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LNCIB
SOP –LCO-1.0

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Issued:
10/01/2019
Revised:

LUNG ORGANOIDS CULTIVATION

Purpose

The purpose of LNCIB SOP 1.0 is to describe how to create, plate and maintain lung organoids from human lung tissues.

Scope

LNCIB SOP 1.0 is intended to cover all resources, personnel and equipment in the lung cancer organoids laboratory.

Introduction

Fresh material from surgically resected lung primary tumors (untreated patients) have been processed by the *Anatomia Patologica Unit of Cattinara Hospital*. The unit performs routine histopathological analysis of the primary tumor. Punch biopsies of fresh lung tumor and normal lung tissue have been passed to LNCIB collaborators to provide material for the cultivation of lung-organoids. Collaborators at LNCIB have used normal and tumor material to establish organoid cultivation protocol as following described.

1. Cell culture media, reagents and solutions

- Human lung samples
- Penicillin G sulfate (Sigma-Aldrich cat. no. P3032)
- Streptomycin sulfate (Sigma-Aldrich cat. no. S9137)
- Primocin (Invivogen cat.no. Ant-pm-1)

- Collagenase II (Sigma-Aldrich cat.no 1088866001)
- FGF7 (Peprotech cat no. 100-19)
- FGF10 (Peprotech cat.no 100-26)
- A83-01 (Tocris cat.no 2939)
- Y-27632 (Stemcell cat. no. 72302)
- SB202190 (Sigma-Aldrich cat. no. S7067)
- B27 supplement (Gibco 17504-44)
- N-Acetylcysteine (Sigma-Aldrich cat. no. A9165-5g)
- Nicotinamide (Sigma-Aldrich cat. no. N0636)
- GlutaMax100x (Invitrogen cat no. 12634-034)
- Hepes (Invitrogen cat no. 15630-056)
- Producer lines for conditioned medium of Noggin and R-spondin-1 (MTA with Calvin Kuo, Stanford)
- PBS-1× (Life Technologies, cat. no. 14190-094)
- DMSO (Sigma-Aldrich, cat. no. D8418)
- FBS (Life Technologies, cat. no. 26010066)
- DMEM-H (Gibco, Carlsbad, CA, cat. no. 11995-065)
- Matrigel (Growth Factor Reduced; BD, cat. no. 354230)
- HBSS Hank's Balanced Salt Solution (Sigma-Aldrich, cat. no. H6648)
- Ham's F-12 Medium (Lonza, cat. no. 12615F)
- Dispase (Stemcell cat. no. 07923)
- TrypLE Express (Invitrogen cat. no. 12605036)

2. Equipment

- Cell culture incubator with 5% CO2, 37 °C
- CKX31 Inverted Microscope, Olympus Life Sciences
- High-speed centrifuge (Eppendorf centrifuge 5810R)
- Pipette aid, serological pipettes (Euroclone, cat. no. EPS05N; EPS10N)
- Conical centrifuge tubes (Euroclone, cat. no. ET5015B; ET5050B)
- Microcentrifuge tubes (Euroclone, cat. no. ET3415)
- 100-µm Cell strainer (Falcon, cat. no. 352360)
- 24-Well adherent culture plate (Thermo Scientific Nunclon, cat. no. 142475)
- 2-ml Cryogenic vials (VWR, cat. no. 479-0287)
- Corning CoolCell LX CellFreezing Container (Sigma-Aldrich, cat. no. CLS432002-1EA)
- Ice

3. Reagent setup

- **Human lung samples** Tissue samples for cancer research are provided by a trained surgeon and pathologist from surgically resected lung tumor tissue. Resected material also contains normal tissue. At least 1 cm^3 of tissue should be collected and processed within 3 hours. The tissue should be stored at 4 °C in Ad-DF+++ medium until processing.
- **Ad-DF+++ medium** DMEM/F12 supplemented with Glutamine 500µg/ml, Primocin 50µg/ml, Penicillin/Streptomycin 5%, Hepes 10mM. Store at 4°C.
- **Tissue digestion solution** 1,5mg/ml collagenase in Ad-DF+++; sterile filtered. This solution should be freshly prepared and used immediately.
- **Cell freezing solution** This solution is prepared by gradually adding DMSO to FBS, to obtain a final solution of 10% of DMSO and 90% FBS.
- **Dispase solution** 5U/ml in Ad-DF+++ medium. This solution should be freshly prepared and used immediately.
- **Medium for the cultivation of lung tissue organoids** ("complete media"):

