Generation of Human Airway Organoids from Primary Cells

Application Note

This Application Note describes the generation of functional 3D airway organoids with apical-out or inward-oriented cilia formation based on Primary Human Bronchial Epithelial Cells (HBEpC) as an *in vitro* model for high-throughput drug screening or large-scale virus-host interaction studies.

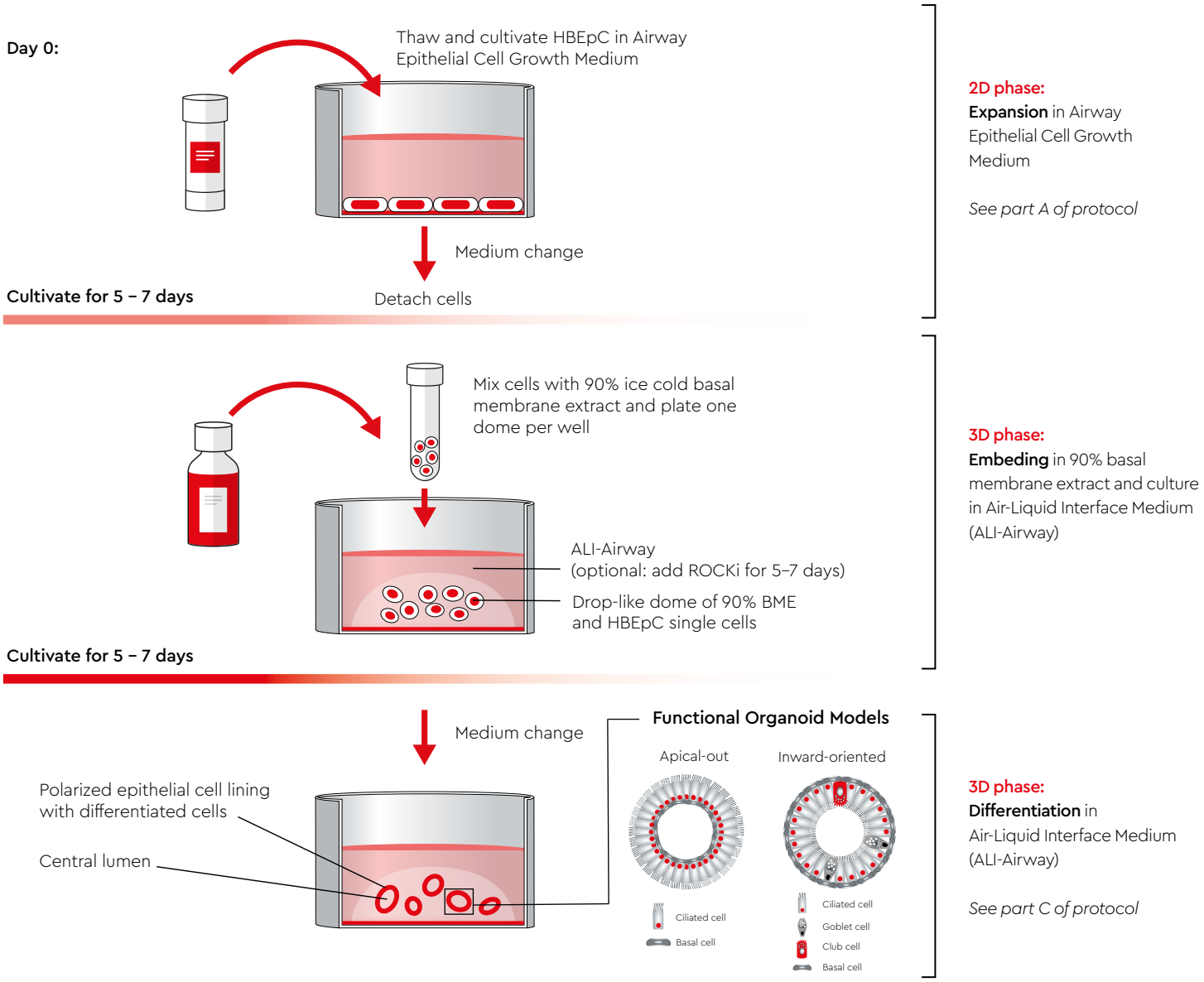

Figure 1: Schematic overview of the generation of lung airway organoids by differentiated HBEpC cultured in our Air-Liquid Interface Medium. *The workflow can be divided into various phases:* ***A,*** *Expansion of HBEpC in 2D cell culture.* ***B,*** *Re-seeding of single-cell HBEpC in a mixture of 90% ice-cold basal membrane extract (BME) and plating of one drop-like cell-containing dome per well in a multiwell plate. Domes are covered with Air-Liquid Interface Medium (ALI-Airway). For increasing the colony forming efficiency, 10 µM ROCKi Y-27632 may be added optional for 5–7 days when reseeded.* ***C****, Differentiation of the organoids. Approximately 2 weeks after re-seeding a central lumen of the 3D cell constructs will become visible and ciliated cells can appear at the outside of the polarized epithelial cell lining with differentiated cells of the organoid (apical-out) or inward oriented towards the lumen (inward-oriented). After 4 weeks of culture in ALI-Airway the differentiation process is complete. For the detailed steps, see the following protocol.*

Background

Primary human airway epithelial cell culture was established in 1982 by Lechner et al. [1]. As the function of a cell is dependent on the structural architecture of the tissue it resides in, there is an advantage in studying cells using 3D cell culture systems [2],[3],[4],[5]. Airway epithelial cells can be successfully cultivated in submerged 2D cultures, as an airlifted monolayer grown on a porous membrane in a multi-well plate (Air-liquid interface [ALI] culture) or as self-organized 3D organoids embedded in a basal membrane extract (BME).

The self-organized 3D structure of functional primary bronchial epithelial cells embedded in an extracellular matrix gel was observed by Rock et al., 2009 [6]. Human basal bronchial epithelia cells were able to form viable self-assembled spheroids after sorting the cells by the progenitor markers Integrin α6 (ITGA2) and nerve growth factor receptor (NGFR). These structures were called bronchospheres and had a basal cell lining on the outside and differentiated ciliated and mucus producing cells oriented to the inner side of the lumen. The polarization of airway epithelial spheres show clear signs of mucociliary differentiation and meaning they can be used as a functional 3D cell culture model [7],[8],[9],[10].

