LUNG ORGANOIDS IMMUNOHISTOCHEMISTRY ANALYSIS

Purpose

The purpose of LNCIB SOP –LCO-3.0 is to describe how to prepare the cells for lung organoids immunohistochemistry analysis.

Scope

LNCIB SOP –LCO-3.0 is intended to cover all resources, personnel and equipment in the lung cancer organoids laboratory.

Introduction

Histological characterization of human lung organoids by immunohistochemistry is fundamental to ensure that organoids resemble the histological characteristics of the original tissue. Collaborators at LNCIB prepare lung organoid samples in agarose plugs for immunoistochemistry. Collaborators at Anatomia Patologica Unit of Cattinara Hospital will perform a routine histopathological analysis of patient tissue and obtained organoids.

1. Reagents and Solutions

- Phosphate buffered saline PBS (Life Technologies, cat. no. 14190-094)
- Fetal bovine serum FBS (Life Technologies, cat. no. 26010066)
- Paraformaldehyde (Sigma-Aldrich, cat. no. 158127) !CAUTION paraformaldehyde contains formaldehyde,

which can cause cancer; handle it using appropriate safety gear.

- Dispase (Stemcell cat. no.07923)
- Anti-p63 primary antibody (Abcam, cat. no. 53039)
- Anti-TTF1 primary antibody (Abcam, cat. no. EP1584Y)
- Anti-NapsinA primary antibody (Abcam, cat. no. KCG1.1)
- Xylene (Sigma-Aldrich, cat. no.247642)
- Ethanol anhydrous denatured (Sigma-Aldrich, cat. no.51976), histological grade (100% and 95%)
- Hematoxylin (Sigma-Aldrich, cat. no.H3136)
- 1% Agarose (Sigma-Aldrich, cat. no.A2576) in H2O: to dissolve agarose, boil the solution in the microwave. If required, add a minimal amount of CDI's Tissue Marking Dye (Cancer Diagnostics cat. no. 0728-3) . This will render agarose block clearly visible in the paraffin wax.
- Wash buffer: 10x Tris Buffered Saline with Tween 20 (Cell Signaling TBST #9997). To prepare 1 L of 1x TBST: add 100 ml 10X TBST to 900 ml dH2O; mix
- SignalStain Antibody Diluent (Cell Signaling #8112)
- TBST/5% normal goat serum: add 250 µl Normal Goat Serum (Cell Signaling #5425) to 4.75 ml 1x TBST
- PBST/5% normal goat serum: add 250 µl Normal Goat Serum (Cell Signaling #5425) to 4.75 ml 1x PBST
- Citrate (Sigma-Aldrich, cat. no.1613859); To prepare 1 L of 10 mM Sodium Citrate Buffer, add 2.94 g sodium citrate trisodium salt dihydrate to 1 L dH2O. Adjust pH to 6.0
- EDTA (Sigma-Aldrich, cat. no.1233508): 1 mM EDTA. To prepare 1 L, add 0.372 g EDTA to 1 L dH2O. Adjust to pH 8.0
- TE: 10 mM Tris/1 mM EDTA, pH 9.0: To prepare 1 L, add 1.21 g Tris base (Sigma-Aldrich, cat. no.T1503): and 0.372 g EDTA to 950 ml dH2O. Adjust pH to 9.0, then adjust volume to 1 L with dH2O
- Pepsin (Sigma-Aldrich, cat. no.P0525000): 1 mg/ml in Tris-HCl, pH 2.0
- 3% hydrogen peroxide (Sigma-Aldrich, cat. no.216763): To prepare 100 ml, add 10 ml 30% hydrogen peroxide to 90 ml H2O.
- Blocking Solution: TBST/5% Normal Goat Serum. To 4.75 ml 1x TBST, add 250 µl Normal Goat Serum (#5425). (Use serum from the same species as the source of the secondary antibody.)
- Detection System: SignalStain® Boost IHC Detection Reagents (HRP, Mouse #8125; HRP Rabbit #8114)
- DMEM-H (Gibco, Carlsbad, CA, cat. no. 11995-065)
- Ham's F-12 Medium (Lonza, cat. no. 12615F)

2. Equipment

- 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)
- Microscopes slides Superfrost® Plus (Thermo Scientific, cat. no. J1800AMNZ)
- Staining jars
- PAP Pen for immunostaining (Sigma-Aldrich, cat. no. Z377821)
- Coverslips
- Microscope
- Ice

