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SOP –UNITS-2.0 | Author: **E. Campaner, A. Zannini** | Approved: **G. Del Sal** | Issued: **12/04/2019** | Revised:

HUMAN BREAST ORGANOID CULTIVATION

Purpose

The SOP-UNITS-2.0 was issued to describe how to generate, plate and maintain organoids from breast cancer samples as well as from healthy tissues derived from the human breast. In addition, this SOP contains the instructions for cryopreservation (freezing as well as thawing) of organoid cultures.

Scope

SOP 2.0 is intended to cover all resources, personnel and equipment needed to generate, maintain and cryopreserve organoids from fresh tissue of breast cancer samples as well as healthy tissue.

Introduction

The aim of this SOP is to provide protocols for the cultivation and long term storage (Live-biobank) of patient-derived tumor-organoids and organoids from the respective healthy tissue to be used for precision cancer medicine.

1. Cell culture media, reagents and solutions

- Human breast samples (cancer and normal tissue)
- Penicillin-Streptomycin Solution 100X (Euroclone, cat. no. ECB3001D)
- Primocin (Invivogen, cat.no. Ant-pm-1)
- Collagenase A (Roche, cat. no. 11088793001)
- FGF7 (Peprotech, cat. no. 100-19)
- FGF10 (Peprotech, cat.,no 100-26)
- Noggin (Peprotech, cat. no. 120-10C)
- Heregulin ß-1 (Peprotech, cat. no. 100-03)
- R-spondin 3 (R&D system, cat. no. 3500-RS-025/CF)
- EGF (Cell Guidance Systems, cat. no. GFH26)

- A83-01 (Tocris, cat. no. 2939)
- Y-27632 (Selleckchem, cat. no. S1049)
- SB202190 (Sigma-Aldrich, cat. no.S7067)
- B27 supplement (Gibco, cat. no. 17504-44)
- N-Acetylcysteine (Sigma-Aldrich, cat. no. A9165)
- Nicotinamide (Sigma-Aldrich, cat. no. N0636)
- GlutaMax100X (Invitrogen, cat. no. 12634-034)
- Hepes (Invitrogen, cat. no. 15630-056)
- 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)
- Matrigel Growth Factor Reduced (BD, cat. no. 354230)
- 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)
- Cell recovery solution (Corning, cat. no. 354253)
- Dispase (Stemcell, cat. no. 07913)
- TrypLE Express 1X (Gibco, cat. no. 12605-010)
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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)
- 100-µm Cell strainer (Falcon, cat. no. 352360)
- 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, 10mM Hepes.
- Digestion solution: 1600 U/mL collagenase A in transport medium. Sterile filter. This solution should be freshly prepared and used immediately. For each sample at least 5mL of this solution should be prepared.
- Ad-DF basal medium: Ad-DF supplemented with 1X Glutamax, 50 µg/mL Primocin, 1X Penicillin/Streptomycin, 10mM Hepes.
- Human breast organoid medium (for normal and cancer specimen): Ad-DF basal medium supplemented with:

Medium component	Final concentration
B27 supplement	1X
Nicotinamide	5 mM
N-Acetylcystein	1.25 mM
R-spondin 3	250 ng/mL
Heregulin ß-1	5 nM
Noggin	100 ng/mL
FGF-7	5 ng/mL
FGF-10	20 ng/mL
EGF	5 ng/mL
A83-01	500 nM
SB202190	500 nM
Y-27632*	5 µM

* if single cells are plated, add it for the first three days of culture

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

4. Procedure

4.1 Collection of fresh biopsies

Fresh material from surgically resected breast primary tumors (untreated patients) and the corresponding healthy tissue are collected from the operating theatre of the hospital. Punch bi-

opsies of fresh breast tumor and normal breast tissue are collected in cold Transport medium and are delivered on ice to the research laboratory. At least 1 cm^3 of tissue should be collected and processed within 6 hours.

4.2 Breast tissues 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) (***Figure 1***). Mince it into 1-3 mm^3 pieces using scalpels (***Figure 2***).

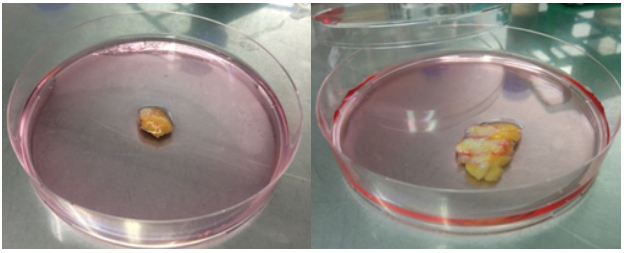

Figure 1. Representative images of normal (left) and tumoral (right) biopsies of human breast tissue.