A newer culture technique means bronchospheres can be stimulated to polarize apical-out, meaning an outward oriented cilia formation. Apical-out may be more relevant for experiments since these cells do not have restricted access to the luminal surface, so nutrients and agents have better access to the cells and cell surface receptors are presented to the medium. Virus infection can occur without microinjection of the organoids, instead virus can simply be added to the culture medium. For example, apical-out airway organoids have been used to study SARS-Cov2 infection by presenting ACE2 on the exposed external surface [11]. Long-term airway organoid culture can be used for disease modeling such as studying drug toxicity and efficacy, lung cancer research or genetic variations [12],[13].

Our Air-Liquid Interface Medium (ALI-Airway) was developed for the culture of Human Bronchial Epithelial Cells (HBEpC) on a porous membrane to form a tight functional epithelial barrier in a serum- and BPE-free environment. The use of porous membrane limits use in high-throughput experiments. As an alternative for small-scale experiments ALI-Airway can be used for the generation of 3D bronchospheres by HBEpC which will form organoid-like structures in BME. Bronchospheres can be used for high-throughput drug screening.

Fully differentiated organoids are morphological characterized with a cell-free center lumen of the organoid which is covered by a polarized epithelial cell layer. Ciliated cells can be found directed to the inner lumen or outward-oriented of the airway organoid, which is also described in the literature [14]. Synchronously beating of outward-orientated cilia may initiate swirling of the organoid in the BME. Addition of the cell survival factor ROCKi (Rho-associated protein kinase inhibitor) Y-27632 [15] to airway organoids was shown to increase colony forming efficiency by around 20% [16]. The first signs of cilia beating in our ALI-Airway can be observed microscopically after 2 weeks. This Application Note describes the protocol for the cultivation of airway organoids with high self-renewal potential for over 4 weeks.

Airway Organoid Culture System Protocol Part A

2D expansion of Primary Human Bronchial Epithelial Cells

The protocol in section A describes the procedure for thawing and expanding of the required amount of ALI pre-screened HBEpC in 2D culture on plastic.

