicillin/Streptomycin, 10 mM Hepes. Store at 4°C.
- Mouse mammary organoid medium: EpiCult-B Mouse Medium supplemented with:

Medium component	Final concentration
Penicillin/Streptomycin	1X
B27 supplement	1X
EpiCult-B supplement	1X
basic FGF	20 ng/mL
EGF	20 ng/mL

- Cell freezing solution: 10% of DMSO in FBS.

4. Procedure

4.1 Collection of mammary tissue

Euthanize the mouse using an appropriate ethically approved method and immerse the mouse in a becker with 70% ethanol to avoid contamination. Collect the inguinal mammary glands avoiding the skin and the surrounding muscle tissue (***Figure 1***). Put the mammary glands in a 50mL tube containing Transport medium.

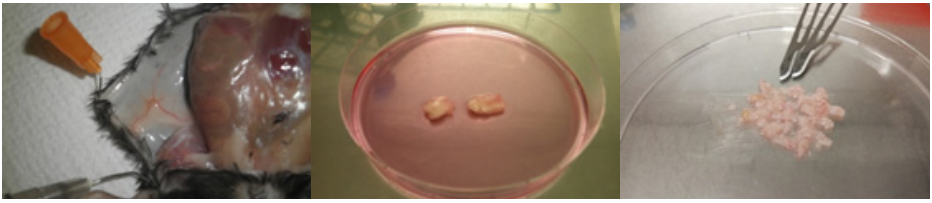

Figure 1. Representative images of inguinal mammary gland location in the mouse (left), surgical resected (middle) and mechanically minced (right) inguinal mammary glands.

4.2 Mouse mammary glands dissociation

!CRITICAL! For the following steps precoat all the tubes, the pipettes and tips with FBS to avoid attachment of the tissue to the plastic.

4.2.1 Transfer the tube containing the breast tissue to the biological safety cabinet. Aspirate the medium and place the tissue in a sterile 100-mm Petri dish (untreated dish to avoid cell attachment). Mince it into 1 mm^3 pieces using scalpels (***Figure 1***).

4.2.2 Transfer the material in a 50mL tube containing 5mL of prewarmed Digestion solution I, pipette up and down several times the medium containing the tissue using a 5mL pipette to promote tissue digestion, and incubate the tissue at 37°C under agitation (200rpm) conditions for 3hrs, if enzymes are diluted 1:5, or 6hrs, if enzymes are diluted 1:10 (***Figure 2***).

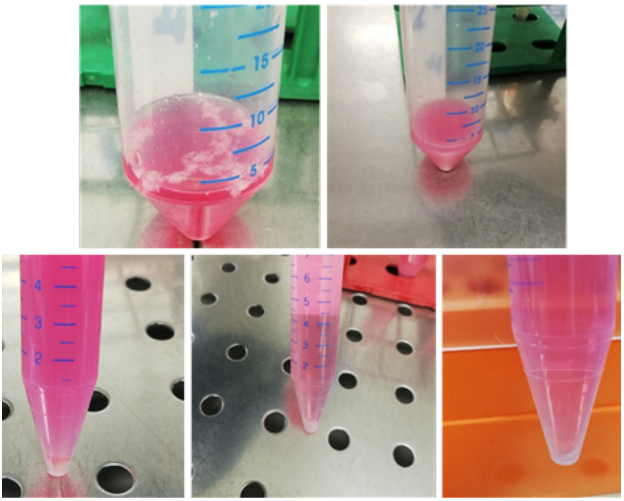

Figure 2. Top, Representative images of minced mammary glands before (left) and after 2 hours of incubation with Collagenase/Hyaluronidase (right). Bottom, Representative images of cells extracted from the mammary glands before (left) and after (middle) red blood cells lysis, and before their seeding (right).

4.2.3 Every 30 minutes of incubation, vortex the mixture for 10 seconds. !CRITICAL STEP! Care should be taken to avoid the over-digestion of the material. Digestion is complete when microscopic examination shows clumps of cells free from the stroma. If the tissue is over-digested the cell viability will be extremely low.

