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Processing of pediatric nasal and bronchial brushing samples for single cell analysis

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Protocol status: Working

We use this protocol and it's working

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Abstract

This protocol describes the collection, processing, and cryopreservation of pediatric nasal and bronchial brushing samples for downstream single-cell analysis.

Guidelines

This is an experimental protocol for the processing of nasal and bronchial brushing samples collected from children. Sample collection must have and be compliant with Human Ethics Committee approval.

Materials

- ⊗ RPMI-1640 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R5886**
- ⊗ Fetal Bovine Serum
- ⊗ 1X PBS (Phosphate-buffered saline)
- ⊗ DMSO (dimethyl sulfoxide) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D8418**
- ⊗ conical tubes, 50ml
- ⊗ conical tubes, 15ml
- ⊗ 1.5 mL Eppendorf tubes
- ⊗ Corning® cell strainer **Corning Catalog #CLS431751-50EA**
- ⊗ FACS Tubes
- ⊗ Acridine Orange/Propidium Iodide stain **Logos Biosystems Catalog #F23991**
- ⊗ PhotonSlide **Logos Biosystems Catalog #L12007**
- ⊗ Human TruStain FcX™ (Fc Receptor Blocking Solution) **BioLegend Catalog #422301, 422302**
- ⊗ Cytology Brush Ring Handle 3.00mm X 120.00cm **Conmed Catalog #149**
- ⊗ Cytology Brush Single-use Respiratory Compatible Channel - 1.2mm , 2mm brush width 6mm length **Olympus Catalog #BC-203D-2006**
- ⊗ cryovial

Flow antibodies:  Flow cytometry panels.pdf 301.2KB

Equipment

Aurora - 5L Configuration	NAME
Spectral Flow Cytometer	TYPE
Cytek Aurora	BRAND
N/A	SKU
5L Configuration	SPECIFICATIONS



Equipment

Luna FL	NAME
Cell counter	TYPE
Luna	BRAND
L20001	SKU
https://logosbio.com/luna-fl/	LINK

Protocol materials

✕ Corning® cell strainer **Corning Catalog #CLS431751-50EA**

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✕ FACS Tubes

✕ RPMI-1640 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R5886**

✕ Fetal Bovine Serum

✕ cryovial

✕ 1X PBS (Phosphate-buffered saline)

✕ DMSO (dimethyl sulfoxide) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D8418**



Safety warnings

- ⚠ Human samples should be processed in a laboratory with appropriate biosafety infrastructure and procedures



COLLECTION OF NASAL AND BRONCHIAL BRUSHINGS

- 1 Prepare collection tubes for nasal and bronchial brushing samples by adding 5mL of pre-chilled RPMI supplemented with 2% heat-inactivated fetal calf serum (referred to as RPMI 2% FCS) to a 15mL tube labelled with the study/patient ID.
- 2 After obtaining informed consent from family and/or patient, collect brushings using a suitable cytology brush.

For nasal brushing:

 Cytology Brush Ring Handle 3.00mm X 120.00cm **Conmed Catalog #149**

For bronchial brushing:

 Cytology Brush Single-use Respiratory Compatible Channel - 1.2mm , 2mm brush width 6mm length **Olympus Catalog #BC-203D-2006**

Note

For guidelines on how to safely perform bronchial/nasal brushings in children, please see:

Citation

McNamara PS, Kicic A, Sutanto EN, Stevens PT, Stick SM (1970)
. Comparison of techniques for obtaining lower airway epithelial cells from children.

<https://doi.org/10.1183/09031936.00162507>

LINK

Citation

Lane C, Burgess S, Kicic A, Knight D, Stick S (1970)
. The use of non-bronchoscopic brushings to study the paediatric airway.

<https://doi.org/>

LINK



- 3 Brushing samples must be placed on ice and processed in the laboratory within



00:30:00 of the procedure.

30m

PROCESSING OF NASAL AND BRONCHIAL BRUSHINGS TO SINGLE CELL SUSPENSION

- 4 Repeatedly pipette media onto brushing to dislodge cells. Do this for at least 1 minute per brushing. Remove cytology brush and top up the cell suspension to 10mL with RPMI 2% FCS
- 5 Filter cell suspension through a 70-120µm cell strainer into a new 15mL tube and centrifuge  300 x g, 4°C, 00:07:00 .
- 6 Discard supernatant and resuspend cell pellet in 3mL RPMI 2% FCS.
- 7 Prepare cell suspension for cell counting. Here, we use AO/PI and the LUNA FL counter. Remove 18µL for cell counting into a microcentrifuge tube. Add 2µL of AO/PI to the count tube and mix well.
- 7.1 Load 10µL of stained cells onto a Luna fluorescent counting slide and count. Record viability, total cell count, and live cell count.

Note

Cell counting can be performed manually using a haemocytometer, or using other automated cell counters.

- 8 Top up remaining cell suspension to 10mL with RPMI 2% FCS, centrifuge  300 x g, 4°C, 00:07:00 and remove supernatant.
- 9 If choosing to run flow cytometry or other single cell assays on fresh cells, here is where you can allocate the required number of cells for downstream processing. For flow cytometry, described below, we allocate 300,000 cells prior to proceeding to cryopreservation for remaining cells.

CRYOPRESERVATION OF NASAL AND BRONCHIAL BRUSHING CELLS

- 10 Resuspend cells at a ratio of 1:1 in RPMI 2% FCS and freeze solution (heat-inactivated FCS + 15% DMSO) such that cells are frozen between 1-10 million cells/mL. Transfer cells



to cryogenic vial.

- 11 Immediately place cryogenic vials into an isopropanol freezing container (e.g. Nalgene® Mr. Frosty) or Cool Cell (Corning) and transfer to -80 °C overnight.
- 12 For long term storage, transfer the vials to liquid nitrogen.

PREPARATION OF FRESH CELLS FOR FLOW CYTOMETRY

45m

- 13 Resuspend cell suspension for fixable viability staining according to manufacturers' instructions (e.g. the LIVE/DEAD™ Fixable Near-IR Stain from Invitrogen/ThermoFisher).

5m

Following the required incubation, stop the reaction by the addition of 1mL staining buffer (2% heat-inactivated FCS in PBMS 2mM EDTA, herein referred to as FACS buffer) and centrifuge at

400 x g, 4°C, 00:05:00

- 14 Resuspend cells in 25µL of FC-block for 00:05:00 at Room temperature .

5m

- 15 The next steps will depend on the requirements for your specific panel. As an example, we have attached our 17-plex spectral cytometry panel that we routinely use on paediatric airway samples, as well as a publication describing the application of this panel. All of the following steps are related to this panel.

nasal_bronchial_BAL_panel.pdf 114.5KB

Citation

Neeland MR, Gubbels L, Tsz Chun Wong A, Walker H, Ranganathan SC, Shanthikumar S (2024)

. Pulmonary immune profiling reveals common inflammatory endotypes of childhood wheeze and suppurative lung disease.

[https://doi.org/pii:S1933-0219\(24\)00020-5.10.1016/j.mucimm.2024.03.001](https://doi.org/pii:S1933-0219(24)00020-5.10.1016/j.mucimm.2024.03.001)

LINK



16 Add 25µL of antibody cocktail made up at 2X concentration and incubate for



00:30:00



On ice .

30m

17 Following staining, wash cells with 2mL FACS buffer, centrifuge at



400 x g, 4°C, 00:05:00

and resuspend cells in 200µL FACS buffer for acquisition on a flow cytometer (here, a Cytex 5L Aurora).

5m

18 Immediately before running the sample, filter the cell suspension through a 35 µm cell strainer (Falcon™ 352235).