Components of "Complete medium"	Supplier	Catalogue number	Final concentration
R-Spondin 1 conditioned medium	*		25% v/v*
FGF 7	Peprotech	100-19	25 ng·ml^{-1}
FGF 10	Peprotech	100-26	100 ng·ml^{-1}
Noggin conditioned medium	**		25% v/v*
A83-01	Tocris	2939	500 nM
Y-27632	Abmole	Y-27632	5 µM
SB202190	Sigma	S7067	500 µM
B27 supplement	Gibco	17504-44	1x
N-Acetylcysteine	Sigma	A9165-5g	1.25 mM
Nicotinamide	Sigma	N0636	5 mM
GlutaMax 100x	Invitrogen	12634-034	1x
Hepes	Invitrogen	15630-056	10 mM
Penicillin / Streptomycin	Invitrogen	15140-122	100 U·ml^{-1} / 100 µg·ml^{-1}
Primocin	Invivogen	Ant-pm-1	50 µg·ml^{-1}
DMEM	Gibco	11995-065	37% v/v
Ham's F-12	Lonza	12615F	25% v/v

* R-Spondin1-conditioned medium was produced from HEK293T-HA-Rspo1-Fc cells; MTA for the use of producer cell line was obtained from Stanford University.

** Noggin-conditioned medium was produced from HEK293-mNoggin-Fc cell line. MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands .

4. Procedure

4.1 Human lung dissociation

4.1.1 Tissue collection. After surgical excision, the tissue should be kept at 4˚C in a 50ml falcon in Ad-DF+++ medium and transported (from the Hospital to research laboratory: time 12 minutes) for processing within 3 hours. !CRITICAL STEP! Precoat all the tubes, the pipettes and tips with FBS to avoid attachment of the tissue to the plastic.

4.1.2 Transfer the falcon containing the lung tissue to the biological safety cabinet. Aspirate the medium and gently pipette up and down with a 10-ml pipette to wash lung tissue in 10 ml of 1X PBS. Aspirate off as much PBS as possible.

4.1.3 Place the tissue in a sterile 100-mm Petri dish. Mince tissue into small pieces using scalpels (See ***Figure 1***).

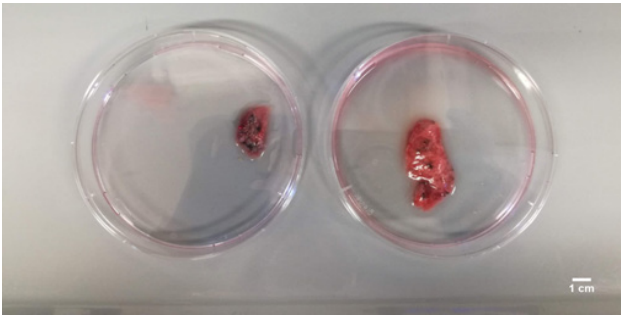


Figure 1. Representative images of tumor (left) and normal (right) lung biopsies. Scale bar 1cm.

4.1.4 Transfer minced tissue into a 50ml tube and incubate tissue with 10 ml of prewarmed digestion solution at 37 ˚C under soft agitation (200rpm) for 2hrs. (See ***Figure 2***).

4.1.5 Every 15 minutes of incubation, vortex the mixture for a few seconds. !CRITICAL STEP! Special attention should be taken in order to avoid the over-digestion of tissue material. Digestion is complete when microscopic examination shows clumps of cells free from attached stroma. If the tissue is over-digested the cell viability will be extremely low.

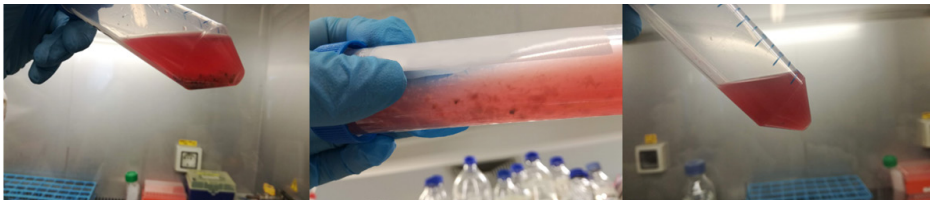


Figure 2. Representative images of minced pieces of lung tissue before (left), after 1 hour (middle) and after 2 hours (right) of digestion.

4.1.6 When digestion is complete (See ***Figure 3***), add 10 ml of Ad-DF+++ supplemented with 10% FBS to neutralize collagenase and pass digested material through a 100-µm cell strainer. Subsequently, pellet the tissue material by centrifuging at 300g for 5 minutes at room temperature.