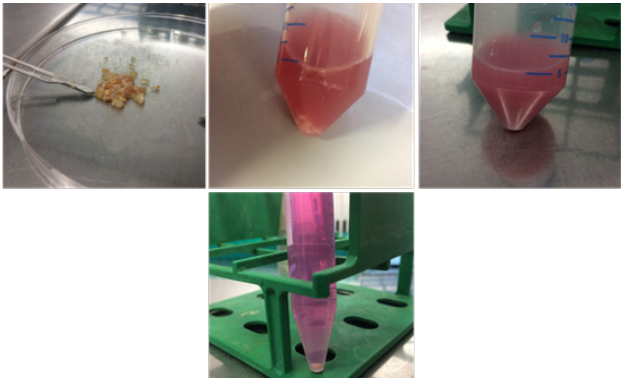

Figure 2. Representative images of i) minced tumor tissue before (top, left and middle) and after 2 hours of incubation with Collagenase A (top, right), and ii) cells extracted from the tissue (bottom).

4.2.2 At this step it is possible to proceed with tissue digestion or minced tissue can be stored at -80˚C (up to 3 months).

For tissue digestion, transfer the material in a 50mL tube and incubate the tissue with 5mL of prewarmed Digestion solution at 37˚C under agitation (200rpm) conditions for 2hrs. For tissue storage, place minced tissue in freezing medium and when desired thaw the tissue in prewarmed Ad-DF basal medium, centrifuge the tube at 200g for 5 min, aspirate the supernatant and proceed with tissue digestion.

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 collagenase, pass the digested material through 100-µm cell strainer and pellet the material by centrifuging at 200g for 5 minutes.

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. This step is not required for minced tissues that have been previously frozen.

4.2.6 Add 10mL of Transport medium to the pellet material and centrifuge at 200g for 5 minutes. Discard the supernatant containing lysed red blood cells.

4.2.7 Resuspend the pellet with 5mL of Ad-DF basal medium and centrifuge at 200g for 5minutes. Remove the supernatant and keep cells on ice.

4.3 Seeding of breast 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).

4.3.2 When the matrix is solidified, add 500µL of Human breast 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 Tumor organoids should be visible within 2-3 days and ready for passaging around 1 week day of culture (***Figure 4***). Normal organoids grow slowly and are smaller than tumor one.

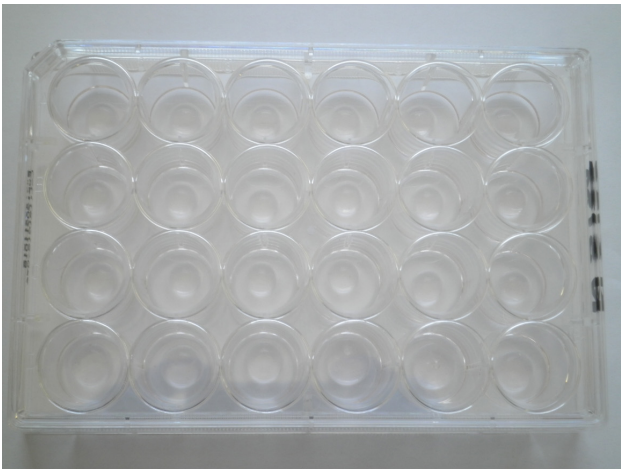

Figure 3. Representative image of drops containing Matrigel-embedded mammary cancer cells plated in the 24-wells plate.

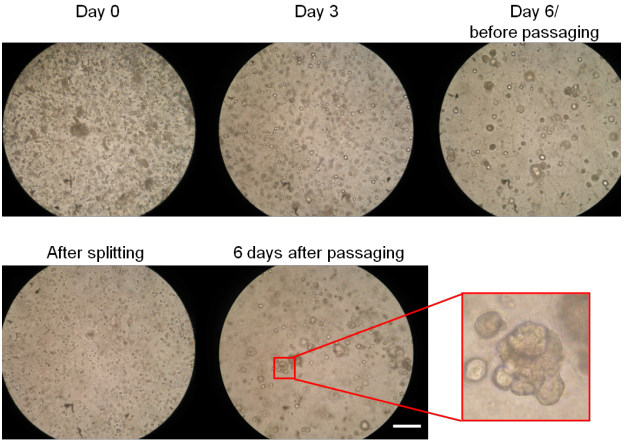

Figure 4. Representative images of breast tumor organoids at different time points. Scale bar (200µm) is indicated.

4.4 Organotypic culture maintenance

Grow the organoids for 7 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 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,pre-warmed Dispase (2,5 U/mL in Ad-DF basal medium, ~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 cryo-conserve organoids for storage or to keep organoids in culture. If organoids freezing is required, follow the procedure described in 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 possible in your experimental setting (8-12 well for each cryovial).

4.5.1 Cryopreservation of organoids

Proceed as described in the paragraph 4.4 and then gently resuspend 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.

4.5.2.1 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 15-mL tube containing basal medium.

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

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

5. References

- Sachs, et al., A living biobank of breast cancer organoids captures disease heterogeneity. Cell 10.1016/j.cell.2017.11.010 (2017)