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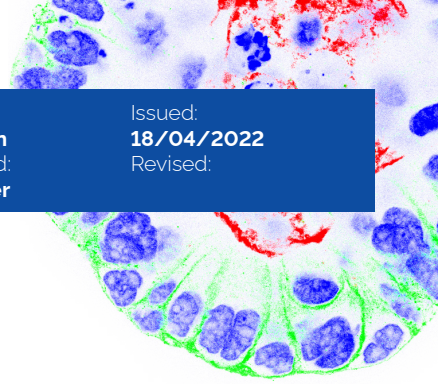
SOP –MUI-1.0	Author: **A. Amann** Approved: **F. Mildner**	Issued: **18/04/2022** Revised:



LUNG SPHEROID CULTIVATION

Purpose

The purpose of SOP 1.0 is to describe how to create, plate and maintain non small cell lung cancer spheroids from human lung tissues.

Scope

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 *Department of Pathology (InnPath, Tirol Kliniken)*. The unit has performed a routine histopathological analysis of the primary tumor. Surgical material of fresh lung tumors were brought immediately into the laboratory of Department of Haematology and Oncology. Collaborators at MUI have used normal and tumor material to establish organoid cultivation protocol as following described.

1. Cell culture media, reagents and solutions

- Human lung tumor samples
- Phosphate-buffered saline (PBS) (Corning, New York, USA)
- GravityPLUSTM microtissue (InSphero AG, Zürich, Switzerland)
- Stempro DMEM low glucose (Lonza Group AG-REG, Switzerland)
- Penicillin G sulfate (PAA laboratories GmbH, Germany)

- Streptomycin sulfate (PAA laboratories GmbH, Germany)
- Primocin (PAA laboratories GmbH, Germany)
- L-Glutamine (PAA laboratories GmbH, Germany)
- Liberase ™ (Sigma-Aldrich cat.no 1088866001)

2. Equipment

- Cell culture incubator with 5% CO_2, 37 °C (Binder C150)
- Inverted Microscope (Olympus CK2)
- High-speed centrifuge (Hettich Rottina 35R)
- Pipette aid, serological pipettes

3. Reagent setup

- Human lung samples: these samples can be obtained for cancer research by a trained surgeon during surgery from the lung. At least 1-2 cm^3 of tissue should be collected and processed within 1 hours. The tissue should be kept cold at 37°C in PBS until processing
- Cultivation medium: Stempro DMEM low glucose (Lonza Group AG-REG, Swiss) supplemented with 20% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-Glutamine, primocin 100 µg/ml (PAA laboratories GmbH, Germany
- Digestion solution: Liberase™ (Sigma Aldrich)/Stempro (without supplements) solution 1:10
- Cell freezing solution: this solution is prepared by gradually adding 10% of DMSO in 90% of FBS

4. Procedure

4.1 Human lung dissociation and generating 3D spheroids

- Tissue collection. After surgical excision, the tissue should be kept at 4°C in a 50ml falcon in cultivation medium and transported (from the Hospital to research laboratory: time 12 minutes) within 1 hours until processing.
- Transfer the falcon containing the lung tissue to the biological

safety cabinet. Aspirate the medium and wash twice with 10 ml of PBS. Gently pipette up and down with a 10-ml pipette to wash in 10 ml of PBS the lung tissue. Remove as much supernatant as possible from the pellet.

- Place the tissue in a sterile 100-mm Petri dish. Mince it into pieces using scalpels. (See *Figure 1*)
- Transfer in a 50ml tube and incubate the tissue 15min at 37°C on a shaker with 2ml Liberase™ (Sigma Aldrich) in PBS 1:10. (See *Figure 1*)
- Then supernatant is transferred into the stop solution (Stem-pro medium with 50 % FBS) to inhibit digestion.
- Repeat incubation process twice.
- Collected supernatant solution was pipetted on top of two consecutive filters (first: 400µM, second: 100µM). (See *Figure 1*)
- For the next steps, the single cell solution (below filter with 100µM) and the tumour pieces between the two filters were used. (See *Figure 1*)
- Single cell suspension was seeded in a density of 2500 cells per well and tumour tissues were seeded alone or with fibro-blasts into hanging drop plates in 40µl. (See *Figure 2*)
- Plates were incubated at 37°C for 7-21 days. With medium re-freshment every 3-5 days.
- Place the tissue in a sterile 100-mm Petri dish. Mince it into pieces using scalpels. (See *Figure 1*)
- Transfer in a 50ml tube and incubate the tissue 15min at 37°C on a shaker with 2ml Liberase ™ (Sigma Aldrich) in PBS 1:10. (See *Figure 1*)
- Then supernatant is transferred into the stop solution (Stem-pro medium with 50 % FBS) to inhibit digestion.
- Repeat incubation process twice.
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5. Figures