I. Materials

- Human Bronchial Epithelial Cells, ALI pre-screened (C-12640)
- Airway Epithelial Cell Growth Medium, containing the Basal Medium and either SupplementMix (Ready-to-use; C-21060) or SupplementPack (Kit; C-21160)
- Water bath at 37°C
- Timer
- Cell counting equipment

II. Protocol

To prepare the medium, thaw the SupplementMix or SupplementPack at 15–25°C. Aseptically mix the supplement by carefully pipetting it up and down. Transfer all components to the 500 ml bottle of Basal Medium. Close the bottle and swirl gently until a homogenous mixture is formed. After addition of the supplement(s) to the Basal Medium, its shelf life (complete Growth Medium) is 6 weeks. Store the complete Growth Medium at 2–8°C.

1

Thaw the HBEpC

Remove the cryovial from liquid nitrogen and transport the vial on dry ice. Under a laminar flow bench, release the pressure of the vial by briefly twisting the cap counterclockwise by a quarter turn and then retightening it. Allow the cell suspension to thaw in a water bath at 37°C for 2 minutes. Rinse the vial with 70% EtOH and place it under a laminar flow bench. Aspirate the ethanol from the threads of the screw cap. Carefully open the cryovial. Transfer the cell suspension to the cell culture vessels containing the prewarmed medium from step 1.

Note: Our cryopreserved cells are frozen in Cryo-SFM, which contains DMSO. Work quickly to prevent a longer incubation of the cell suspension in Cryo-SFM, because the cells are very sensitive after thawing.

2

Incubate the cells

Gently swirl the vessel containing the cell suspension and place it in an incubator (37°C, 5% CO_2). After 16–24 hours, check the cell adherence under a microscope and replace the Growth Medium. There should only be a few floating cells.

3

Cultivate cells and regularly change the medium

Change the medium every two to three days (e.g., Monday, Wednesday, Friday). Use prewarmed complete Airway Epithelial Cell Growth Medium (180 µl medium/cm²). Regularly check the confluence of the cells. Once they reach 70–80% confluence, passage the cells.

Note: Avoid confluence >90% for HBEpC. The cells can become contact-inhibited, resulting in slower proliferation after passaging.

4

Subcultivate the expanded cells

Once the cells have reached 70–80% confluence, subcultivate the cells described in section B. The required confluence is typically reached 5–7 days after thawing. The morphology of the cells should typically present as a cobblestone pattern of epithelial cells (see Figure 2).

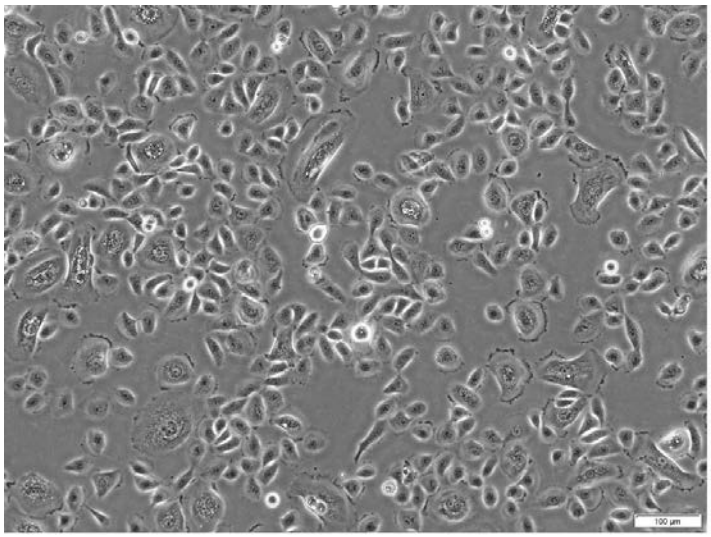

Figure 2: Morphology of HBEpC in passage 2 cultured in 2D on plastic. *Image shows Human Bronchial Epithelial Cells cultured for six days in Airway Epithelial Cell Growth Medium after thawing and seeding with 5,000 cells/cm². Scale bar = 100 µm.*

Airway Organoid Culture System Protocol Part B

Embedding of HBEpC into basal membrane extract (BME)

This section describes the detachment from 2D culture and embedding of HBEpC into BME for 3D culture.

