May 08, 2024

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

DOI
dx.doi.org/10.17504/protocols.io.5jyl8p379g2w/v1

Liam Gubbels[1], Shivanthan Shanthikumar[1], Melanie R Neeland[1]

[1]Murdoch Children's Research Institute

earlyAIR

Liam Gubbels
Murdoch Children's Research Institute

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Protocol Citation: Liam Gubbels, Shivanthan Shanthikumar, Melanie R Neeland 2024. Processing of pediatric nasal and bronchial brushing samples for single cell analysis. **protocols.io https://dx.doi.org/10.17504/protocols.io.5jyl8p379g2w/v1**

Protocol status: Working
We use this protocol and it's working

Created: March 13, 2024

Last Modified: May 08, 2024

Protocol Integer ID: 96676

Keywords: single-cell, flow cytometry, respiratory, Paediatric, nasal brushing, bronchial brushing

Funders Acknowledgement:
Chan Zuckerberg Initiative
Grant ID: 2021-237883

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 **Sigma Aldrich Catalog #**R5886
- Fetal Bovine Serum
- 1X PBS (Phosphate-buffered saline)
- DMSO (dimethyl sulfoxide) **Sigma Aldrich Catalog #**D8418
- conical tubes, 50ml
- conical tubes, 15ml
- 1.5 mL Eppendorf tubes **Contributed by users**
- Corning® cell strainer **Corning Catalog #**CLS431751-50EA
- FACS Tubes **Contributed by users**
- 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 **Contributed by users**

Flow antibodies: Flow cytometry panels.pdf 301KB

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

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

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 (2008). Comparison of techniques for obtaining lower airway epithelial cells from children..

LINK

https://doi.org/10.1183/09031936.00162507

CITATION

Lane C, Burgess S, Kicic A, Knight D, Stick S (2005). The use of non-bronchoscopic brushings to study the paediatric airway..

LINK

https://doi.org/

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 . 7m

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. 7m

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 114KB

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..

LINK

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

16 Add 25µL of antibody cocktail made up at 2X concentration and incubate for 00:30:00 30m
On ice .

17 Following staining, wash cells with 2mL FACS buffer, centrifuge at 400 x g, 4°C, 00:05:00 5m
and resuspend cells in 200µL FACS buffer for acquisition on a flow cytometer (here, a Cytek 5L Aurora).

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