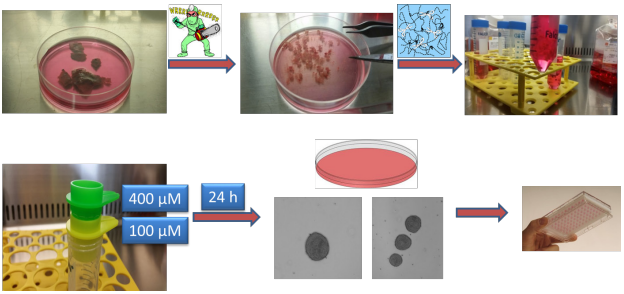

Figure 1. Representative images of of how tumor tissues were minced, digested and processed further for cultivation

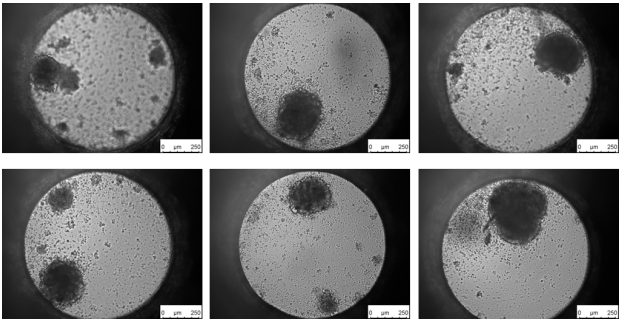

Figure 2. Representative images of NSCLC spheroids after three days

6. Preparation of drug library working concentrations

The PRESTWICK CHEMICAL LIBRARY is a library of 1,520 off-patent small molecules, mostly approved drugs by FDA and EMA. The compounds are provided at a concentration of 10 mM in 100% DMSO. Each well contains 25 µl of drug. The objective of the library preparation is to dilute the compound to reach a final drug concentration of 10µM/1µM in the complete medium without exceeding 0.2% DMSO

- Add 25µl of DMSO to each well of library using STARlet automated liquid handling station thus reaching a concentration of 5mM in 50 µl (100% DMSO).
- Store 384 plates at -20°C until use.
- At the day of drug screening 5 µl of the library (5 mM) to in 45 µl of RPMI 1640 medium without FBS serum, prepared in a new 384 well plate using STARlet automated liquid handling station. Thus, the concentration of the drug library is reduced to 500 µM in 10% DMSO (in RPMI 1640 medium).
- Transfer 5 µl of library (500 µM in 10% DMSO; in RPMI 1640 medium) to 45 µl of RPMI 1640 medium without FBS serum, prepared in new 384 well plates using STARlet automated liquid handling station. The concentration of the drug library is now 50 µM in 1% DMSO. These dilution plates are used in the subsequent screening. Briefly, in the screening 10 ul of library dilution (50 µM in 1% DMSO) is added to experimental cells grown in 40 µl of complete medium, thus reaching a final drug concentration of 10 µM in 0,2% DMSO; complete medium.

7. Drug repositioning screening procedure

- Plate resistant (750 cells/well) and sensitive (500 cells/well) H1299 cells in 40 µl of complete medium in 384 well plates using a Multidrop™ Combi Reagent Dispenser (Thermo Fisher Scientific). Cell numbers indicated in SOP-UNITS-1 refer to adherent H1299 cell. For any other cell type, cell number titration is recommended to determine the optimal cell seeding density.
- Incubate cells overnight at 37°C
- The next day, the drug library is diluted to 50 µM; 1% DMSO using the STARlet automated liquid handling station (Hamilton), as indicated in point 6 of this SOP.
- Add 10 µl of drug library (50 µM; 1% DMSO) on top of cultivated experimental cell. Thus, a final drug concentration of 10 µM in 0.2% DMSO is reached.

- After 72 hours of cultivation in a cell culture incubator, 40 µl complete medium is removed using an aspirator vacuum pump (ELx405 Select Deep Well Washer). Remaining 10 µl of complete medium should be considered for the calculation of the concentration of resazurin working solutions.
- Prewarm resazurin working solution (0,35 mg/ml) at 37°C and dilute 1:8 in prewarmed complete medium to obtain a final concentration of 0,042mg/ml.
- Add resazurin solution (40 µl) at a final concentration of 0.042 mg/ml in complete medium on top of the cells still covered by 10µl of complete medium.
- Incubate 384 well plates with H1299 cells (resistant and sensitive) for 3 hours in a standard cell culture incubator. For any other cell type, incubation time needs to be optimized and can be in the range of 1-6 hours
- Measure the relative fluorescent units (RFU) using a plate reader Envision 2104 Multilabel Reader (Perkin Elmer). Excitation wavelength = 560 nm, Emission wavelength = 590 nm.

8. Read out

In the drug repositioning screening ATR inhibitor resistant cells will be treated with 1520 drugs and an ATR inhibitor (VE-822). From each plate select the drugs that re-sensitize resistant cells to ATR inhibitor treatment according to fluorescence intensity.

Controls in the experiment are resistant cells, only treated with ATR inhibitor and sensitive, parental cells serve as controls. Finally, order the fluorescence intensity of interesting drugs according to the Z-score. For data analysis, please check SOP –UNITS-5.0.

NOTES