Immuno staining for fresh nasal cells\_version4

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Preparation:

1) Coordinate with the patient or volunteer; **2) Reserve the biological hood;** 3) Pre-warm (the medium+supplement); 4) Prepare 10 ml medium, put into 15ml centrifuge tubes, 3 ml each tube; 5) Put the tube in a cup filled with 37 ℃ water, put the cup in a bag, visit Sickkids clinic (Room5521 for Julie and Room4A08 for Sharon)

1. Get the cells.

The cells are fresh nasal cells fetched directly from a volunteer or patient’s noses. Cells from both left and right noses are needed. This step is performed by Nurse Julie or Doctor Sharon. The patient or volunteer is asked to clean his/her nose using the tissue paper first. The tiny brush is dropped into 15 mL centrifuge tube, immersed in 3 ml pre-warmed (37 ℃) medium. The centrifuge tube is taken to tissue culture room (Samples should be kept in warm water). The brush is whirled to wash down the cells (after whirling, using a pipette, blow and absorb, 30~40 times) For the immunofluorescence, drop 30 µl into round coverslip and check under the microscope for cell density, adjust concentration if the cell density is too high or too small(experience based). Then one drop of cell suspension are dropped onto round coverslip (Electron Microscopy Sciences, Cat.#72230-01 #1-1/2 Micro Coverglass-12 mm Dia Lot# 160722, uncleaned). Coversilps are then put into small dish (Corning, 60mmx15mm style. FEF:430196). 5 coverslips for each dish. Dry the cells on coverslip.

Put the petri-dish that holds all the coverslipin the fume hood. Wait until the samples dry on the coverslip. Extra samples can be stored in -80 ℃ (Petri-dish is sealed with parafilm membrane).

1. Fixation.

For FPA fixation: Fix with 4% paraformaldehyde in PBS for 15 min (freshly made, PFA stock (Electron Microscopy Sciences, PARAFORMALDEHYDE 16% SOLUTION, EM GRADE 15710))

Samples are transferred to 12 well dish (Falcon, REF: 353043), one well, one coverslip, 50 µL fixation buffer for each well..

For methanol fixation: Fix with precooled anhydrous methanol (-20 °C) in -20 °C for 30 min (Methanol stock (Fisher Chemical, Methonal Histological Grade LOT 160371)). 50 µL fixation buffer for each petri-dish. After fixation, samples are transferred to 12 well dish, one well, one coverslip.

1. Reducing(optional)

Discard PFA, reduce with 0.1% sodium borohydride (Sigma Aldrich, Lot# MKBT3048V) in PBS for 7 min. 50 µL each well. The sodium borohydride solution needs to be prepared immediately before use to avoid hydrolysis. Washing with 1x PBS is better.

For methanol fixation, reducing is not needed.

1. Blocking.

Discard reducing solution,

For FPA fixation: Block with 3% BSA and 0.2% Triton-X100 in PBS(stored in 4°C) for 30 min. 50 µL each well.

For methanol fixation: Block with 3% BSA and 0.05% tween in PBS(stored in 4°C) for 30 min. 50 µL each well.

1. Primary antibody.

Stain with primary antibody in **blocking buffer (notice: different between PFA and methanol fixation!)** for 1 hour at room temperature or overnight at 4°C, 50 µL for each sample on parafilm. Flip the coverslip to ensure the sample have full contact with primary antibody. Then wash with PBS for three times (each with 50 µL PBS, 7 min each). The washing step is performed on the parafilm to reduce the wash volume. For some specific antibodies, 3 hours incubation time is required.

1. Secondary antibody.

Stain with secondary antibody in blocking buffer (notice: different between PFA and methanol fixation!) for 30 min at room temperature, 50 µL for each sample on parafilm. Flip the coverslip to ensure the sample have full contact with secondary antibody. Then wash with PBS for three times (each with 50 µL PBS, 7 min each).

(Optional) use 50 µL HOESCHT solution (stored in room temperature, shielded from light) in the first wash if nucleus DNA labeling is needed. Finally wash with 50% glycerol.

1. (Optional) Directly labeled antibody. For instance, anti-Centriolin-555(should be stored in 4 °C rather than -20°C)

Stain with directly labeled primary antibody in **blocking buffer (different between PFA and methanol fixation!)**

1. Mount the sample with mounting medium.

Put ~5 µL mounting medium onto a glass slide(VWR Micro Slides, Cat.No. 48312-400), cover the medium with the coverslip, Seal the sample with nail polish.

1. Label the specimen with a marker pencil. Check it under the microscope or store it in 4°C shielding from light for future use.

Before checking, clean the coverslip with ddH2O, after checking, clean the oil on the coverslip with anhydrous alcohol.

Notes:

* For the fresh medium, the step 1 below is ready. We only need to warm 1 Falcon tubes, 50x supplement and Hydrocortisone, mix them according to step 2 below before use.

Fresh medium:

How to prepare Pneumacult-Ex

BEGM/Ex 500 ml bottle

1. Add antibiotics currently stored at -20C in the TC to the bottle and aliquote in 49 ml Falcon tubes

Tobramycin 80 ug/ml

Vancomycin 100 ug/ml

Gentamycin 50ug/ml

Anti-Anti 100x (5ml)

**2. When ready to use add:**

**- 1ml of 50x BEGM/Ex supplement (stored at -20C)**

**- 50 ul of Hydrocortisone stock solution (200x) (stored on the chemical bench, filter before use)**

**For 10~14 days.**

For DNAH11 antibody, always use fresh nasal cells not frozen, always use fresh 4% PFA not old.

For DNAH11, add an extra permeation step between reducing and blocking. Permeation with 3% BSA and 0.2% Triton-X100 in PBS for 12 minutes. For the following steps, avoid any detergent, just use 3% BSA in PBS for blocking and primary secondary mixture.

4% PFA should always be fresh. 16% PFA stock should be kept in dark and shielded from air. Opened stock can be stored for 2 weeks.

For DNAH5, DNAH11, CCDC39 antibody, At least 3 hours incubation at room temperature is recommended to get a better results. CCDC39 works well by methanol fixation

* A table as below is quite useful for future check

