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HUMAN LUNG ORGANOID CULTURE PROTOCOL

This protocol provides a procedure for subculturing and expanding human lung organoids. This protocol was modified from methods described in Sachs, N. *et al.* (2019) EMBO J. **34**:e100300.

The protocol provided below is intended to culture organoids from normal human lung tissues using Cultrex Organoid Qualified Basement Membrane Extracts as a scaffold. The majority of reagents used in this protocol were sourced from the Bio-Techne brands of R&D Systems and Tocris Bioscience.

EQUIPMENT

1. Cell culture incubator (37 °C, 5% CO_2)
2. Cell culture hood with laminar flow
3. Centrifuge with refrigeration and swinging bucket rotor
4. 37 °C water bath
5. Ice bucket
6. Laboratory refrigerator
7. Pipet aid and serological pipettes (5 mL)
8. Micropipettes and tips (2–200 µL)
9. Conical tubes, 15 mL and 50 mL, sterile
10. 24-well plate, tissue-culture treated, sterile
11. Vacuum pump
12. Medium filtration unit, 0.1 µm, 500 mL, sterile
13. Syringe, 50 mL, sterile
14. Syringe filter, 0.2 µm, sterile
15. Cell culture waste container

OTHER REQUIRED REAGENTS

1. Distilled (DW) or deionized water (DI)
2. Phosphate buffered saline (PBS)

MATERIALS

PRODUCT NAME	SUPPLIER	CATALOG #
A 83-01	Tocris Bioscience	2939
Cultrex Organoid Harvesting Solution	R&D Systems	3700-100-01
Cultrex UltiMatrix Reduced Growth Factor Basement Membrane Extract	R&D Systems	BME001-05
Advanced DMEM/F-12 Cell Culture Medium		
Glutamine	Tocris Bioscience	5823
HEPES	Tocris Bioscience	3173
N21-MAX Supplement	R&D Systems	AR008
N-Acetylcysteine	Tocris Bioscience	5619
Penicillin/Streptomycin	R&D Systems	B21210
SB 202190 (p38 MAPK Inhibitor)	Tocris Bioscience	1264
Nicotinamide	Tocris Bioscience	4106
Y-27632 dihydrochloride (Rho Kinase Inhibitor)	Tocris Bioscience	1254
Recombinant Human R-Spondin 1	R&D Systems	4645-RS
Recombinant Human Noggin	R&D Systems	6057-NG
Recombinant Human FGF-10	R&D Systems	345-FG
Recombinant Human FGF-7	R&D Systems	251-KG

TABLE 1. Materials needed for human lung organoid culture

REAGENT PREPARATION

Use aseptic technique at all times during this protocol. This protocol is optimized for human lung organoids; organoids from other tissues may have different culture requirements.

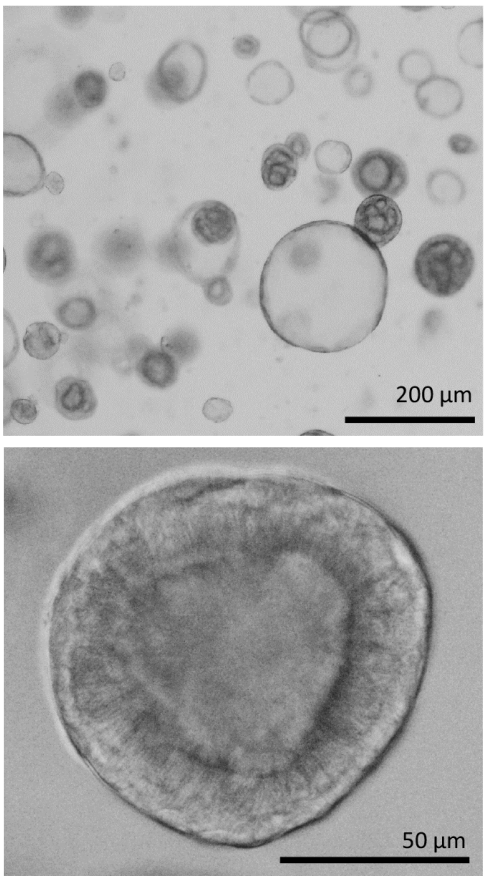
1. Thaw Cultrex UltiMatrix RGF Basement Membrane Extract (BME) on ice for four hours or overnight at 2 - 8 °C (on ice in the refrigerator).
2. Prepare Lung Organoid Expansion Medium, as indicated in TABLE 2:
3. Sterile filter the media

REAGENT NAME	[FINAL]
Advanced DMEM/F-12 Cell culture Medium	NA
N21-MAX Supplement	1X
Glutamine	2 mM
HEPES	10 mM
Penicillin/Streptomycin	1X
Nicotinamide	5 mM
A 83-01	0.5 µM
SB 202190	0.5 µM
Y-27632	5 µM
N-Acetylcysteine	1.25 mM
Recombinant Human Noggin	100 ng/mL
Recombinant Human FGF-10	100 ng/mL
Recombinant Human FGF-7	25 ng/mL
Recombinant Human R-Spondin 1	0.5 µg/mL

TABLE 2. Preparation of Human Lung Organoid Expansion Medium.