3. Procedure immunohistochemistry on lung organoids

3.0 Sample preparation Stock solution

- Grow organoids for 5-7 days following procedure described in LNCIB SOP –LCO-1.0. Organoids derived from 3-6 wells of a 24-well plates are generally sufficient to perform immunohistochemistry analysis. !CRITICAL For the following steps precoat all the tubes, the pipettes and tips with FBS to avoid attachment of the tissue to the plastic.
- Keep the plates on ice.
- Remove the medium from 24 well, add 500 µl of room temperature 1X PBS to wash organoids. Aspirate the supernatant.
- Add 500 µl of ice-cold 1X PBS, recover organoid-matrigel mixture and transfer to a 15ml tube. Centrifuge at 200 – 300 x g for 5 minutes at 2-8°C. Remove supernatant.
- Gently resuspend organoids in 1 ml of Dispase for 10 minutes at 37 °C in order to digest remaining Matrigel. This liberates cells from remaining Matrigel with minimal cell damage.
- Add 10 ml of DMEM/F12 (1:1) supplemented with 10% FBS and centrifuge organoids at 200 – 300 x g for 5 minutes at 2-8°C.
- 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.
- Add 3 mL of cold PBS and let organoids settle under gravity.

- Carefully aspirate the supernatant.
- Fix organoids by adding 3mL of 4% Paraformaldehyde for 20 min at RT (shaking slowly). Organoids will settle under gravity.
- Carefully aspirate the supernatant and add 3mL of PBS and let organoids settle under gravity. Repeat this step.
- Cut off and discard the conical bottom of 1.5mL microcentrifuge tube and cap the tube.
- Carefully aspirate the supernatant and resuspend organoids pellet in 200µL of 1% agarose pre-dissolved in microwave using a cut tip. Transfer them in the cap of the previously prepared microcentrifuge tube (the cylinder of agarose will be prepared in the cap of the tube) and put the tube immediately on ice. This step allows the embedding of organoids in agarose blocks, which can be easily embedded in paraffin wax.
- Extract the agar cylinder from its base and process them into paraffin blocks using standard tissue processing.
- From paraffin blocks, cut thin slices (4-6 µm) on a microtome, and float sections in a water bath.
- Mount sections on to charged slides and dry overnight. Using charged slides helps the section to adhere to the slide.

3.1 Sample preparation Stock solution

To perform immunohistochemistry, paraffin wax must be removed from the sample and the sample must be rehydrated

!CAUTION Do not allow slides to dry at any time during this procedure as this can lead to inconsistent staining.

- To remove paraffin wax, place sections in xylene containing jars for three times for 5 minutes each. Fresh xylene should be used as incomplete deparaffinization can also lead to inconsistent staining.
- To start rehydration, place sections in 100% ethanol containing jars for two times for 10 minutes each.
- Place sections in 95% ethanol containing jars for two times for 10 minutes each.
- To complete the rehydration process, wash sections two times in dH2O for 5 minutes each.

3.2 Antigen Unmasking

Tissue pre-treatment with the antigen retrieval reagents breaks the protein cross-links formed by formalin fixation and thereby allows the uncover of hidden antigenic sites. !CAUTION Consult product datasheet for antibody-specific recommendation for the unmasking solution.

- For Citrate: Bring slides to a boil in 10 mM sodium citrate buffer, pH 6.0; maintain at just below boiling point for 10 minutes. Cool slides at RT for 30 minutes.
- For EDTA: Bring slides to a boil in 1 mM EDTA, pH 8.0; maintain at just below boiling point for 10 minutes. Cool slides at RT for 30 minutes.
- For TE: Bring slides to a boil in 10 mM Tris/1 mM EDTA, pH 9.0 (See 4.1.1 and 4.1.2); maintain at just below boiling point for 18 minutes. Cool at room temperature for 30 minutes.
- For Pepsin: Digest for 10 minutes at 37 °C.

3.3 Staining

This protocol is for chromogenic staining. !CAUTION Consult product datasheet for recommended antibody diluent.