4.2.4 When digestion is complete (***Figure 2***), add 10mL of Transport medium to dilute enzymes and transfer the suspension in a 15mL tube and centrifuge for 5 minutes at 200g. After centrifugation, a fat layer on the top, an aqueous layer in the middle and a red pellet on the bottom are visible (***Figure 2***). Discard the supernatant (the fat and the aqueous layers) without disturbing the pellet.

4.2.5 Resuspend the pellet in 3mL of 4:1 mixture of ammonium chloride and cold modified HBSS for 3 minutes on ice to eliminate red blood cells. Add 8mL of Transport medium and pellet at 200g for 5 minutes (***Figure 2***). Discard the supernatant.

4.2.6 Resuspend with modified HBSS and pellet (200g, 5min) twice the cell suspension to remove all the bivalent ions to facilitate break down of cell-cell contacts. Remove as much of the supernatant as possible.

4.2.7 Add 2mL of pre-warmed Digestion solution II to the cell suspension and pipette up and down with a p1000 tips for 2 minutes. Add 8mL of modified HBSS supplemented with 2%FBS to neutralize trypsin. Centrifuge the cells at 200g for 5 minutes and remove as much of the supernatant as possible.

4.2.8 Add 2mL of pre-warmed Digestion solution III and pipette for 1 minute with a p1000 tip. Add 8mL of Transport medium, centrifuge the cells (200g, 5min), aspirate the supernatant and resuspend the cells with 10mL of Transport medium. Count viable cells using Trypan Blue. In general, from 1 mice, $1x10^6$ of mammary epithelial cells (MECs) are extracted. For an optimal growth, consider $25\text{-}50x10^3$ MECs/well of a 24 multi-well plate. Transfer the desired amount of MECs in a new 15mL tube, centrifuge the cells (200g, 5min) and remove the supernatant. Resuspend the pellet with 10mL of cold Ad-DF basal medium, centrifuge the cells (200g, 5min, 4°C) (***Figure 2***) and remove as much of the supernatant as possible.

4.3 Seeding of mouse mammary epithelial cells to generate organoids

!CRITICAL! Matrigel is stored at -20°C and requires thawing on ice at 4°C for 2-3 hours. Freeze-thaws should be minimized by aliquoting into one time use aliquots. Matrigel will solidify at room temperature, so work quickly and keep the basement matrix cold through the process.

4.3.1 By gently pipetting resuspend cell pellet in Matrigel and seed 50µL drops of Matrigel-cell suspension on pre-warmed 24-well plates (1 drop/well), avoiding formation of air bubbles (***Figure 3***). Incubate the plate 1hr at 37°C (until the matrix is solidified).

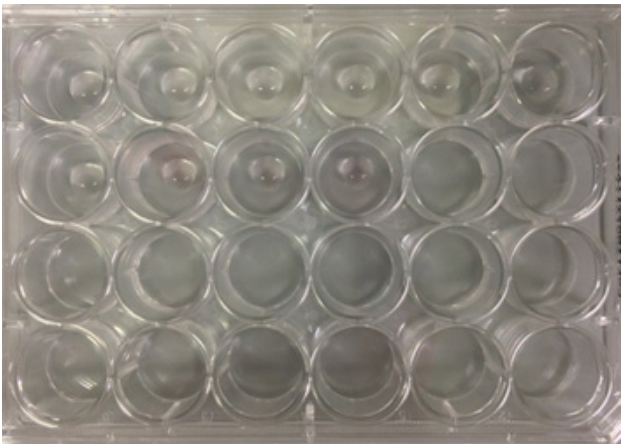

Figure 3. Representative image of drops containing Matrigel-embedded mammary epithelial cells plated in the 24-wells plate.