I. Materials

- Phosphate Buffered Saline without Ca^{++}/Mg^{++} (PBS, C-40232)
- 0.04% Trypsin/0.03% EDTA (Trypsin/EDTA, C-41010)
- 0.05% Trypsin Inhibitor, 0.1% BSA (TNS, C-41110)
- Airway Epithelial Cell Growth Medium, containing the Basal Medium and either SupplementMix (Ready-to-use; C-21060) or SupplementPack (Kit; C-21160)
- Air-Liquid Interface Medium (ALI-Airway) (C-21080)
- Gentamicin-sulfate solution with a final concentration of 50 µg/ml in the medium
- 96-well plate for suspension cells (e.g., 96-Well Suspension U-bottom Plate #650185 Cellstar® Greiner Bio-One) other tested cell culture plastic is listed in table 2
- Growth factor-reduced basal membrane extract of Engelbreth-Holm-Swarm mouse sarcoma (e.g., 3D Cell Culture Matrix BME Kit, BioVision Inc. #K518)
- Optional: 10 mM Y-27632 stock solution (e.g., FUJIFILM Wako Chemicals, #030–24021)

II. Protocol

1

Preparation of a 10 mM Y-27632 stock solution (Optional)

For optimal seeding results of airway organoids, the ALI-Airway can be optionally supplemented with 10 µM ROCKi Y-27632. Addition of ROCKi can increase the colony forming efficiency and can lead to larger 3D constructs of the cells. Prepare a 1,000x stock solution and aliquot and store at -20°C until the day of use. To prepare a 10 mM stock solution dilute 1 mg Y-27632 powder in 312 µl of DMSO. Vortex the solution until it is completely dissolved. Aliquot and store aliquots at -20°C. Avoid thawing-and freezing of aliquots.

2

Preparation of ALI-Airway for use as airway organoid seeding medium

Take an aliquot of the ALI-Airway (e.g., 50ml) and bring it to room temperature. We recommend the addition of gentamicin-sulfate solution with a final concentration of 50 µg/ml in the medium. Optionally, add 10 µM of Y-27632 (e.g., 50 µl of 10 mM Y-27632 per 50 ml of ALI-Airway). The airway organoid seeding medium can be used for 6 weeks when stored at 4–8°C.

Note: The addition of Y-27632 to the airway organoid seeding medium is not necessary for revealing of cilia beating airway organoids, but it can improve the colony forming efficiency if it is added for 5–7 days after seeding in the BME.

3

Preparation of basal membrane extract aliquots

BME stock solution is stored at –20°C. It gels at room temperature. Avoid longer exposure times to temperatures >7°C. We recommend generating BME aliquots and storing at –20°C. Therefore, the BME stock solution can be thawed overnight at 4–7°C. Keep the vial on ice when putting it under the laminar air flow. Work fast to prevent gelling. Pipet an aliquot of BME in fresh precooled vials and keep the aliquots on ice and store them at -20°C. Perform the airway organoid assay on ice throughout.

4

Wash the cells

Approximately 5–7 days after thawing in 2D culture, the HBEpC should reach 70–90% confluence. Aspirate the medium and wash the cells by adding an equal volume of PBS without Ca^{++}/Mg^{++}.

Note: Allow the PBS without Ca^{++}/Mg^{++} to reach room temperature before adding to the cells.

5

Detach the cells

Aspirate the PBS without Ca^{++}/Mg^{++} from the vessel and add pre-warmed Trypsin/EDTA (100 µl/cm²) to the cells. Gently swirl the vessel to ensure that the cells are completely covered with Trypsin/EDTA. Place the vessel in an incubator (37°C, 5% CO_2) for 3–4 minutes. Check detachment under a microscope. The cells should be rounded. To encourage detachment, you can gently tap the vessel horizontally against the tabletop. Return the vessel to the laminar flow bench and add an equal amount of TNS to the cells. Gently swirl the vessel.
Note: Epithelial cells stick tightly to plastic because of the large number of adherens junctions. If the cells do not round up after four minutes of incubation at 37°C, you can place the vessel in the incubator for one additional minute. Do not over-trypsinize them. If they are still sticking after one minute of incubation, use a 1,000 µl pipette to wash them down.