PROTOCOL

1. Prepare a suspension of isolated and dissociated human lung tissues as detailed in Sachs, N *et al*. (2019) EMBO J. **34**:e100300
2. Resuspend lung tissue cell pellet in Cultrex UltiMatrix RGF Basement Membrane Extract (BME) and aliquot into wells and dispense 50 µl of the Cultrex UltiMatrix RGF BME/ cell suspension mixture in the center of each well of a 24-well plate. **Note:** *The Cultrex UltiMatrix RGF BME-contained organoids should not touch the sides of the well.*
3. Incubate the plate in the cell culture incubator for 15 minutes to polymerize the Cultrex UltiMatrix RGF Basement Membrane Extract.
4. Add the appropriate volume of Lung Organoid Expansion Medium needed (approximately 500 µL/well of a 24-well plate).
5. Return plate containing organoid cultures to the cell culture incubator to promote growth.
6. The culture medium should be aspirated from each well and replaced with fresh Lung Organoid Expansion Medium every 2-3 days. **Note:** *Medium should be gently aspirated from and pipetted into the corner of the well away from the Cultrex UltiMatrix RGF BME/ organoids to prevent their disruption.*
7. Organoids can be passaged for continued culturing (see Passaging Lung Organoids section).



Human Lung Organoids Grown in Cultrex UltiMatrix RGF BME. Representative images of lung organoids, derived from lung biopsy adult stem cells, embedded in Cultrex UltiMatrix RGF BME and cultured in Lung Organoid Expansion Media. Images show organoids at day 52 of culture.

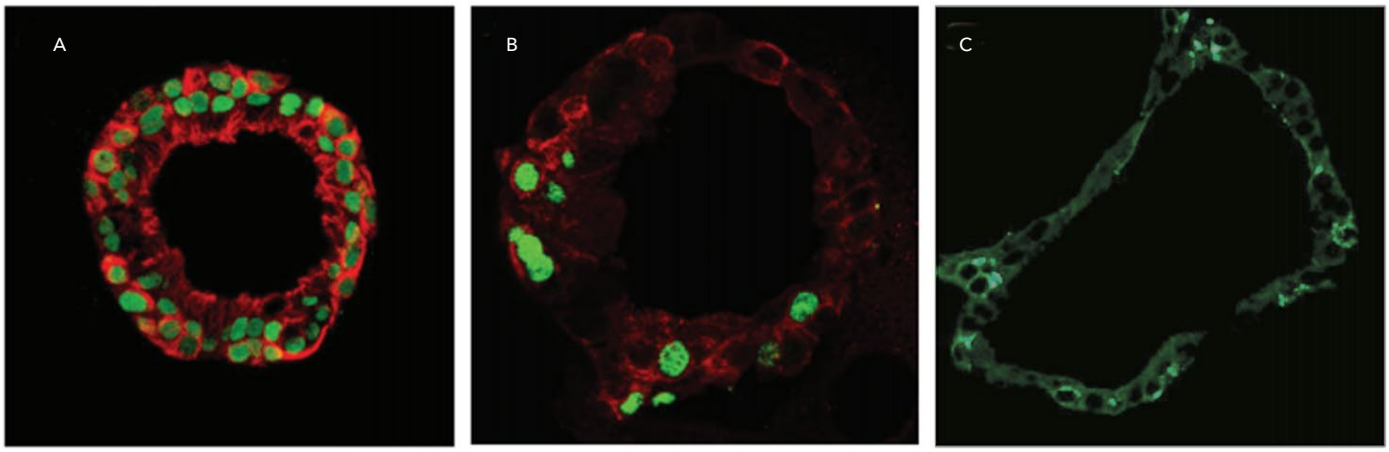


FIGURE 2. Characterization of Human Lung Organoids. Representative images of human lung organoids for tissue-specific cell types. (**A**) Expression of Sox2 (green; R&D Systems, Catalog # AF2018) and Acetylated Tubulin (red; Novus Biologicals, Catalog # NB600-567). (**B**) Expression of p63/TP73L (green; R&D Systems, Catalog # AF1916) and Cytokeratin 10 (red; Novus Biologicals, Catalog # NBP2-61736). (**C**) Expression of Podoplanin (green; R&D Systems, Catalog # AF3670) as a marker of type 1 alveolar cells.