- Wash sections in dH2O three times for 5 minutes each using staining jars.
- To quench endogenous peroxidase activity in samples, which may lead to high background staining, place sections in 3% hydrogen peroxide for 10 minutes.
- Wash sections in dH2O two times for 5 minutes each using staining jars.
- Wash sections in wash buffer for 5 minutes.
- Use the PAP Pen to make an hydrophobic barriers on glass slides around the sample, taking care not to touch the sample. This allows to use small amounts of reagents in the following steps.
- To prevent non-specific binding of the antibody to the tissues, block each section with few drops of blocking solution for 1 hour at RT in a humidified chamber.
- Remove blocking solution and add 100–400µL of primary antibody solution to each section. Incubate overnight at 4˚C in a humidified chamber
- Equilibrate SignalStain Boost Detection Reagent to room temperature.
- Remove antibody solution and wash sections in wash buffer three times for 5 minutes each using staining jars.
- Cover sections with 1–3 drops SignalStain Boost Detection

Reagent as needed. Incubate in a humidified chamber for 30 minutes at RT.

- Wash sections three times with wash buffer for 5 minutes each using staining jars.
- Add 1 drop (30µL) SignalStain DAB Chromogen Concentrate to 1mL SignalStain DAB Diluent and mix well before use.
- Apply 100–400µL SignalStain DAB to each section and monitor closely until a brown reaction product is yielded. 1–10 minutes generally provides an acceptable staining intensity.
- Immerse slides in dH2O.
- For better visualization of tissue morphology, counterstain sections with hematoxylin. This reagent has a deep blue-purple colour which stains the cell nuclei and provides a contrast to the brown reaction product of the DAB chromogen.
- Wash sections in dH2O two times for 5 minutes each.

3.4 Dehydrating and Mounting Sections

SignalStain DAB Chromogen is compatible with either aqueous or non-aqueous mounting medium. If choosing non-aqueous, the sections must be dehydrated again prior to coverslip mounting.

- Place sections in 95% ethanol containing jars for two times for 10 minutes each.
- Place sections in 100% ethanol containing jars for two times for 10 minutes each.
- Place sections in xylene containing jars for two times for 5 minutes each.
- Mount sections with coverslips using mounting medium, being careful to avoid introducing air bubbles.
- Allow mounting medium to set and view slides on a microscope (Figure 1).

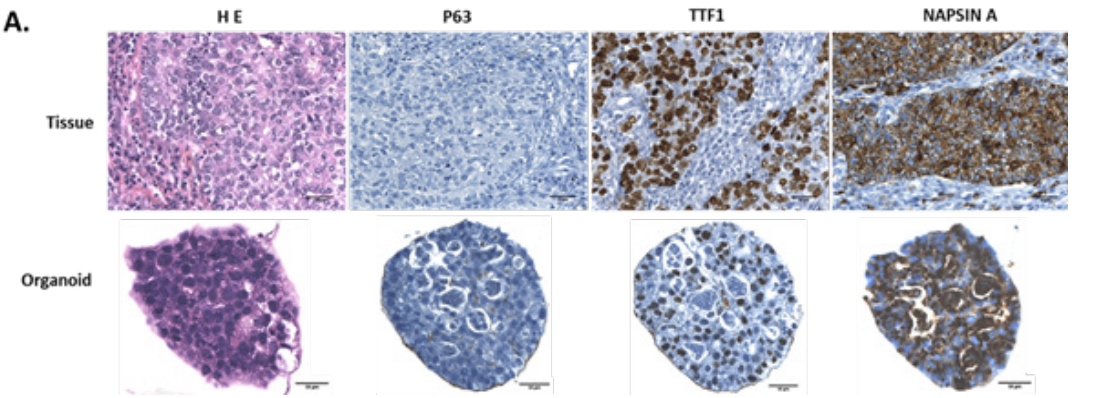

Figure 1. Organoids recapitulate histological features of original tumors (A) H&E and immunohistochemistry on sections of the primary tumor of patient1 (top panel) and tumor organoids derived from the primary tumor (bottom panel). Immunohistochemistry was performed using antibodies for P63, TTF1 and Napsin A. The status of original lung cancer adenocarcinoma is maintained in tumor organoid cultures. Scale bar 50 µm.

4. Applicable references to LNCIB SOPs

LNCIB SOP –LCO-1.0, LNCIB SOP –LCO-2.0

5. Applicable references

1. Long-term expanding human airway organoids for disease modelling. Normal Sachs et al. BioRxiv, 2018.

2. Differentiated human airway organoids to assess infectivity of emerging influenza virus. Zhou J. et al, 2018.