6

Collect the cells and determine the cell number and viability

Transfer the cell suspension to a 15 ml conical tube. To collect all remaining cells, add complete Airway Epithelial Cell Growth Medium to the vessel and transfer into the same 15 ml conical tube. Examine the vessel under a microscope to check if all cells have been collected. Use an appropriate volume of detached cell suspension to determine the cell number. Use your standard methods for cell counting and viability assessment. Spin down the cells (3 minutes at 300 x g) and aspirate the supernatant. Transfer Airway Epithelial Cell Growth Medium to the pellet and resuspend the cells by pipetting them up and down. Keep the cells under the laminar flow bench until you seed them.
Note: Resuspended cells in Airway Epithelial Cell Growth Medium can be further subcultivated for other experiments. HBEpC cultivated in Airway Epithelial Cell Growth Medium can be passaged at least for 15 population doublings following the methods in our cell culture instruction manual for airway epithelial cells.

7

Calculate a cell suspension

We recommend a cell suspension concentration ranging from 1–2 x 10^6 cells/ml. If you start with HBEpC in passage three for the first time we recommend starting with a concentration of 2 × 10^6 cells/ml. After cell counting calculate the preparation of a cell suspension with 2 x 10^6 cells/ml. Resuspend the cells in 500 µl ALI-Airway (optional: add 10 µM Y-27632 to the ALI-Airway) at a concentration of 2 × 10^6 cells/ml and keep the cell suspension on ice under the laminar air flow.
Note: In this protocol the seeding density of 10.000 cells/50 µl cell-matrix for one 96-well plate was optimized for HBEpC in passage three. If you use cells in higher passage the seeding density may be altered.

8

Prepare the matrix and embed cells in the 90% BME gel

Take an aliquot of the matrix from -20°C and thaw it on ice. Keep on ice throughout. Multiwell plates should be preheated at 37°C in an incubator for at least two hours before use to increase the speed of matrix gelling. If you use a 96-well plate, take 500 µl of your prepared cell suspension (e.g., 2 × 10^6 cells/ml) and carefully dilute it with 4,500 µl of matrix on ice to reveal a 90% BME-cell solution. Mix gently and add 50 µl of the cell-matrix mix to each well in the preheated plate which will result in 10,000 cells/well. We strongly recommend the use of U-bottom suspension plates otherwise cells may adhere and proliferate on the plastic bottom. Avoid air bubbles when pipetting the cell-matrix mix to the wells and work fast <60 seconds. Afterwards do not swirl the plate. Put the plate into an incubator (37°C, 5% CO_2) to solidify the cell-matrix mix for 30 minutes. Do not move the plate during that time. After 30 minutes check the gelled cell-matrix under a microscope. Cells should be spread through the whole well. Put the plate back under the laminar air flow and add ~200 µl of ALI-Airway (optional incl. 10 µM Y-27632) to it. The high volume of the medium should cover ~90% of the well. Place the plate into an incubator (37°C, 5% CO_2) and let the cells grow.
Note I: If you wish to use less wells in the 96-well suspension plate, mix 5 µl of your cell suspension (e.g., 2 × 10^6 cells/ml) with 45 µl of BME and pipet the whole 50 µl to one well of the preheated plate to get 10,000 cells per well.
Note II: If you wish to use a 24-well format, you can use 24-well suspension plates (see Table 2 for recommended plastic). It is important to preheat the 24-well plate in a 37°C incubator at least two hours to enhance a fast-gelling process when pipetting the 50 µl domes in the well. If the plate is not preheated, the domes will not form a proper 3D "drop-like" architecture and melt. Use a 1,000 µl pipette for pipetting each 50 µl cell-matrix mix. Pipet the 50 µl cell-matrix mix in the center of one well by holding the pipet vertically. Do not touch the prewarmed plastic bottom of the well with the pipet tip to avoid gelling in the tip. Gently dispense the cell-matrix mix and avoid air bubbles. After pipetting one dome per well quickly incubate the plate for 30 minutes at 37°C in an incubator. Do not move the plate. After that time check for proper gelling. Afterwards add 500 µl of ALI-Airway (optional: incl. 10 µM Y-27632) to each well and incubate at 37°C and 5% CO_2 in a humidified incubator.