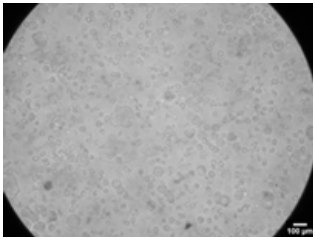


Figure 3. Representative images of lung tissue cells after 2 hours of digestion. (Phase contrast microscope image; scale bar 100 µm).

4.1.7 In order to eliminate red blood cells, resuspend the pellet in 5ml of 4:1 mixture of ammonium chloride and cold modified HBSS for 3 minutes on ice.

4.1.8 Add 10 ml of Ad-DF+++ supplemented with 10% FBS and centrifuge at 300g for 5 minutes. Discard the supernatant containing lysed red blood cells.

4.1.9 Resuspend the pellet with 10 ml of room temperature modified HBSS to maintain pH, osmotic balance and to provide cells with water and essential inorganic ions. Centrifuge at 300g for 5minutes; remove supernatant. Repeat procedure. This procedure removes all the bivalent ions to facilitate break down of cell-cell contacts, allowing the seeding of cells in step 4.2 Seeding of lung cells to generate organoids.

4.2 Seeding of lung cells to generate organoids

4.2.1 !CRITICAL STEP! Matrigel is stored at -20°C and requires thawing on ice at 4 °C for around 2-3 hours. Matrigel will solidify at room temperature. Thus, it is necessary to work quickly and to keep the basement matrix at low temperatures throughout the process of cell manipulation.

4.2.2 Resuspend cell pellet in matrigel (300.000 cells in 30 µl) and add a droplet of matrigel (30µl) to the center of each 24 well, avoiding formation of air bubbles (See ***Figure 4***). Incubate the plate 30 min at 37 °C to allow solidification of the matigel. Note: all available cell material will be plated in series of wells of a 24 well plate.

4.2.3 When the matrix is solidified (after 30-40 minutes incubation of 24 well plate at 37°C), add 400 µl of complete medium.

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

4.2.5 Change complete media every 4 days.

4.2.6 Small tumor organoid structures should be visible within 2-3 days and reach sufficient size for passaging around 1 week day of culture. Normal organoids tend to grow more slowly and become visible after ca. 2 weeks (See ***Figure 5***).

Figure 4. Representative image of drops containing Matrigel-embedded lung epithelial cells plated in the 24-wells plate.

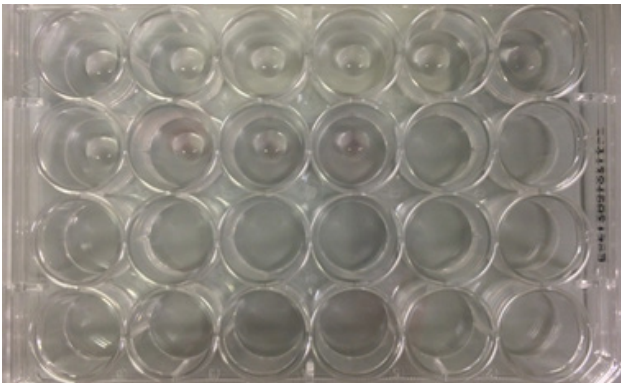
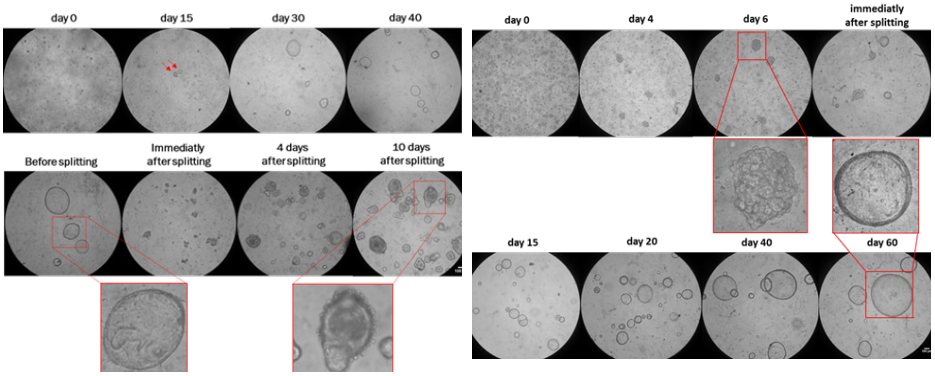


Figure 5. Representative phase contrast micrograph image of normal and tumor lung organoids derived from a patient tissues. (Phase contrast microscope image; scale bar 100 µm).



4.3 Cell culture: passaging organoids for maintenance

4.3.1 Cultivate organoids for 7 days. The recommended split ratio used for organoids in a Matrigel drop (in a 24 well) is 1:2. This depends however also on the number and size of the organoids.