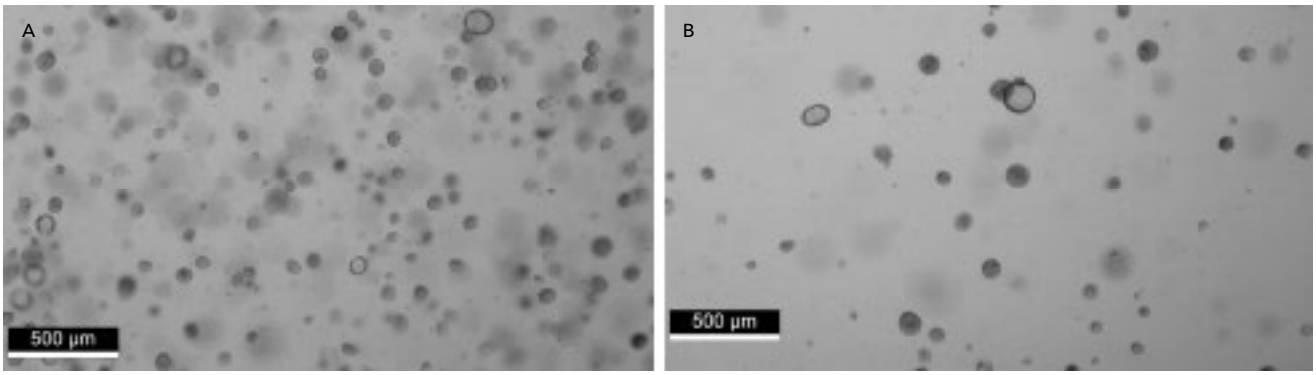


FIGURE 3. Density of Lung Organoids Following Expansion and Passaging. Lung organoids were expanded using Lung Organoid Expansion Medium for 25 days prior to passaging. Representative brightfield images demonstrating the density of human lung organoids before (**A**) and after passaging (**B**).

PASSAGING LUNG ORGANOIDS

1. View lung organoids under the microscope. Each well should contain approximately 500 organoids for optimal growth. Organoid cultures exhibiting rapid growth may be split 1:4 during passaging, while slow growing cultures may benefit from a 1:1 split. Make this determination prior to harvesting to estimate reagent needs prior to starting. **Note:** *Organoid density is important for optimal growth; too many organoids will strain culture resources, while too few organoids lack paracrine signaling necessary to sustain growth.*

2. Transfer the 24-well plate containing lung organoids from the cell culture incubator to the cell culture hood.

3. Aspirate the medium without disturbing the Cultrex UltiMatrix RGF BME-contained organoids at the bottom of the tube.

4. Gently wash each well with 10 volumes of cold (2-8 °C) PBS (TABLE 1). Be careful not to disrupt basement membrane matrix containing organoids.

5. Aspirate the PBS, and add 10 volumes of cold (2-8 °C) Cultrex Organoid Harvesting Solution to each well (TABLE 1).

PLATE TYPE	VOLUME OF BASEMENT MEMBRANE MATRIX	VOLUME OF PBS AND ORGANOID HARVESTING SOLUTION
96-well plate	5 µL	50 µL
48-well plate	25 µL	250 µL
24-well plate	50 µL	500 µL

Table 1. Suggested volumes of PBS and Organoid Harvesting Solution

6. Incubate the plate at 2-8 °C or on ice for 30–90 minutes with moderate shaking. This incubation is complete when the basement membrane matrix dome is no longer visible at the bottom of the well and the organoids are seen floating at the bottom of the well. **Note:** *Dislodging the dome with a cell scraper or pipet may accelerate this process.*

7. Once the matrix depolymerizes, transfer contents of the well into a tube on ice. Single wells may be transferred to a microtube while multiple domes may necessitate a 15 mL or 50 mL conical tube.

8. Centrifuge the tube at 500 x g for 5 minutes at 2-8 °C in a swinging bucket rotor to pellet the organoids. Aspirate the supernatant.

9. Wash organoids with 10 volumes of cold (2-8 °C) PBS, and repeat centrifugation at 500 x g for 5 minutes at 2-8 °C in a swinging bucket rotor to pellet the organoids. Aspirate the PBS. Add fresh ice-cold Lung Organoid Expansion Medium.

10. Pipet up and down three times with a serological pipette to mix the organoids.

11. Centrifuge the tube at 500 × g at room temperature for 3 minutes.

12. Aspirate medium, but be careful not to disturb the organoid pellet.

13. Resuspend organoids in Cultrex UltiMatrix RGF Basement Membrane Extract, and dispense 50 µL of the mixture in the center of each well of a 24-well plate to form domes. Follow the density/splitting ratios recommended in Passaging Protocol Step 1. **Note:** *The Cultrex UltiMatrix RGF BME domes should not touch the sides of the well.*

14. Incubate the plate in the cell culture incubator for 15 minutes to polymerize Cultrex UltiMatrix RGF Basement Membrane Extract.

15. Add 500 µL of Lung Organoid Expansion Medium per well. **Note:** *Medium should be gently pipetted into the corner of the well away from the Cultrex UltiMatrix RGF BME/organoids to prevent their disruption.*

16. Return plate containing organoid cultures to the cell culture incubator to promote organoid growth. Follow Lung Organoid Expansion Protocol.

Learn more | rndsystems.com/organoids

View Our Full Cultrex UltiMatrix Video | rndsystems.com/products/cellculture