Airway Organoid Culture System Protocol Part C

Differentiation and cultivation of airway organoids

This section describes the culture and differentiation of HBEpC in 3D culture.

I. Materials

- Air-Liquid Interface Medium (ALI-Airway) (C-21080)
- Gentamicin-sulfate solution with a final concentration of 50 µg/ml in the medium
- Optional: Y-27632 stock solution (e.g., FUJIFILM Wako Chemicals, #030–24021)

II. Protocol

1

Change medium and let the cells differentiate

Bring the ALI-Airway medium (optional: incl. 10 µM Y-27632) to room temperature. If using a 96-well plate format, change the medium every day. Carefully aspirate ~180 µl of the medium. Be careful not to touch the cell-matrix mix with the pipet tip. Gently dispense ~180 µl of ALI-Airway medium (optional: incl. 10 µM Y-27632) to each well. Spheroid structures are formed during the first three days (see Figure 3). If you added the optional 10 µM Y-27632 to the ALI-Airway, you can switch to ALI-Airway 5–7 days after seeding.
Note: Do not remove the medium by vacuum pump because aspiration can lead to loss of samples. Be careful during medium

changes as the cell-matrix mix is very soft and can easily get aspirated by a 1,000 µl pipet tip. If you are not sure about the two phases of cell-matrix mix and culture medium on top, aspirate less medium. Due to daily medium changes of a 96-well plate format, the volume of fresh medium is less crucial. To cover the weekend, change the medium on Friday afternoon and on Monday morning. It is important to change the medium every day because of high metabolism of the cells.
If you use a 24-well plate format, a medium change every 2–3 days is required (e.g., Monday, Wednesday, Friday). Carefully remove the medium using a 1,000 µl pipette and add 500–750 µl ALI-Airway. A 5–7 day optional incubation with 10 µM Y-27632 can increase colony forming efficiency. After the incubation, switch to ALI-Airway and change the medium every 2–3 days using 750 µl medium.

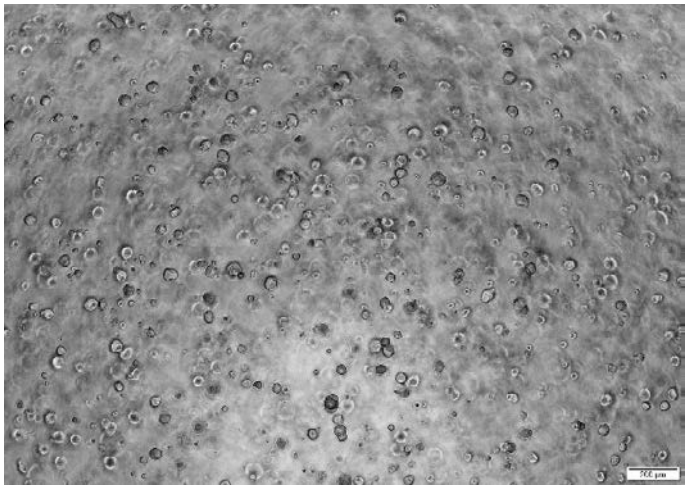

Figure 3: Three days after embedding HBEpC in BME and culture in ALI-Airway medium spheroid formation can be observed. *HBEpC were mixed with 90% BME and 10.000 cells/well were seeded in a 96-well U-bottom suspension plate. Medium changes with airway organoid seeding medium were performed daily. Note the consistent distribution of spheroids through the matrix, observed using analysis of different plains by microscopy. Scale bar = 200 µm*

2

Differentiation can be observed after two weeks of organoid culture

After approximately two weeks of organoid culture ciliated cells become visible, which is a sign of differentiation. Ciliated cells can be located at the outside of the polarized epithelial cell lining (apical-out) or located at the inner side of the lumen of the organoids. After more time, a central lumen will become visible which can be analyzed by confocal microscopy. Organoids with apical-out located ciliated cells tend to rotate in the matrix because of powerful synchronously beating cilia. Differentiation is completed after four weeks of 3D culture.