4.3.2 Remove the medium from 24 well, add 500 µl of room temperature 1x PBS to wash organoids. Aspirate the supernatant.

4.3.3 Add 500 µl of ice-cold 1x PBS, recover organoid-matrigel mixture and transfer to a 15ml tube. Pipette up and down 10 times with a 200 µl pipette tip in order to mechanical disrupt organoids. Centrifuge at 200 – 300 x g for 5 minutes at 2-8°C. Remove supernatant.

4.3.4 Gently resuspend organoids in 3 ml of Dispase solution for 10 minutes at 37 °C in order to digest remaining Matrigel. This liberates cells from remaining Matrigel with minimal cell damage.

4.3.5 Add 10 ml of Ad-DF+++ supplemented with 10% FBS and centrifuge organoids at 200 – 300 x g for 5 minutes at 2-8°C.

4.3.6 Wash cell pellet by gentle resuspension in 10 ml of ice-cold 1X PBS and centrifuge at 2-8°C, aspirate off the supernatant. Repeat washing step.

4.3.7 Optional for large organoids: Add 500µl to 1ml of TrypLE Express to cell pellet and resuspend organoids by pipetting up and down 5 times in order to obtain an enzymatic digestion of the cells. Incubate cell pellet for 5 minutes at 37°C. Note: perform enzymatic digestion of large organoids only to split them. Do not perform enzymatic digestion of the cells if you desire to do a) cryoconservation of organoids; b) immunofluorescence of organoids; c) immunohistochemistry analysis of organoids.

4.3.8 Add 10 ml of Ad-DF+++ supplemented with 10% FBS and centrifuge organoids at 200 – 300 x g for 5 minutes at 2-8°C. Aspirate off supernatant completely.

4.3.9 Wash cell pellet by gentle resuspension in 10 ml of ice-cold 1X PBS and centrifuge at 2-8°C, aspirate off the supernatant.

4.3.10 At this step, it is possible to cryo-conserve organoids for storage or to seed organoids for further amplification.

4.3.11 For cryo-conservation, follow the procedure 4.4 Cell culture: passaging organoids for maintenance. For re-seeding organoids follow paragraph 4.2 Seeding of lung cells to generate organoids.

4.3.12 For performing immunofluorescence or immunohistochemistry, follow respectively the LNCIB SOP-LCO-2.0 or LNCIB SOP-LCO3.

4.4 Cell culture: passaging organoids for maintenance

4.4.1 Cryopreservation of organoids

4.4.1.1 Pre-cool Corning CoolCell LX CellFreezing Container filled with isopropanol at 4 °C.

4.4.1.2 Prepare cell material as described in the paragraph 4.3 Passag-

ing organoids for maintenance. Gently resuspend the cell aggregates in ice-cold freezing medium (use 1 ml of freezing medium to cryo-conserve organoids recovered from 6 wells of a 24 well plate). Transfer the suspension to cryovials and place them in the pre-cooled freezing container. Transfer freezing container to –80 °C overnight. Subsequently transfer cryovials to liquid nitrogen vapor for long-term conservation.

!CRITICAL STEP! The freezing medium contains DMSO, which is toxic to the cells at room temperature, work quickly and do not exceed a total time of 5 minutes between adding the freezing medium to the organoids and transferring them to –80 °C.

4.4.2 Thawing of organoids

4.4.2.1 Pre-cool Corning CoolCell LX CellFreezing Container filled with isopropanol at 4 °C.

4.4.2.2 Prewarm 10 ml of Ad-DF+++ medium in a 15 ml tube to 37 °C.

4.4.2.3 Place cryovial in the 37 °C water bath, until cells are almost completely thawed.

4.4.2.4 Pipet up and down and transfer cells to the prewarmed Ad-DF+++ medium.

4.4.2.5 Centrifuge the tube at 300g for 5 min.

4.4.2.6 Aspirate off supernatant.

4.4.2.7 Seed organoids following the procedure described in the paragraph 4.2 *Seeding of lung cells to generate organoids.*

Note: plate frozen organoids obtained from 6 wells of a 24 well plate into 3 wells.

5. Applicable references to LNCIB SOPs

LNCIB SOP –LCO-2.0, LNCIB SOP –LCO-3.0

6. Applicable references

Long-term expanding human airway organoids for disease modelling. Normal Sachs et al. EMBO J; 38(4); 2019. doi: 10.15252/embj.2018100300.

Differentiated human airway organoids to assess infectivity of emerging influenza virus. Zhou J. et al; Proc Natl Acad Sci U S A; 115(26), 20182018. doi: 10.1073/pnas.1806308115.