4.3.2 When the matrix is solidified, add 500µL of Mouse mammary organoid medium.

4.3.3 Incubate the material under standard tissue culture conditions (37°C, 5% CO_2).

4.3.4 Change media every 4 days.

4.3.5 Organoids should be visible within 2-3 days and ready for passaging around 1 week day of culture (***Figure 4***).

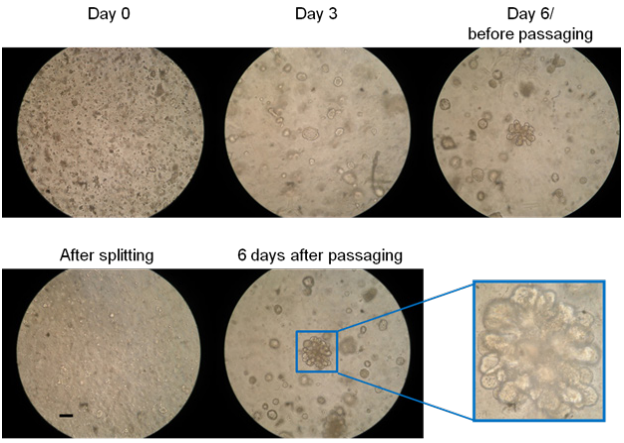

Figure 4. Representative images of mouse mammary organoids at different time points. Scale bar (100µm) is indicated.

4.4 Organotypic culture maintenance

Grow the organoids for 7-10 days. The general split ratio used for a 24 well is 1:2 depending on the number and size of the organoids. !CRITICAL! For the following steps precoat all the tubes, the pipettes and tips with FBS to avoid attachment of the tissue to the plastic.

4.4.1 Keep the plates on ice. Remove the medium from each 24 well, add 500µL of cold PBS to wash organoids. Aspirate PBS and add 500µL of cold PBS.

4.4.2 Using p1000 pipette tips displace Matrigel from the wells and collect the material (PBS and organoids in Matrigel) in a 15mL tube and centrifuge it at 200g for 5 minutes at 4°C.

4.4.3 Remove the supernatant and add Cell Recovery solution (~100µL each well). Incubate organoids in this solution for 1 hr at 4°C in order to gentle digest Matrigel and release cells with minimal cell damage.

Alternatively, 2,5 U/mL pre-warmed dispase (~100µL each well) can be used for 10 minutes at 37°C followed by addition of 10mL of cold PBS.

4.4.4 Centrifuge organoids at 200g for 5 minutes at 4°C and aspirate supernatant.

4.4.5 Add 5mL of cold PBS and centrifuge organoids at 200g for 5 minutes at 4°C to wash cells.

4.4.6 Aspirate the supernatant completely. At this step, it is possible to freeze the organoids for storage or to keep organoids in culture. If organoids freezing is required, follow the procedure described in the next paragraph.

4.4.7 For organotypic culture expansion, further digestion can be performed using pre-warmed TrypLe solution (~100µL each well) for 5 minutes at 37°C followed by gentle pipetting (p1000). This step is not required if organoids are smaller than 50 µm.

4.4.8 Add 5mL of Ad-DF basal medium and centrifuge 200g for 5 minutes.

4.4.9 Remove supernatant and wash cells by adding 5mL of Ad-DF basal medium and centrifuge 200g for 5 minutes at 4°C.

4.4.10 Remove supernatant and proceed with step 4.3.

4.5 Cryopreservation and thawing of organoids

!CRITICAL STEP! The freezing/thawing procedure is not efficient because of decreased viability of thawed organoids. For this reason, freeze the highest quantity of organoids in your experimental setting as possible (8-12 well for each cryovial).

4.5.1 Cryopreservation of organoids

Proceed as described in the paragraph 4.4 and then gently re-suspend the organoids in 1mL of cold freezing medium (4.4.6). Transfer the suspension to cryovials and place them into ice bucket. Transfer the vials into a Cell Freezing Container at -80°C for 24 hrs, and then transfer them into liquid nitrogen vapor. !CRITICAL STEP! The freezing medium contains DMSO, which is toxic to the cells at room temperature, work quickly and do not exceed a total of 5 min between adding the freezing medium to the organoids and transferring them to –80 °C.

4.5.2 Thawing of organoids

4.5.2.1 Pre-warm a water bath and 10mL of Ad-DF basal medium in a 15mL tube to 37°C.

4.5.2.2 Incubate the cryovial in the 37°C water bath, stopping when the ice is almost completely thawed. Quickly transfer the cells to the pre-warmed 15mL tube containing basal medium.

4.5.2.3 Centrifuge the tube at 200g for 5 min and aspirate the supernatant.

4.5.2.4 Seed organoids following the procedure described in the paragraph 4.3.

5. References

- Shackleton et al., Generation of a functional mammary gland from a single stem cell. Nature (2006)
- Jaimeson et al., Derivation of a robust mouse mammary organoid system for studying tissue dynamics. *Development* (2017)