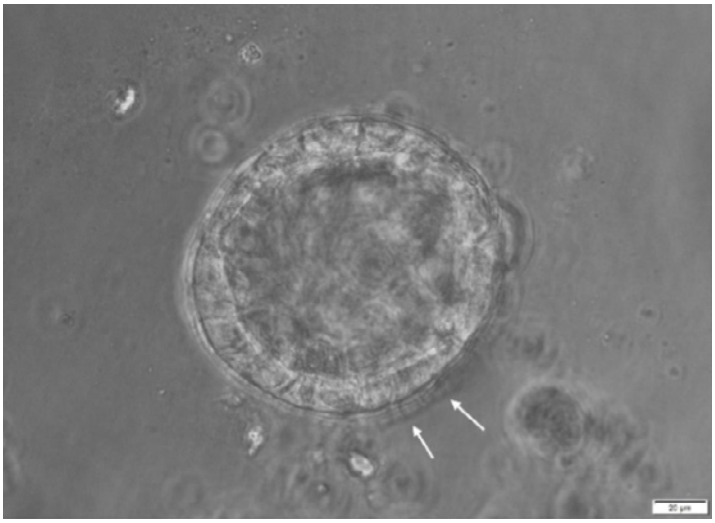

Figure 4: Differentiation of HBEpC after 16 days of 3D culture in ALI-Airway. *Bright-field microscopy shows the polarized epithelial cell lining of the organoids and the inner central lumen. Arrows indicate differentiated, outside oriented ciliated cells. Scale bar = 20 µm. A movie of the cilia beating is available on our website www.promocell.com.*

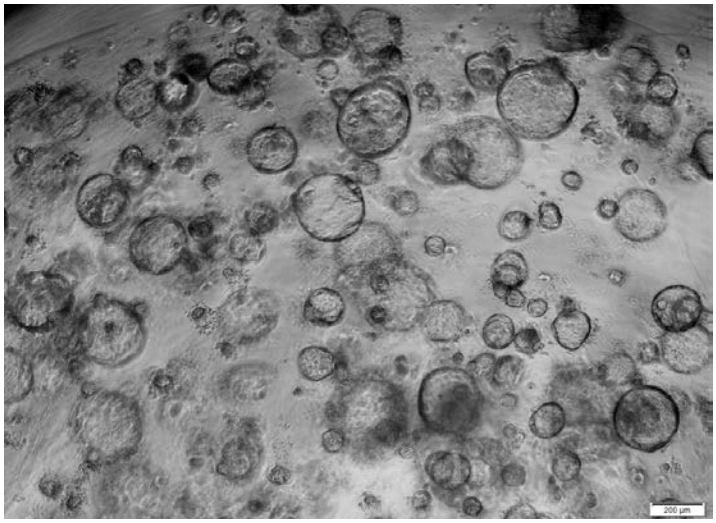

Figure 5: Airway organoids with self-renewal potential can be used for long-term culture. *HBEpC were seeded with 10.000 cells/well in airway organoid seeding medium containing 10 µM Y-27632 in 90% BME. Organoids were cultured for over 38 days in ALI-Airway. Note the visible central lumen of the polarized organoids. Scale bar = 200 µm*

Material

Product	Size	Catalog Number
ALI pre-screened Human Bronchial Epithelial Cells (HBEpC)	500,000 cryopreserved cells	C-12640
Airway Epithelial Cell Growth Medium (Ready-to-use)	500 ml	C-21060
Airway Epithelial Cell Growth Medium Kit	500 ml	C-21160
Air-Liquid Interface Medium (ALI-Airway)	500 ml	C-21080
Phosphate Buffered Saline without Ca^{++}/Mg^{++}	500 ml	C-40232
Trypsin/EDTA (0.04% (w/v) Trypsin/0.03% (w/v) EDTA)	125 ml	C-41010
Trypsin Neutralizing Solution (0.05% (w/v) Trypsin Inhibitor, 0.1% (w/v) BSA)	125 ml	C-41110

Additional products recommended for airway organoid culture

Product	Size	Catalog Number
3D Cell Culture Matrix BME Kit (BioVision Inc.)	100 assays	K518–100
Y-27632 (FUJIFILM Wako Chemicals)	1 mg	030–24021
96-Well Suspension U-bottom Plate (Cellstar® Greiner Bio-One)	–	650185
24-well plate Nunclon™ Sphera™ (Thermo Scientific)	–	174930

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